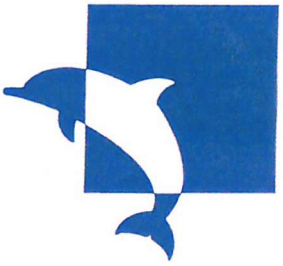

University of Southampton

Respiratory Infections and the Immune Response to Respiratory Syncytial Virus in the First Year of Life

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ABSTRACT

UNIVERSITY CHILD HEALTH
SCHOOL OF MEDICINE

Doctor of Medicine

Respiratory Infections and the Immune Response to Respiratory Syncytial Virus in the First Year of Life

by Julian Peter Legg

Respiratory syncytial virus (RSV) bronchiolitis is a major cause of childhood morbidity and mortality. Most infants are infected with respiratory syncytial virus in the first year of life, but a minority develop bronchiolitis for reasons unknown. The primary aim of this thesis was to determine the importance of type 1 and type 2 immunity in determining the outcome of RSV infection during infancy. Previous studies have established that antigen-specific priming of foetal T-cells can occur *in utero* from 22 weeks gestation. An additional aim of this thesis was to study the potential occurrence of prenatal sensitisation to RSV and it's immunological consequences. This thesis also sought to establish the relative incidence of respiratory pathogen infection during infancy.

36 cord blood specimens were obtained from randomly selected newborn infants. Cord peripheral blood mononuclear cells were separated and cultured in the presence of cells containing UV-inactivated RSV. Proliferation was assessed by tritiated thymidine incorporation. Supernatant cytokine levels were measured using ELISA. Significantly higher proliferative response rates were demonstrated to UV-inactivated RSV in those infants exposed *in utero* to the RSV epidemic after 22 weeks gestation (P=0.035). UV-inactivated RSV stimulation induced significantly higher interferon-γ production from specimens with a positive proliferative response (primed) than from those with a negative response (not primed, P= 0.039).

Subsequently, 88 babies with at least one atopic, asthmatic parent were prospectively studied through their first winter. Nasal lavage (NL) specimens were collected with each Upper Respiratory Tract Infection (URTI) and analysed for eight respiratory pathogens using Reverse Transcription Polymerase Chain Reaction (RT-PCR). Pathogens were detected in 83% of episodes with dual pathogen infections identified in 18%. Picornaviruses were the most frequent pathogen (44% of episodes) followed by RSV (26%) and the parainfluenza viruses (13%). 28 infants had an URTI where RSV was detected and 9 developed signs of acute bronchiolitis (AB). NL specimens were obtained from all 28 infants at days 1-2 and 5- 7 of the URTI. NL specimens were assayed for interferon-γ (IFN-γ), interleukin (IL-) 4, IL-10 and IL-12 and the RSV load determined by quantitative PCR. mRNA was extracted from stimulated peripheral blood mononuclear cells collected on days 5-7 and IFN-γ, IL-4, IL-12 and IL-18 mRNA levels determined by PCR. Cytokine profiles were analysed in relation to clinical outcome. The IL-4/IFN-γ ratio for infants with AB was elevated in nasal lavage fluid on both days 1-2 (p=0.014) and days 5-7 (p=0.001) of the illness compared with infants with URTI alone. Those with AB demonstrated a higher IL-10/IL-12 ratio (p=0.0015) on days 1-2. IL-18 mRNA levels were reduced (p=0.019) and the IL-4/IFN-γ ratio elevated (p=0.01) in stimulated PBMCs from infants with AB. There was no difference in initial RSV load.

It is concluded that: (a) immune priming to RSV can occur antenatally if the mother is exposed at the appropriate stage of gestation and that this exposure is associated with a type 1 response, and (b) RSV bronchiolitis is associated with a marked imbalance in type 1/type 2 cytokines in favour of a type 2 response. These data combined suggest that priming of foetal T cells to RSV may result in a reduced severity of subsequent RSV disease by augmenting the infant's own type 1 specific cellular immune response to RSV. This may explain much of the clinical diversity of RSV disease and raises the possibility that RSV infection/immunisation of mothers after 22 weeks gestation might represent a new strategy for prevention of RSV bronchiolitis.

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Associated Publications

Legg JP, Jones CA, Warner JA, Johnston SL, Warner JO

A hypothesis: Antenatal sensitisation to respiratory syncytial virus in viral bronchiolitis.

Archives of Disease in Childhood. 2002; **86**(6): 431-433

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RSV Infection - Determinants of Disease Severity

Clinical Pulmonary Medicine. 2002; **9**(5): 256-263

Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO

Type 1 and Type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis

American Journal of Respiratory and Critical Care Medicine 2003; **168**(6): 633-639

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Abbreviations

AB	Acute Bronchiolitis
ANCOVA	Analysis of Covariance
AOM	Acute Otitis Media
APC	Antigen Presenting Cell
CBL	Cord Blood Lymphocytes
CD40-L	CD40 Ligand
cDNA	Copy Deoxyribonucleic acid
CBMC	Cord Blood Mononuclear Cells
CCD	Charged Coupled Device
CLD	Chronic Lung Disease
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic acid
Dpm	Disintegrations per minute
E.Coli	Escherichia Coli
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
FAM	6-carboxy-fluoroscein
FEF_{25-75}	Expiratory Flow at 25%-75% of maximal lung volume
FEV_1	Forced Expiratory Volume in the 1st second of exhalation
FI-RSV	Formalin Inactivated-Respiratory Syncytial Virus
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
gmr	Geometric mean ratio
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
IFN-	Interferon-
Ig	Immunoglobulin
IL-	Interleukin-
LB-	Luria Burtani-
LPS	Lipopolysaccharide
LRT	Lower Respiratory Tract
LRTI	Lower Respiratory Tract Infection

LTF	Lymphocyte Transformation Activity
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Chemotactic Protein-1α
MCP-1	Monocyte Chemotactic Protein-1
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
NK Cells	Natural Killer Cells
NREVSS	National Respiratory and Enteric Virus Surveillance System
NW	Nasal Wash
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemaglutinin
PIV-	Parainfluenza virus
RANTES	Regulated upon Activation, Normal T-cell Expressed and presumably Secreted
RAST	Radio Allergosorbent Test
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rVV	Recombinant Vaccinia Virus
SP-	Surfactant Protein-
SPT	Skin Prick Test
TAMRA	6-carboxy-tetramethyl-rodamine
$TCID_{50}$	50% tissue culture infective dose
Th	T Helper
TNF-α	Tumour Necrosis Factor-α
TT-Ag	Tetanus Antigen
UHQ-	Ultra High Quality-
UNG	Uracil-N-glycosylase
URTI	Upper Respiratory Tract Infection
UV-	Ultraviolet-
V_{max}FRC	Maximal flow with reference to functional residual capacity

Chapter 1

Introduction

Respiratory Infections in Infancy

Epidemiology

Acute infections of the respiratory tract are the most common illness of childhood accounting for approximately 50% of all illnesses in children under the age of 5 years and approximately 75% of illnesses in young infants (Dingle 1964). The annual incidence of respiratory infection reduces with increasing age. Adults usually have four to five respiratory infections per year with a similar rate observed in children over the age of 8. Children between the ages of 1 and 6 contract an average of seven to nine such infections per year, with three of these episodes associated with constitutional disturbance (Dingle 1964). During infancy, the rate of infection is highest in the second 6 months of life with up to 10 respiratory infections per child year compared to 8 respiratory infections per child year in the first six months (Loda 1972) (Figure 1.1).

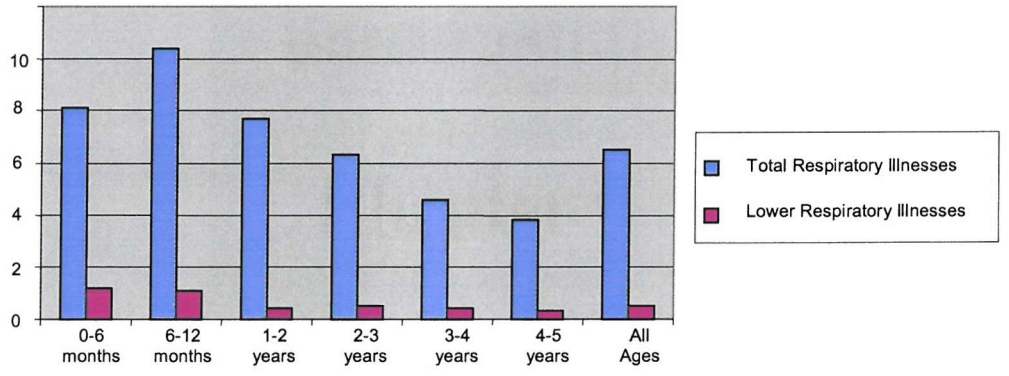

Figure 1.1 Frequency of Respiratory Illnesses by Age in Day Care, Chapel Hill

The vast majority of respiratory infections involve the upper respiratory tract alone. Less than 10% of respiratory infections involve the lower respiratory tract although this proportion is higher in infancy, with a reported incidence of 240 lower respiratory tract infections (LRTI) per 1000 infants per year (Glezen 1973). This rate falls dramatically after the first year of life with an incidence of less than 50 LRTIs per 1000 children per year over the next 4 years (Loda 1972).

Classification of Acute Respiratory Infections

Acute respiratory infections present with a wide variety of clinical features and it has proved helpful to classify these into several clinical syndromes. Such classification enables a clearer understanding of the underlying pathogenic mechanisms and helps to identify the likely causative agents since many have been associated with particular combinations of clinical features. A useful division is to separate the respiratory tract into upper (above the epiglottis) and lower (below the epiglottis) tracts. Table 1.1 summarises the recognised clinical syndromes of acute respiratory infection and their reported association with respiratory pathogens.

	Clinical Syndrome	Rhino-virus	RSV	Corona-virus	Para-influenza	Influenza	Adeno-virus	Entero-virus
Upper Respiratory Tract Infection	Common	+++++	+++	++++	++	+++	+	++
	Acute Pharyngitis & Tonsillitis	+	+	+++	++	++	+++++	+++
	Acute Otitis Media	+++	+++	++	+	+	++	+
	Acute Paranasal Sinusitis	++			+	+	+	
Lower Respiratory Tract Infection	Epiglottitis / Supraglottitis		+		+			
	Croup	+	++	+	+++++	+++	++	+
	Acute Bronchitis	++++	++	++	+++	++++	+	+
	Non-Bacterial Pneumonia	+	++++	+	++	+++++	++	+
	Bronchiolitis	+	+++		+++	+		+

Table 1 Reported associations of respiratory viruses with respiratory clinical syndromes

Upper Respiratory Tract Infections (URTI)

The Common Cold

The vast majority of upper respiratory tract infections present as the so-called *common-cold syndrome*. Throat irritation, sneezing and nasal stuffiness are the primary complaints on the first and second day of illness; rhinitis, watering eyes and sometimes hoarseness and cough follow on the second to fourth days of illness. Typically the illness lasts for 6 to 7 days with cough and nasal discharge being the most persistent symptoms. Nasal symptoms tend to be more prominent and throat and systemic symptoms less prominent in upper respiratory tract illness caused by rhinovirus compared to that caused by other viruses.

In infancy, the above symptoms are frequently associated with fever, irritability and restlessness. Nasal obstruction often leads to disturbed sleeping and feeding in this age group.

The common cold can be complicated by extension of infection to include the lower respiratory tract and an increased local susceptibility to bacterial infection may result in subsequent sinusitis or otitis media. Furthermore, 80-85% of asthma exacerbations in children in the community are associated with upper respiratory tract infection due to the viruses primarily responsible for the common cold (Johnston 1995).

The common cold is now recognised to be associated with more than 200 viruses, occasional bacteria, protozoa and mycoplasma. Table 1.2 represents the most common aetiologic agents and their relative prevalence in adults.

Virus	Incidence of Isolation (%age) (Using Antigen Detection, Culture, PCR and Serology)
Rhinovirus	52.5 %
Influenza A Virus	5 %
Influenza B Virus	1 %
Adenovirus	1 %
Parainfluenza virus type 1	0.5 %
Parainfluenza virus type 2	1 %
Parainfluenza virus type 3	1.5 %
RSV	2 %
Enterovirus	0.5 %
Coronavirus OC43	3.5 %
Coronavirus 229E	5 %
Mycoplasma Pneumoniae	0.5 %
Chlamydia Pneumoniae	2 %
Other Bacteria	1.5 %

Table 1.2 Isolation rates of viruses and bacteria from patients with the common cold

Both host and environmental factors play a considerable part in determining the likely cause of the common cold. For example, disease due to respiratory syncytial virus (RSV) is most common and most severe in children less than 1 year of age and causes disease almost exclusively during the winter months.

Acute Pharyngitis and Tonsillitis

Pharyngitis, by definition, is inflammation of the mucous membranes and underlying structures of the throat whilst tonsillitis refers to inflammation of the palatine tonsils. Pharyngitis can be usefully divided according to whether it is accompanied by nasal symptoms.

The main virus associated with nasopharyngitis is adenovirus; influenza and parainfluenza being the other major viral agents. RSV and rhinovirus are infrequently associated with pharyngeal disease.

Pharyngitis (including tonsillitis and tonsillopharyngitis) without nasal symptoms has a far broader range of possible aetiological agents. The most commonly identified agents include group A β-haemolytic streptococcus, adenovirus, influenza viruses A and B, parainfluenza viruses, Epstein-Barr virus, enteroviruses and M*ycoplasma pneumoniae.*

Pharyngitis typically presents with a sore throat. Variable degrees of fever and other constitutional symptoms are often seen depending on the causative agent, environmental factors and host factors such as age and immune status. Children may often complain of headache, nausea, vomiting and, occasionally, abdominal pain. Younger children and infants often suffer with dysphagia and poor feeding. Physical examination reveals pharyngeal erythema and tonsillar enlargement together with varying degrees of cervical lymphadenopathy. Recent evidence suggests that clinical findings are limited in establishing the likely aetiological agent (Schwartz 1998).

Acute Otitis Media

Acute otitis media (AOM) refers to suppurative middle ear infection of relatively sudden clinical onset.

Bacteria may be isolated from the middle ear fluid in over 65% of patients with AOM. The primary pathogens are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Branhamella catarrhalis* and group A S*treptococcus*. Studies in recent years have highlighted the important role that respiratory viruses play in the pathogenesis and clinical features of AOM. Using a combination of viral culture, antigen detection and serologic tests, Heikkinen *et al* found evidence of viral infection in 41 percent of 456 children with AOM (Heikkinen 1999c). 81% of the infections were caused by five viruses (RSV, parainfluenza, influenza, enteroviruses and adenoviruses). Of those middle ear fluid specimens analysed for bacteria 43 (65%) of 66 had a combined bacterial and viral infection. Whilst this data suggests that respiratory viruses primarily act as antecedents to bacterial infection in otitis media, the finding of viruses as the only detectable microorganism in certain cases may point to viruses playing a more active part in both the pathogenesis and clinical manifestations of AOM.

AOM typically presents with rapid onset of fever, severe ear pain and hearing loss in a child with an URTI. However, these classical symptoms are rarely observed in the infant

and younger child where non-specific symptoms such as irritability and poor feeding are more frequently seen. Examination reveals a hyperaemic, opaque tympanic membrane with distorted or absent light reflex and indistinct landmarks. Occasionally there may be evidence of tympanic membrane perforation with purulent otorrhoea.

Infants and young children are at highest risk for development of otitis media with peak prevalence between 6- and 24- months of age (Schappert 1992). At 1 year of age approximately 75% of children will have experienced at least one episode of AOM with this rate increasing to over 90% by 2 years (Paradise 1997). Risk factors for otitis media include low socio-economic status, exposure to tobacco smoke, low birth weight and exposure to other children either at home or in day care (Paradise 1997).

Acute Paranasal Sinusitis

At birth there are 3 pairs of paranasal sinuses- the ethmoid, maxillary and sphenoid sinuses. The frontal sinuses begin to develop from the anterior ethmoids by 1-2 years of age but are not seen radiographically until 5-6 years of age. Acute viral infections commonly spread to the paranasal sinuses causing an increase in nasal secretions with headache and pain localised in the area of the sinus. Viral infection causes mucosal swelling and impairs normal mucociliary transport leading to ostial obstruction and stagnation of secretions within the sinuses. The trapped secretions become an ideal culture medium for bacteria leading to acute bacterial sinusitis. Bacteriological studies of sinus secretions have demonstrated a similar group of responsible pathogens to those seen in otitis media.

The clinical presentation of acute sinusitis in childhood is inconstant, varying considerably with age. Most commonly, the signs and symptoms of a cold become persistent with ongoing cough and nasal discharge beyond 7-10 days. In this form of presentation, fever, facial swelling and headache are uncommon symptoms. A less common presentation of sinusitis is the child with severe cold symptoms, high fever, purulent nasal discharge and often periorbital swelling or facial pain.

The exact incidence of sinusitis in infancy is difficult to establish. By defining sinusitis as an URTI lasting more than 2 standard deviations over the mean duration, Wald *et al* estimated that between 8 and 11% of all URTIs were complicated by sinusitis in the first year of life (Wald 1991). However, the symptoms of acute sinusitis in infancy have not

been well documented and accurate diagnosis is difficult in the absence of a direct, non-invasive measure of sinus infection.

Lower Respiratory Tract Infections

Epiglottitis / Supraglottitis

Supraglottitis refers to inflammation of the structures above the glottis including the epiglottis, aryepiglottic folds and arytenoids. The most common site of involvement is the epiglottis.

Haemophilus influenzae type b causes over 90% of cases of epiglottitis with other bacteria such as *Staphylococcus aureus* and *Streptococcus pneumonia* being less frequently associated. Viruses appear to cause epiglottitis in only a small proportion of cases with reports implicating both RSV (Alligood 1989) and parainfluenza (Goldhagen 1989). Presentation is acute with high fever, inability to swallow and slow, laboured respiratory effort. Epiglottitis typically occurs in children between 2 and 7 years of age.

Croup

Croup is a syndrome of upper airway obstruction characterised by a barking cough, a hoarse voice and stridor. Croup can be divided according to the causative organism and the anatomical distribution of inflammation. Viral laryngitis, laryngotracheitis and laryngotracheobronchitis represent degrees of viral respiratory tract infection which are most easily considered together and which together cause the vast majority of croup in childhood.

The vast majority of cases in childhood are caused by parainfluenza types 1 and 2 (Henrickson 1994) but respiratory syncytial virus, influenza A and B, adenoviruses, enteroviruses (particularly in summertime) and *Mycoplasma pneumoniae* may also cause croup. Following transmission of the virus to the nasopharynx by either direct droplet spread or hand-mucosa inoculation, virus replication occurs producing nasal symptoms. The infection spreads locally to involve the larynx and trachea. Cellular infiltration of the lamina propria, submucosa and adventitia in these areas produces mucosal oedema and consequent airway obstruction primarily in the subglottic area.

Clinically the child develops signs and symptoms of a common cold with coryza, cough and sore throat. This is followed over the next 24-48 hours by a harsh, "barking" cough with varying degrees of stridor, which is accentuated when the child is disturbed. Signs of

more severe obstruction include tachypnoea, subcostal and intercostal recession and cyanosis.

Croup in the first year of life generally runs a more severe clinical course than in later years. It is relatively less common in infancy than in the second year of life. In one large study of LRTIs during the first year of life, croup accounted for 23% of all LRTIs with an incidence of 7.5 per 100 children (Wright 1989).

Acute Bronchitis

The term acute bronchitis refers to a febrile illness with cough and usually wheeze on auscultation. As a clinical entity, it has been beleaguered by inexact definition, multiple synonyms and, consequently, lack of research.

Acute bronchitis accounts for between 10-40% of acute lower respiratory infections in childhood. Its peak incidence is during the second year of life. By the age of 5 years, more than 20% of all children will have had at least one episode of acute bronchitis. In pre-school children RSV and parainfluenza are the predominant pathogens. In older children and adults influenza A and B viruses, adenovirus and *M. pneumoniae* are more common.

The child typically presents with fever, dry cough and upper respiratory congestion following a prodromal period of 24 to 48 hours characterised by fever and rhinitis. The cough becomes increasingly productive and may be accompanied by gagging and vomiting leading to poor oral intake and dehydration. There is a relatively slow recovery phase of 1 to 2 weeks characterised by a persistent cough. Examination reveals tachypnoea with diffuse wheezes and occasionally crackles on auscultation.

As a diagnosis, acute bronchitis has considerable overlap with both asthma and bronchiolitis. A sizeable proportion of children presenting with symptoms and signs of acute bronchitis will subsequently be diagnosed as asthmatic. Whether this therefore represents the first episode of asthma is debatable. Historically, the term wheezy bronchitis was used to describe children with recurrent episodes of presumed bronchitis. However, it is now recognised that children diagnosed with wheezy bronchitis are clinically indistinct from those with asthma and respond to anti-asthma drugs (Speight 1983, Williams 1969). Thus, children with wheezy bronchitis appear to be part of the asthma phenotype and do not represent a distinct clinical entity.

The differentiation of acute bronchitis from bronchiolitis is often arbitrary since, in strictly pathological terms, there may well be inflammation of the bronchioles as well as the small bronchi during acute bronchitis. This can lead to confusion and has convinced many of the

need for tighter, more clearly defined diagnostic guidelines (see below). In practical terms this differentiation may be of little consequence since management of both conditions is largely similar. However, this is not the case with regard to research studies of drug therapy and longitudinal outcomes since their relevance to the general population relies heavily on clear diagnostic inclusion criteria.

The diagnosis of acute bronchitis in infancy is difficult in the light of the above discussion. Many would contend that bronchiolitis is the diagnostic term of choice in infants presenting with cough and wheeze.

Pneumonia

By definition, pneumonia refers to infection of the alveoli and surrounding lung interstitium that may involve one or more lobes or segments.

In childhood, viruses and *Mycoplasma* are a far more common cause of pneumonia than bacteria. Age has a significant influence on the incidence of infection with the various pathogens. In the first 3 years of life most pneumonias are viral in origin (Ray 1993, Denny 1986). RSV is the most common pathogen particularly in those pneumonias associated with bronchiolitis. Parainfluenza viruses (particularly types 1 and 3) and adenoviruses are less frequent aetiological agents. Whilst infection with *M. pneumoniae* is frequent in this age group it usually takes the form of a mild infection without pneumonia (Anon. 1991). In the older child viruses are less frequent causes of pneumonia with *M.pneumoniae* and pneumococcus becoming progressively more important.

Acute, nonbacterial pneumonia in the infant or younger child generally follows 1 or 2 days of coryza, decreased appetite and low-grade fever. This is followed by gradually increasing fretfulness, respiratory congestion, vomiting, cough and fever. Examination reveals signs of respiratory distress with tachypnoea, nasal flaring, chest recession and use of accessory muscles (sometimes represented by head nodding in the infant). Chest examination may reveal scattered crackles but the classical findings of bronchial breathing and dull percussion are infrequently present. The younger the child the less likely it is to find specific signs of pneumonia (Singhi 1994) and occasionally there may be no auscultatory findings. Differentiating between the various pathogens is often difficult purely on clinical grounds. *Mycoplasma* infection has an insidious onset with dry cough and marked constitutional upset and fever whereas pneumococcal infection has a very rapid onset. Viral pneumonias are often associated with wheeze on auscultation although the specificity of this sign has been called into question (Isaacs 1989a).

Bronchiolitis

Acute bronchiolitis is the most common lower-respiratory tract infection of infancy. The clinical history typically consists of a 2 to 5 day period of coryza and nasal congestion followed by signs and symptoms of lower respiratory tract infection. Typical features include tachypnoea, dyspnoea and subcostal / intercostal retractions with wheeze and crackles on chest auscultation. Bronchiolitis is usually self-limiting lasting between 4 to 7 days. However, severe cases may require intravenous therapy for poor feeding and intensive care support for respiratory failure. Approximately 10-20% of all children develop bronchiolitis in the first year of life with 1-2% requiring hospitalisation as a consequence (Phelan 1994). Certain groups of children are at risk of severe bronchiolitis and have higher rates of hospitalisation and need for intensive care. These include infants with underlying cardiac or pulmonary disease or immunosuppression and those infants who are born prematurely (Wang 1995).

Bronchiolitis has been associated with a wide range of pathogens including parainfluenza virus, influenza viruses, adenovirus and certain picornaviruses (Phelan 1994). However, one virus accounts for the vast majority of bronchiolitis cases, being isolated in over 80% of cases during annual epidemics - the respiratory syncytial virus.

Respiratory Syncytial Virus

Respiratory syncytial virus is a medium sized, enveloped, single stranded RNA virus. It is a member of the pneumovirus subfamily of the *Paramyxoviridae* family which includes the parainfluenza viruses and the causative agents of measles and mumps. It was first isolated in 1956 from a chimpanzee with coryza (Morris 1956) and was subsequently recovered from 2 children in Baltimore, one with the clinical diagnosis of pneumonia and the other with croup (Chanock 1957). It was a further four years before RSV was associated with bronchiolitis (McClelland 1961).

RSV is now recognised as a ubiquitous pathogen in all age groups resulting in significant morbidity and healthcare cost worldwide. In the USA alone it has been estimated to result in over 54000 hospitalisations and 2700 deaths annually in children less than one year old (Institute of Medicine 1985).

Structure

The RSV virion consists of a central nucleocapsid surrounded by a lipid envelope. When visualised by electron microscopy the virions are irregular in both shape and size (120-300 nm) although, despite this, each has been shown to contain a single copy of the genome.

The nucleocapsid takes the form of a symmetrical helix with a diameter of 12 to 15 nm. The lipid envelope is derived from the host cell and bears the transmembrane surface glycoproteins that give RSV its characteristically "spikey" appearance on electron microscopy (Figure 1.2).

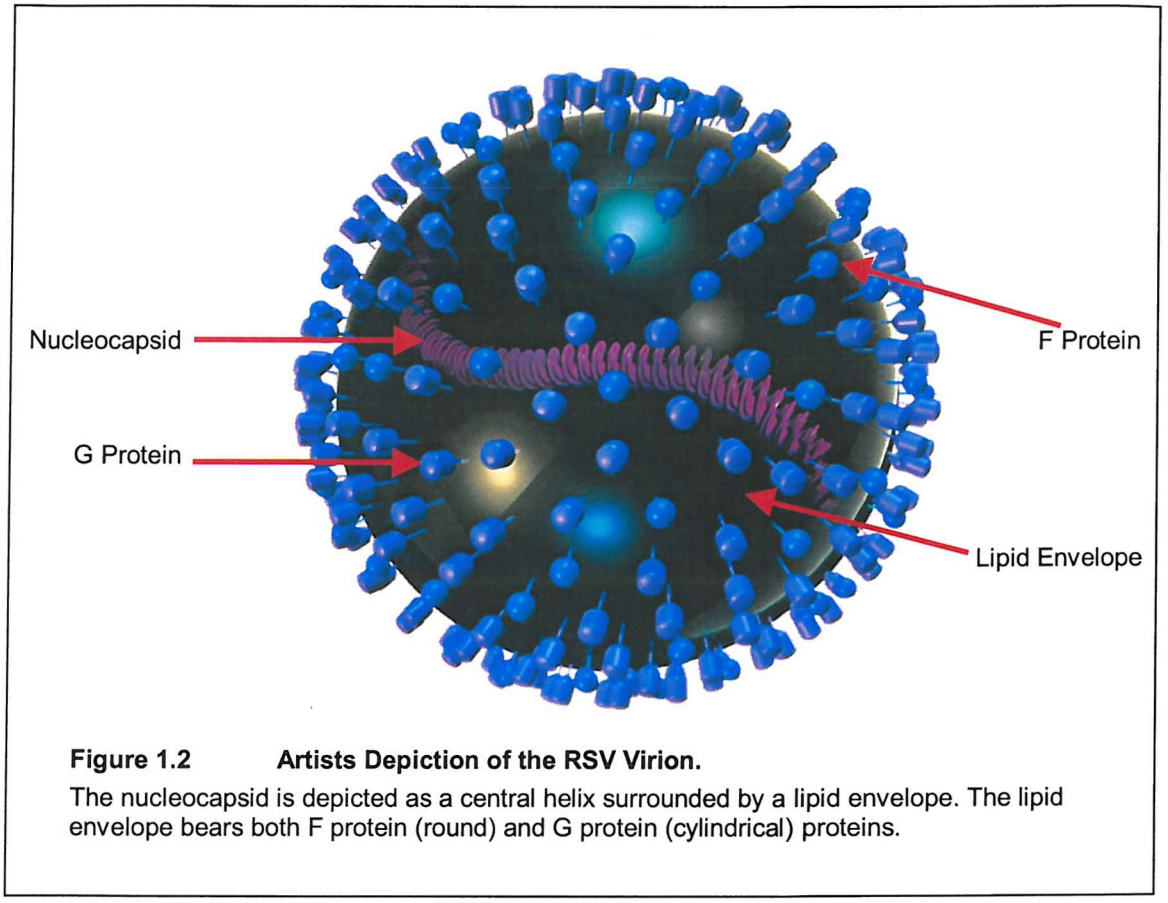

Figure 1.2 Artists Depiction of the RSV Virion.
The nucleocapsid is depicted as a central helix surrounded by a lipid envelope. The lipid envelope bears both F protein (round) and G protein (cylindrical) proteins.

Genome

RSV has a negative polarity RNA genome composed of 15,000 nucleotides with an estimated weight of 500 kilodaltons (kDa). The genome has now been sequenced in its entirety and encodes for 10 separate genes with 2 overlapping open reading frames (Figure 1.3).

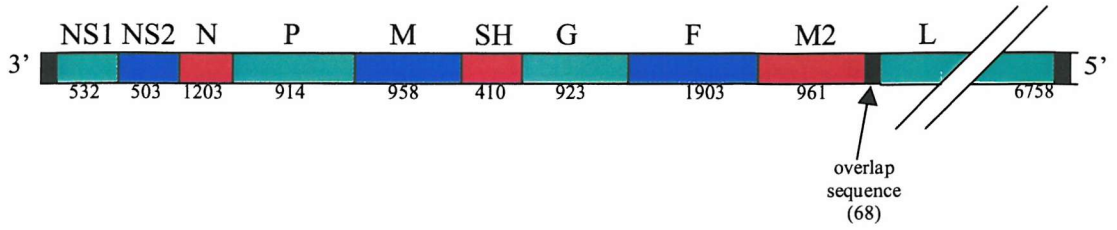

Figure 1.3 The RSV Genome.

Gene sequences for the 10 RSV proteins are depicted. Numbers beneath each gene represent the nucleotide length. The overlap between M2 and L nucleotide sequences is identified.

The gene sequences NS1 (532 nucleotides) and NS2 (503 nucleotides) encode for so-called nonstructural proteins whose function is unknown. The nucleocapsid gene, N (1,203 nucleotides), codes for the major nucleocapsid protein which forms the nucleocapsid core of RSV together with the major viral phosphoprotein, encoded by the P gene (914 nucleotides) and the protein product of the polymerase (L) gene (6578 nucleotides). Two matrix proteins are encoded in the genome. The first matrix protein M (958 nucleotides) is associated with the host cell membrane and is involved in targeting viral proteins and nucleocapsids to the site of viral budding. The second matrix protein, M2 (961 nucleotides) appears to be localised to the nucleocapsid although its exact function is unclear.

The RSV genome also codes for three surface proteins: the fusion or F protein (1903 nucleotides), the glyco- or G protein (923 nucleotides) and the SH protein (410 nucleotides).

Fusion F Protein

The F, or fusion protein, has a coding sequence of 1903 nucleotides which codes for a protein of 574 amino acids in length, having a molecular weight of 63kDa. The F protein mediates membrane fusion, which is responsible for viral penetration and syncytium formation (Collins 1996). The F protein is synthesised as a single strand, F0, which is cleaved intracellularly by a trypsin-like protease into the two subunits F1 and F2 that remain linked by disulfide bridges (Collins 1984). The F0 protein also undergoes post-

transcriptional glycosylation, though to a much lesser extent than the G protein. The F1 subunit is hydrophobic, while the smaller F2 sub-unit is hydrophilic.

Attachment G Protein

The gene coding for the G protein is 923 nucleotides long giving a relatively small protein of 298 amino acids. It is anchored in the membrane by a signal/anchor sequence near the N-terminus such that the large C-terminal domain is extracellular. It is heavily glycosylated containing several *N*-linked carbohydrate side chains and approximately 24 or 25 *O* –linked chains (the ectodomain contains more than 70 acceptor sites). Glycosylation occurs in the host Golgi compartment. Since the carbohydrate side-chains are derived from the host, it is possible that they might conceal the underlying virus-specific polypeptide chain from the host's immune system. Approximately 15% of the G protein formed is soluble and formed without the anchor domain so that it is slightly smaller at 82kD (Hendricks 1987). Significant deletions in the anchor domain appear to block glycosylation of the G protein suggesting that the anchor domain is important for association with the Golgi apparatus.

Antibodies to the G protein (but not those to the F protein) interfere with the adherence of RSV to the cell surface (Levine 1987). This observation has confirmed the importance of the G protein in the initial virus-host cell interaction and attachment. The G protein is not involved in syncytia formation, however, as antibodies against the G protein do not disrupt syncytia formation during RSV infection.

Small Hydrophobic SH Protein

The SH protein is a short integral membrane protein whose precise role is yet to be determined. Its location within the membrane suggests it's possible involvement in attachment or penetration and a recent study has demonstrated its ability to augment membrane fusion formation when co expressed with both the F and G protein (Heminway 1994).

Antigenic Subgroups

Through the application of monoclonal antibody technology, two major antigenic groups of RSV have been identified – the A and B subgroups. The majority of the antigenic differences between the two subgroups are attributable to variation in the G glycoprotein.

This has been quantified by analysis of post-infection sera from infants using enzyme-linked immunoassay with purified F and G protein. The F proteins were shown to be 50% related between the two subgroups whilst the G proteins were only 1-7% related (Hendry 1988).

Genetic analysis has also confirmed the existence of two distinct RSV subgroups. Nucleotide sequencing of the G protein region of the genome has demonstrated extensive differences between viruses from the two subgroups. Moreover, nucleotide sequences are very similar between viral strains of the same subgroup (Johnson 1987).

Antigenic and genetic variation also exists within each subgroup although to a lesser extent. The G protein has been demonstrated as the greatest source of intragroup variability with up to 12% amino acid difference noted in one study (Sullender 1991).

The single stranded, non-segmented nature of the RSV genome precludes the genetic rearrangements that typify the dramatic antigenic shifts of the influenza virus (Scholtissek 1995). However, there is considerable potential for genomic mutation given the inability of RNA polymerase to proof-read during replication of the genome. This provides an opportunity for antigenic drift to occur under the influence of selective pressures from the environment. The current variability of the G protein between RSV strains may therefore be explained by the progressive accumulation of change together with survival and extinction of particular genotypes (Cane 1995).

Epidemiological studies have revealed that the two major antigenic groups have existed for over 20 years and have a worldwide distribution (Anderson 1985). Both groups appear to circulate concurrently with geographical and temporal clustering frequently reported (Tsutsumi 1989, Hendry 1989, Storch 1987, Morgan 1987, Hendry 1986). Genetic studies have assessed strain variability within the two major groups primarily through analysis of the G protein gene (Peret 1998, Cane 1995, Cane 1994, Sanz 1994, Garcia 1994). Subsequent phylogenetic analyses have identified multiple lineages within both group A and group B viruses with marked similarities observed between strains from different locations and time periods (Coggins 1998, Peret 1998). Several distinct RSV strains appear to cocirculate within an individual community during each annual epidemic with the predominant strain varying year to year (Seki 2001, Coggins 1998, Peret 1998).

Virus Biology

RSV initiates infection by binding to a cellular receptor that remains to be identified. The G glycoprotein has been shown to be essential for the entry of RSV into the host cell. Following penetration the viral envelope fuses with the host plasma membrane as

demonstrated by immunofluorescent staining of viral surface proteins (Routledge 1987). Subsequent genome expression and replication are entirely cytoplasmic without nuclear involvement. The incorporation of RNA polymerase with the nucleocapsid enables transcription to occur even in enucleated cells.

Transcription occurs rapidly in the 3' to 5' order from a single promoter near the 3' end. Under the influence of viral polymerase, viral mRNA is produced sequentially guided by gene-start and gene-stop signals. The resultant series of subgenomic mRNAs are first detected in the cytoplasm within 4 to 6 hours of infection. RSV protein synthesis rapidly follows mRNA production. Peak RSV mRNA production occurs 16 hours post-infection and peak protein production occurs between 18 and 20 hours following infection of the host cell. RSV proteins are produced in quantities according to their relative position within the genome. The distance of the gene from the 3' end of the genome is inversely proportional to the total protein translated, a process assumed to be due to diminishing polymerase activity during sequential transcription.

In addition to subgenomic mRNA production, RNA replication occurs with the production of a complete positive-sense RNA complement of the genome called the antigenome. This positive-strand copy is produced in quantities 10- to 20- fold less than the original negative-strand genome. The antigenome in turn acts as a template for synthesis of progeny genomes.

The replicated RNA gathers with nucleocapsid and polymerase proteins at the plasma membrane in areas of coalesced viral envelope proteins. These areas are visible in infected cells as diffuse threadlike aggregates below the plasma membrane. Filamentous viral processes bud from the cytoplasmic membrane protruding to a final length of 10μm. Filaments extend at a rate of 110 to 250 nm/s and shedding of the complete virus takes less than 1 minute. Up to 90% of the assembled virions do not leave the cell surface due to faulty packaging and remain associated with the host cell.

Epidemiology

RSV infection causes significant morbidity and mortality in both young infants and the elderly. Rates of RSV infection vary considerably through the year with epidemics occurring on a seasonal basis throughout the world. In temperate climates outbreaks occur yearly during wintertime whilst peak rates of infection arise during the rainy season in tropical climates. In the United States, RSV activity is monitored by the National Respiratory and Enteric Virus Surveillance System (NREVSS), a voluntary, laboratory-

based system. RSV activity is considered widespread by NREVSS when 1) >50% of participating laboratories report one or more RSV detections for at least 2 consecutive weeks, and 2) >10% of all specimens tested for RSV during a surveillance week are positive. Over recent years, RSV has been widespread between early November and late March (Figure 1.4).

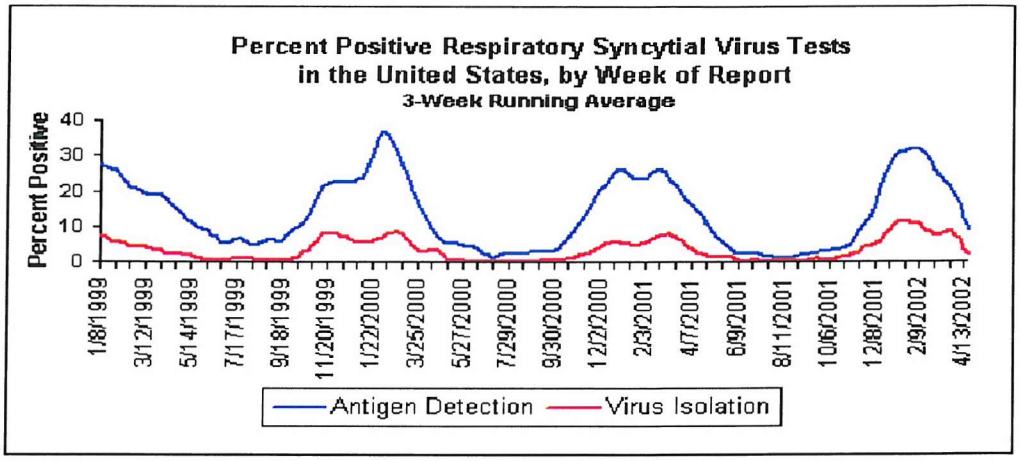

Figure 1.4 Percent Positive Respiratory Syncytial Tests in United States by Week
(Source: NREVSS website http://www.cdc.gov/ncidod/dvrd/nrevss as of May 2002)

Respiratory syncytial virus (RSV) is the most important viral pathogen that infects young children. Approximately two thirds of infants are infected during the first year of life with one third of those infected developing lower respiratory tract disease, 2.5% requiring hospitalisation, and a 0.1% mortality rate (Holberg 1991). Ukkonen *et al* identified antibodies to RSV in 87% of children by age 18 months and almost all children by age 3 years (Ukkonen 1984). Reinfection occurs frequently at all ages and previous infection does not prevent subsequent infections. Family studies performed in Houston (Texas, USA) demonstrated RSV infection in 69% of children monitored from birth during their first year with approximately half reinfected during their second year (Glezen 1986). Rates of reinfection are even higher in the day care setting with an attack rate for first infection of 98 per cent and reinfection rates in the second and third years of life of 74% and 65%, respectively (Henderson 1979).

In developed countries, most infants acquiring RSV are mildly affected and experience only symptoms of the common cold. However, approximately 33% of those infected develop signs and symptoms of lower respiratory tract disease. Certain groups of infants

have been shown to be more likely to develop lower respiratory tract disease including infants with predisposing conditions such as chronic lung disease (CLD), prematurity without CLD, congenital heart disease, cystic fibrosis and immunodeficiency. In addition, certain environmental factors appear to be associated with more severe disease - these include a crowded home, low socio-economic status, lack of breast feeding and exposure to tobacco smoke.

The healthcare costs of RSV infection are substantial with the economic burden having been predominantly evaluated in North America. In 1988, the Institute of Medicine concluded that RSV disease accounted for approximately 91,000 paediatric hospitalisations per annum with an estimated cost of $300 million per annum in the United States alone (Heilman 1990). A study of Canadian hospitals accounting for approximately 80% of available paediatric tertiary beds, estimated the annual cost of RSV-associated illness to these institutions as almost $18 million with the vast majority of direct expenditure attributable to inpatient care (Langley 1997). More recently, the overall annual cost of RSV disease was determined in the United States for the years 1993, 1994 and 1995 (Howard 2000). Using a cross-sectional analysis of the Healthcare Cost and Utilization Project National Inpatient database, hospital charges for RSV pneumonia–associated episodes were estimated at $295 million in 1993; $392 million in 1994; and $296 million in 1995.

The importance of RSV as a cause of respiratory infection in developing countries is far less clear. A recent article has reviewed all published data from developing and tropical countries (Weber 1998). RSV has been found in all climactic and geographical areas from which it has been sought. Earlier studies that relied on viral culture alone appear to have underestimated the impact of RSV probably due to the fragility of RSV. More recent studies have incorporated both culture and immunofluorescence and have demonstrated significant rates of RSV infection. In hospital-based studies, RSV has been shown to be the predominant viral cause of LRTI being identified in an average of 65% of cases (range 27-96%). The age distribution appears similar to the developed world, with the majority of cases identified in the first year of life. Very few studies have reported on mortality rates although the limited data that exists does not seem to indicate a dramatically higher rate than that reported for developed countries.

RSV appears to be spread primarily by large droplets or through fomite contamination as opposed to small-particle aerosols (Hall 1981b). Spread may occur through close contact with an infected individual or as the result of physical contact (usually the hand) with fomites from surfaces contaminated with infected respiratory secretions. Rubbing of the

eyes or nose then transfers RSV to the conjunctiva or nasal mucosa - areas known to be susceptible to RSV infection (Hall 1981b). RSV is highly contagious and can rapidly spread among family members. Older siblings, who are less likely to have severe disease, are the most likely to introduce the virus into the family with almost half of all family members and two thirds of infants being subsequently infected (Hall 1976b).

Nosocomial infection is a major problem, particularly during the yearly RSV outbreaks when large numbers of infected infants are inevitably admitted onto the same paediatric units as other vulnerable children. The rate of hospital-acquired infection during community outbreaks of RSV has been reported to range from 26-47% in newborn units and from 20-40% in paediatric inpatient units. The situation is exacerbated by the increasing numbers of those children most likely to develop severe disease when infected with RSV. Advances in immunosuppressive cancer therapy, cardiac surgery and neonatal intensive care medicine have seen a rapidly growing population of immunosuppressed oncology patients, children with congenital heart disease and premature infants. The consequences of this are highlighted by studies of RSV infection in high-risk populations. A large prospective Canadian study followed children hospitalised with RSV infection over a 4-year period. The mortality rate was found to be significantly increased in children with cardiac disease or chronic lung disease of prematurity when compared to the group as a whole (3.4%, 3.5% and 1% respectively). In addition those children with underlying cardiac or lung disease were shown to require longer hospital stays and need longer periods of oxygen supplementation (Navas 1992).

The contribution of hospital staff to the spread of nosocomial infection is significant. Rates of infection in healthcare workers exposed to large numbers of infants during outbreaks are extremely high, ranging from 30-60% (Agah 1987, Hall 1975b). Various strategies to reduce the risk of cross-infection have been examined. It is well acknowledged, that hand washing is the single most effective means of interrupting the spread of RSV in the health care setting (Murphy 1981). However, compliance appears to be a major obstacle to its success. Other interventions that have been shown to be efficacious include cohorting of infected infants and their carers (Snydman 1988) and the use of gloves and gowns by staff in close contact with infected patients (Leclair 1987).

Pathogenesis of RSV infection

The epithelium of the respiratory mucosa acts primarily as a protective barrier against harmful inhaled stimuli. RSV initially affects the respiratory epithelium at the point of entry (generally the nares), following viral attachment to epithelial cells through an

interaction between the G protein and cell surface molecules believed to be glycosaminoglycans (Martinez 2000). Rapid replication occurs within the epithelial cells with high viral titres detectable in the nasopharynx during early infection (Hall 1976a). The mechanism by which there is subsequent spread of infection to the lower respiratory tract is unclear. One possibility is that cell-to-cell spread occurs along the epithelium primarily as a consequence of virus mediated cellular fusion although this process has only been convincingly demonstrated *in vitro*. A second possibility is the migration of infected cells of the monocyte-macrophage lineage from the upper to lower respiratory tract. However, whilst such cells can be infected with RSV *in vitro* (Becker 1992) the necessary transfer from nose to lungs remains unproven.

Due to the low mortality rate and a consequent lack of tissue for study, most reports on the pathological changes seen in the lungs during human RSV bronchiolitis are over 30 years old. Acute RSV infection is associated with disruption or destruction of the epithelial layer and loss of ciliated epithelium in the bronchioles between 75 and 300μm in diameter. In severe cases there is widespread necrosis of the bronchiolar epithelium together with peribronchiolar mononuclear cell infiltrates, submucosal oedema and bronchorrhoea. Physiologically, these processes result in bronchiolar obstruction leading to areas of atelectasis and collapse together with areas of compensatory emphysema (Aherne 1970). In some infants, a pneumonic process ensues with mononuclear cell infiltration of the interalveolar walls and fluid infiltration of the alveolar spaces.

Milder cases of bronchiolitis are presumed to be characterised by similar, if less severe, pathological processes. In addition, other properties of RSV may be important such as its ability to induce rapid and near complete ciliostasis when placed in culture with respiratory epithelial cells (Tristram 1997).

It is not clear whether RSV spreads outside the respiratory tract during infection of the immunocompetent host. RSV has been identified in organs such as the kidney, heart and liver in cases of fatal infection in severely immunocompromised infants and adults (Milner 1985, Padman 1985). Whilst RSV has not been cultured from the blood of infected individuals, viral and transcribed RNA has been identified by RT-PCR in arterial blood from infants with RSV bronchiolitis (O'Donnell 1998). This finding would suggest ongoing viral replication within the blood and points strongly to the spread of RSV beyond the confines of the lungs during infection. Further studies are required to confirm this finding.

Clinical Features of RSV infection

Infection in Infants and Young Children

Upper Respiratory Tract Infection

The average incubation period for RSV infection is 5 days, with a reported range of between 2 and 8 days (Sterner 1966). Primary RSV infection leads to signs and symptoms of an upper respiratory tract infection alone in the majority of infants. Asymptomatic infection appears to be rare. After the initial incubation period typical symptoms of rhinorrhoea with rhinitis develop. There is subsequent progression over the ensuing days to nasal obstruction, pharyngitis and low-grade fever together with systemic symptoms such as poor feeding and irritability. In cases not progressing to LRT involvement there is a gradual resolution of symptoms with complete recovery often taking up to 14 days from the time of peak symptoms.

Lower Respiratory Tract Infection

The first symptom of LRT involvement is often a worsening cough that develops insidiously after several days of upper respiratory symptoms. As the disease progresses, tachypnoea and dyspnoea and signs of respiratory distress develop manifested by intercostal and subcostal retraction together with cyanosis in severe cases. Auscultatory findings include diffuse inspiratory and expiratory crackles and polyphonic wheeze. Upper respiratory symptoms often remain with profuse rhinorrhoea, intermittent fever and otitis media.

There is very poor correlation between clinical findings and disease severity. Mulholland *et al* found that only cyanosis and crackles were closely related to disease severity with other clinical features such as chest wall recession, tachypnoea and tachycardia correlating poorly (Mulholland 1990). A further confounding factor is the large degree of interobserver variability when assessing most clinical features except possibly cyanosis (Mai 1995). Hypoxaemia is a consistent finding in hospitalised infants with one study finding average oxygen saturations of only 87% at the time of admission (Hall 1979). Furthermore, admission pulse oximetry appears to be the best predictor of subsequent progress in hospital (Mulholland 1990) and would, therefore, currently seem to be the best single tool for initial assessment of disease severity.

The characteristic clinical course of LRT disease is one of slow improvement over a period of 7 to 12 days (Hall 1979). Those infants that have required hospitalisation usually follow a similar course with an average hospital stay of 7 days (Wang 1995). A small proportion will require admission to intensive care with approximately 9% of all hospitalised infants requiring ventilatory support (Wang 1995). Despite clinical improvement, significant hypoxaemia has been shown to persist at the time of discharge with return to normal several weeks later (Hall 1979).

The percentage of infected infants that progress to have symptoms and signs of LRT involvement varies considerably between studies. Much of this variation can be accounted for by the differences in patient demographics, diagnostic criteria and community characteristics. For example, a high incidence of LRT disease (80%) was found during an outbreak of RSV disease in a welfare institution in the District of Columbia, USA (Kapikian 1961). However, children up to the age of 50 months were included in the analyses and LRT disease was inexactly defined as inspiratory crepitations on auscultation together with cough, coryza, fever, listlessness or cough. By contrast, a LRT disease rate of 33% was identified in the Houston Family Study - a community-based longitudinal study of respiratory disease in childhood (Glezen 1986). In this study 125 infants were followed through their first year of life and specific diagnostic criteria were used to define LRT disease. However, clinical examinations were performed less frequently and RSV surveillance was less intensive than during the District of Columbia study. It seems likely, therefore, that the true percentage of LRT disease in RSV infected children lies somewhere between these rates.

Although the majority of children who develop LRT infection have only mild disease, symptoms are severe enough in approximately 2% of all infants to necessitate hospitalisation (Kim 1973).

Complications

Approximately 20% of hospitalised infants have apnoea as a predominant symptom. The apnoeas may occur before other respiratory symptoms have become evident. Those most at risk appear to be premature infants with a gestation of 32 weeks or less, those of young postnatal age and those with a history of apnoea of prematurity (Church 1984). Among these infants, approximately 30% ultimately require ventilation. The apnoeas are non-obstructive in nature and typically occur within the first few days of illness. Affected infants do not appear to be predisposed to further apnoeas later in life (Church 1984).

Concomitant otitis media has been increasingly recognised in children infected with RSV. Heikkinen *et al* found that middle ear fluid collected from children with an RSV respiratory tract infection and otitis media was positive for RSV in 74% of cases (Heikkinen 1999c). Other respiratory viruses were identified in the middle ear fluid less frequently. Furthermore, when virus was isolated from the middle ear fluid, co-infection with one or more bacteria was detected in 65% of cases. A combination of physical damage to respiratory cells as a result of viral infection and enhanced bacterial adherence to cells infected with virus has been proposed as an explanation for these findings (Hament 1999).

Except for otitis media, the incidence of secondary bacterial infection appears to be extremely low with a rate of 1.2% in one large study (Hall 1988). Interestingly, those children that received parenteral antibiotics as part of their treatment had a significantly higher rate of subsequent bacterial infection compared to those who received no antibiosis.

Another important and frequent complication is the substantial long-term respiratory morbidity following RSV infection. This will be discussed in detail below.

Infection in Older Children and Adults

Immunity following RSV infection is only effective for a matter of months before the individual is once again susceptible to reinfection (Hall 1991). Consequently, reinfection occurs throughout life. In normal children over three years of age, reinfection primarily causes upper respiratory symptoms and fever. In addition, otitis media with middle ear effusion is a common complication in this age group (Henderson 1982).

Reinfection in adults is generally mild, although fever, pharyngitis and cough are common sequelae (Hall 1975b). Pulmonary function testing following artificial infection of volunteer adults has demonstrated increased airway reactivity for up to 8 weeks after initial exposure (Hall 1978b). Recently, the true clinical burden of RSV infection in previously healthy adults has been established. Hall *et al* found that 7% of all adults acquired RSV infection during a 20-year study period (Breese 2001). Of these infections, 84% were symptomatic with upper respiratory tract symptoms only in 74% and lower respiratory tract involvement in 26%. RSV infection resulted in absence from work in 38% of RSV infections. At the same time, there has been an increasing recognition of the role of RSV in severe respiratory disease in certain adult groups.

Three adult populations seem particularly at risk of severe RSV infection:

(1) solid organ or bone marrow transplant recipients receiving immunosuppressive therapy (Wendt 1995).

(2) the elderly and particularly residents of long term care facilities (Falsey 1998).

(3) adults with chronic pulmonary disease (Glezen 2000)

Factors Influencing the Severity of RSV Disease

RSV infection is associated with a wide spectrum of disease severity in infancy. As detailed above, the majority of infants develop signs and symptoms of an upper respiratory tract infection alone. However, a significant proportion will develop lower airways disease with some requiring hospitalisation. The reasons for this diverse individual response to infection are debatable. The following factors have all been proposed as potential explanations:

Viral Factors

RSV Type and Genotype

The two major antigenic groups of RSV, groups A and B, have been shown to circulate concurrently during an RSV epidemic with the dominant group varying year to year (Hall 1990). Using various genetic techniques, including restriction endonuclease and nucleotide sequence analysis, it has been possible to assess strain variability within the two major groups. During the course of each RSV "season", several distinct strains from groups A and B have been shown to co-circulate (Peret 2000, Coggins 1998).

The link between the major antigenic groups of RSV and disease severity is unclear. Early studies found group A RSV infection to be associated with greater clinical severity as evidenced by an increased need for intensive care (Hall 1990) and mechanical ventilation (McConnochie 1990). More recently, this association has been called into question. Kneyber *et al* studied 232 children over 3 RSV epidemics and found no evidence of an association between disease severity and subtype (Kneyber 1996), whilst a recent Danish study has observed an increased illness severity scores in infants infected with group B RSV (Hornsleth 1998).

Three studies have attempted to determine whether particular RSV genotypes are associated with illness severity. Using restriction endonuclease analysis of G- and N-

protein gene cDNA, Fletcher *et al* studied the relationship between RSV genotype and response to RSV infection as classified on a 4-point severity score. Whereas the N-protein genotype had no apparent relation to severity score, there was a significant association between the SHL2 RSV G genotype and moderate to severe disease (Fletcher 1997). In a similar study, Hornsleth and colleagues identified 8 type A and 5 type B RSV genotypes co-circulating during three consecutive winter epidemics. Type B genotype B1122 was found to produce more severe disease than type A genotype A2311 (Hornsleth 1998). Two recent studies, however, have failed to identify an association between particular RSV genotypes and disease severity. Brandenburg *et al* identified 28 RSV group A isolates using G protein sequencing over three successive epidemics from 232 infants (Brandenburg 2000b). Clinical severity was evenly distributed over the different isolates. Similarly, Smyth *et al* found no association between N- and G-protein genotype and disease severity in a cohort of 276 infants hospitalised with RSV infection (Smyth 2002).

The possibility of different RSV genotypes having distinct infective properties is an attractive explanation for the diverse severity of RSV disease that merits further study. However, there is a great variation in the individual clinical response to the same genetic strain of RSV (Brandenburg 2000b, Hornsleth 1998) indicating the importance of host factors.

Host Factors

Predisposing Airway Geometry

As part of a much larger study of childhood wheezing, Martinez *et al* performed lung function on 125 infants before any lower respiratory tract illness had occurred (Martinez 1995). Using the "squeeze" technique, maximum expiratory flow at functional residual capacity was measured ($V_{max}FRC$). This measurement is believed to reflect the size of intrapulmonary airways. On subsequent follow up, those infants who developed transient early wheezing (at least one lower respiratory tract illness with wheezing during the first three years of life with no wheezing at six years of age) were found to have significantly lower $V_{max}FRC$. Similar findings were made in a cohort of 246 healthy infants in whom $V_{max}FRC$ was measured at 5 weeks of age (Young 1995). 17 (7%) of the infants developed doctor-diagnosed bronchiolitis in the first two years of life. Virology was only available on the two infants that required hospitalisation – both were RSV positive. $V_{max}FRC$ was reduced in those infants developing bronchiolitis.

Both studies therefore suggest that congenitally small airways may predispose infants to wheezing in early childhood. Since wheeze is regarded as one of the cardinal signs of

bronchiolitis, these findings may also relate to RSV bronchiolitis. However, neither study identified RSV infection virologically and further studies are necessary before the importance of pre-existing airway geometry in RSV disease can be fully established.

Atopic Predisposition

The close clinical similarity between RSV bronchiolitis and allergic asthma has led to the hypothesis that an atopic constitution may predispose an individual to bronchiolitis following RSV infection. This hypothesis, however, has proved to be a controversial one with evidence both for and against a link.

A recent Swiss study examined the families of 172 infants with a history of a respiratory illness associated with RSV infection (as diagnosed by immunofluorescence of nasopharyngeal secretions) (Trefny 2000). The clinical course of infection was analysed in relation to family history of atopy as established by questionnaire. Those infants with an atopic family history were found to have a significantly increased risk of bronchiolitis and a more protracted inpatient stay. On the other hand, Koh *et al* studied the parents of 122 children with a history of bronchiolitis and 120 control children with no such history (Koh 2000). Their atopic status was established using a combination of bronchial challenge, aeroallergen skin prick testing and total serum IgE. There was no difference in atopic status, as assessed by the prevalence of atopy (at least one positive response to the allergens tested) or by serum total IgE levels, between index and control parents. However, there was a higher incidence of bronchial hyperresponsiveness in the parents of children with a history of bronchiolitis than in control parents. Other smaller studies have also made contradictory findings (Wennergren 1992, Murray 1992, Carlsen 1987). Unfortunately, the studies reported to date all suffer with design limitations such as retrospective analysis (Trefny 2000), poor (or no) RSV detection techniques (Koh 2000, Wennergren 1992, Carlsen 1987) and limited evidence for atopy (Trefny 2000, Wennergren 1992). Therefore, in the absence of a truly definitive study, the influence of an atopic predisposition on clinical response to RSV infection remains unclear.

Host Immune Factors

Host immunity has a considerable influence on disease outcome in many infectious diseases. Recent interest has focussed on the role of the host immune response in the pathogenesis of bronchiolitis. This is discussed in detail below.

Immune Response to Infection

The immune response to infection can be divided into two arms:

(1) Innate Immunity – this involves host mechanisms that are present and ready to attack an infecting organism at any time. These mechanisms occur rapidly and do not rely on clonal expansion for their efficacy.

(2) Adaptive Immunity – this is required when the infection has eluded the innate defence system and generates a threshold dose of antigen. Adaptive immunity requires the clonal expansion of antigen-specific lymphocytes – a process taking several days to complete. This response produces a level of immunological memory that enables the immune system to respond more rapidly and effectively to pathogens that have been encountered previously.

The Innate Immune Response to Infection

Epithelium

The nasal epithelium is generally the primary point of contact between RSV and the host. In addition to its function as a physical barrier, the epithelium also affects various aspects of innate and adaptive immunity by releasing a variety of proinflammatory mediators and cytokines. A number of *in vitro* studies have now demonstrated the production of Interleukin- (IL-) 1, IL-6, IL-8, IL-11, Tumour Necrosis Factor-α (TNF-α), RANTES (Regulated upon Activation, Normal T-cell Expressed and presumably Secreted), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) (Jiang 1998, Olszewska-Pazdrak 1998a, Elias 1994, Arnold 1994) by infected human epithelial cells. Similar cytokines have been found in nasal lavage fluid collected from infected individuals. Noah has demonstrated an increase in nasal lavage levels of IL-8, MIP-1α and MCP-1 following artificial RSV infection of adults (Noah 2000) and the same group has previously shown markedly elevated levels of IL-1 β, IL-8, IL-6, and TNF-α in nasal lavage fluid from children with an acute RSV URTI (Noah 1995).

The release of these mediators has a significant effect both on the early inflammatory response as well as later immunological events. For example, RANTES, MIP-1α and MCP-1 are potent chemotactic and activation factors for monocytes and eosinophils as well as being chemotactic for $CD4^+$ T cells. Interestingly, TNF-α production has significant antiviral effects as evidenced by its ability to inhibit RSV replication in human epithelial cells (Merolla 1995).

IL-8 has been shown to have chemotactic and activating effects on neutrophils. Genetic studies have recently identified an association between a common polymorphism of the IL-8 gene promoter region and RSV disease severity (Hull 2000). Furthermore, this polymorphism was associated with increased IL-8 production by lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMC). These are intriguing results having identified a genetic susceptibility determinant for RSV disease whilst hinting at high IL-8 production as a possible risk factor for developing bronchiolitis. However, further prospective studies are required before a definitive causal link between IL-8 production and severe RSV disease can be confirmed.

In addition to cytokine production other epithelial cell factors are likely to contribute significantly to the host immune response. RSV infection of epithelial cells is associated with increased cell surface expression of intercellular adhesion molecule (ICAM)-1 which is an important ligand for neutrophil and eosinophil adhesion to respiratory epithelium and is felt to contribute to airway inflammation in asthma (Stark 1996). RSV infection of epithelial cells also upregulates Class-I Major Histocompatibility Complex (MHC) expression and this may have a significant influence on the development of adaptive immunity through the importance of this molecule in the presentation of processed virus to T cells (Garofalo 1996).

Interferon and Natural Killer Cells

The type I interferons (Interferon- (IFN-) α and IFN-β) are produced by cells in response to viral infection and make several contributions to host defence against viruses. IFN-α and IFN-β bind to a common cellular receptor and induce the synthesis of various host proteins including enzymes that degrade viral RNA and inhibit viral replication. In addition, the interferons upregulate cellular expression of the MHC Class I molecule and strongly activate natural killer cells. RSV infection appears to be a poor stimulant of IFN production *in vitro* when compared to other viruses such as influenza (Roberts, Jr. 1992) and nasopharyngeal aspirate studies during RSV infection have detected only low levels of IFN-α in approximately 50% of patients (Nakayama 1993).

Natural killer (NK) cells are important effectors in the innate immune response and play a key role in the first line of host defence against viral infection. Thus far, there has been no direct measurement of NK activity in RSV-infected humans. Murine studies have demonstrated that NK cells are the major lymphocyte population present in the lung during the first days of primary RSV infection and that activated NK cells can significantly

reduce disease severity (Hussell 2000). However, the exact role of NK cell activity in the pathogenesis and defence against RSV remains to be characterised.

Neutrophils

Neutrophils have been shown to accumulate in large numbers in the respiratory tract of children with RSV infection (Everard 1994). IL-8 is a potent neutrophil chemoattractant and is produced both *in vitro* from RSV infected epithelial cells and *in vivo* with increased quantities detected in nasal aspirates from RSV infected children (Noah 1995). Furthermore, RSV infection of epithelial cells effects increased expression of cell surface adhesion molecules including ICAM-1 (Stark 1996) and neutrophils have been shown to adhere avidly to RSV-infected epithelial cells *in vitro* (Faden 1984). Therefore, not only are neutrophils attracted to sites of RSV infection but, once there, they are retained and activated to mediate inflammatory responses.

Neutrophil elastase levels are increased in the nasal lavage fluid of infants with acute RSV infection suggesting the possible contribution of neutrophil degranulation in the pathophysiology of RSV infection (Abu-Harb 1999). Furthermore, recent *in vitro* evidence suggests that the epithelial damage and detachment caused by RSV infection is significantly augmented by neutrophils in a dose-dependent manner (Wang 1998b). However, the exact contribution of neutrophils to viral clearance and/or disease pathogenesis in RSV infection remains to be clarified.

Macrophages and Surfactant Protein-A

The *in vitro* effect of RSV infection on macrophages has been extensively studied. Macrophages appear to be relatively resistant to infection and intracellular virus replication is extremely limited (Becker 1992). Following infection with RSV, macrophages respond rapidly with the production of a wide range of immunological mediators including TNF-α, IL-1, IL-6, IL-8 and IL-10. The cytokine profile induced by RSV infection of macrophages suggests that these cells may have a potential immunomodulatory role and has led some to propose a central role for macrophages in the host immune response (Panuska 1995).

Macrophages also have an important phagocytic role, killing bacteria and viruses in association with cellular activation and release of intracellular proteases and reactive oxygen species. The process of trapping and engulfment by macrophages relies on opsonisation of the target pathogen with antibody, complement and various other proteins

including the collectins. The collectins are a family of proteins believed to be involved in innate host defence against various bacterial and viral pathogens. They have been shown to bind to surface oligosaccharides on a wide variety of pathogens and to thus mediate phagocytosis and killing by phagocytic cells (McNeely 1993). Recent interest has concentrated on the role of one of the collectins, Surfactant Protein-A (SP-A), in the immune response to RSV.

LeVine et al studied the effects of RSV infection in SP-A knockout mice (LeVine 1999). They found greater polymorphonuclear lymphocyte infiltration and markedly elevated levels of proinflammatory cytokines in the lungs of SP-A knockout mice compared to controls. In addition, RSV clearance was impaired and alveolar macrophage oxygen radical production was reduced in SP-A negative mice. The exogenous administration of SP-A reduced both inflammation and enhanced viral clearance signifying the importance of SP-A in pulmonary host defence against RSV possibly through alveolar oxygen radical production and viral killing. A further study has demonstrated that SP-A enhances uptake of RSV by both PBMCs and macrophages *in vitro* (Barr 2000). Interestingly, this enhanced uptake is accompanied by increased production of the proinflammatory cytokine TNF-α and reduced production of the anti-inflammatory cytokine IL-10 pointing to a possible immunomodulatory role for SP-A.

The Adaptive Immune Response to Infection

The adaptive immune response is essential for both recovery and protection from RSV infection. It can largely be divided into two arms:

(i) the humoral immune response consisting of serum and secretory antibodies as well as maternally derived antibodies in young infants

(ii) the cell-mediated immune response represented primarily by major histocompatibility complex class I restricted cytotoxic T lymphocytes

Humoral Immunity

The developing foetus actively acquires maternal IgG antibodies through the placenta. This process occurs initially from approximately 30 weeks of gestation. The transfer rate gradually increases to a maximum between 38 and 40 weeks of gestation. Following birth, levels of neutralizing antibody slowly diminish with a mean half-life of 26 days (Brandenburg 1997). The amount of antibody transferred appears to be important in

protecting infants against RSV infection. Maternally acquired antibody levels have been shown to correlate closely with both age at time of first infection (Glezen 1981) and severity of disease following infection (Kasel 1987).

Infants also acquire antibodies from the mother through breast feeding and there is some evidence to suggest that levels of RSV specific antibodies in breast milk correlate with protection against bronchiolitis (Downham 1976). Normal breast milk has been shown to contain high levels of RSV-specific IgA in the immediate post partum period with negligible / undetectable levels of specific IgM or IgG. Whilst these levels appear to drop in the first 9 months following birth, maternal exposure to RSV during this period appears to cause a boost in breast milk antibody production (Fishaut 1981). Interestingly, the antibodies produced by such an exposure are exclusively of the IgA class.

There is considerable variability in the humoral response of infants to primary infection. During the first year of life there is rapid maturation of the immune system and this may account for much of this variability. In primary infection infants respond with both secretory and serum neutralizing antibody directed at the F and G surface glycoproteins and internal proteins (Vainionpaa 1985). The nature of this antibody response varies considerably with age. In the first 6 months of life the serum IgA response is greater than that of IgG (Watt 1986). In older children, there is a predominant rise in IgG levels with IgG1 and IgG3 subclasses accounting for the major part of this response (Watt 1986), whereas IgG1 and IgG2 subclasses form the bulk of the adult response (Wagner 1987).

Whilst there are qualitative differences in antibody response with age, there is also quantitative variation with generally very low titres of antibody produced in early infancy. The antibody titres against both the F and G surface proteins of RSV in infancy are 10 to 12 times lower than those observed in children over 12 months of age (Murphy 1986b). This blunted response appears to be caused by a combination of factors including the presence of residual maternal antibody and immaturity of the immune system (Murphy 1986a). With increasing age and repeated infections, antibody titres rise to a relatively constant level through childhood and young adulthood before a subsequent decline in old age (Falsey 1998).

The importance of adequate serum antibody levels in preventing reinfection is debatable. In one study adults who had had a proven RSV infection were challenged repeatedly with RSV at 2, 4, 8, 14, 20 and 26 months following the initial infection. The risk of developing an RSV infection was 25% at each challenge, though 50% were infected at 2 months, rising to 66% by 8 months. By the end of the study 73% of test subjects had two or more infections, while 43% had three or more. The development of high titres of antibodies

against F and G proteins was associated with some protection, though even with the highest antibody titres the reinfection rate was reduced by only 25% (Hall 1991). In another study, higher serum titres of IgG anti-F antibodies were shown to reduce the risk of reinfection in a group of 34 children followed from birth to 3 years of age (Kasel 1987). Furthermore, children with low levels of RSV-specific IgG1 are at a higher risk of developing bronchiolitis (Carlsen 1993). The most convincing evidence of the protective properties of RSV specific antibody has come from trials of polyclonal and monoclonal RSV antibodies in infants. Studies have shown intravenous respiratory syncytial virus immunoglobulin and the anti-F protein monoclonal antibody palivizumab to be effective in decreasing the number of hospitalizations and hospital days attributable to RSV (Anon 1998, Hemming 1995). However, whilst there is a reduced risk of RSV reinfection in the presence of high neutralizing antibody titres, there is much evidence that reinfection can occur in the face of this strong humoral protection (Johnson 1962).

A similar partially protective role has been established for mucosal antibody. IgA comprises the vast majority of the mucosal antibody response to RSV infection, with a detectable rise in 56-65% of RSV infections during the first six months of life (McIntosh 1978b). Several investigators have demonstrated an inverse relationship between infection rates in adults after experimental challenge and the level of pre-existing RSV-specific nasal antibody (Watt 1990, Mills 1971). However, as with serum antibody, RSV infections can still occur despite high titres of nasal wash neutralizing antibody (Hall 1991).

Murine studies have not found a major role for RSV-specific antibodies in the resolution of RSV infection with no apparent temporal relation between RSV antibody generation and subsequent viral clearance (Anderson 1990). However, mice depleted of B cells, and hence with no antibody response, experience greater disease severity (Graham 1991a). Similarly, higher neutralizing antibody titres in children are associated with a lower incidence of severe lower respiratory tract disease once infected with RSV. A large Canadian study has shown that the rate of lower respiratory tact infection is significantly lower among children with RSV-neutralising antibody titres greater than 1:100 than among those with lower titres (Law 1997).

In summary, humoral immunity appears to be important in protection against severe RSV disease although it's contribution (or lack of it) to RSV clearance in humans has yet to be studied.

Cell Mediated Immunity

The cellular arm of the adaptive immune response is orchestrated primarily by cytotoxic T lymphocytes (CTLs) and T helper (Th) lymphocytes. The importance of cell mediated immunity is highlighted by the severe clinical effects of RSV infection in individuals with congenital/acquired impairment of cellular immunity (Hall 1986, Fishaut 1980).

Cytotoxic T Lymphocytes

Activated CTLs are very effective at destroying target cells, especially virus-infected cells and tumour cells. The killing happens in three steps. Firstly, through the interaction of the T cell receptor complex and the MHC I-antigen, the target cell and the CTL form a conjugate. Immediately following the formation of the conjugate, granules in the CTL start to move through the cytoplasm towards the end nearest the target cell and then release their contents, including cytokines, enzymes and a molecule called perforin, causing direct damage to the target cell membrane. Subsequently, the CTL induces target cell death both through lysis secondary to membrane damage and through the process of apoptosis.

Murine studies have indicated an important role for CTLs in clearing RSV. A significant CTL response has been demonstrated in the lungs of experimentally infected mice and this is temporally related to a decrease in viral shedding (Anderson 1990, Taylor 1985). Moreover, athymic mice which are unable to produce RSV-specific CTLs become chronically infected with RSV (Taylor 1985) with resolution of this infection effected by the injection of the mice with primed RSV-specific CTLs (Cannon 1987). However, CTLs may also contribute to disease pathogenesis when produced in large numbers. Whilst low numbers of RSV-specific CTLs clear virus from chronically infected immunosuppressed mice, larger numbers are associated with acute respiratory disease and, in many cases, death (Cannon 1988). The possible pathogenic role of CTLs is discussed further below.

Few studies have investigated the role of RSV-specific CTLs in man. Of 22 infants with acute RSV bronchiolitis, Isaacs *et al* found RSV-specific CTLs in only 4 (Isaacs 1987). Interestingly, all 4 infants with detectable CTLs had a mild clinical course. Chiba *et al* also found evidence of significant cellular toxicity to RSV in infants, with a peak approximately one week following infection (Chiba 1989). Similarly, adult studies have identified RSV specific CTLs in the peripheral blood following infection (Cherrie 1992) and further analysis has ascertained those RSV proteins which serve as targets for human CTL epitopes. Human CTLs were tested for their ability to lyse a series of cell lines infected with recombinant vaccinia viruses expressing various RSV surface proteins

(Cherrie 1992). Whilst the N, SH and F proteins proved to be the major antigenic targets for CTLs, there was no significant recognition of the G protein.

In contrast to their importance for virus clearance in primary infection, the role of memory CTLs in preventing infection appears to be limited. Murine studies have shown that there is almost complete resistance to subsequent RSV challenge following infection with vaccinia virus expressing the RSV F, G or M2 protein. The resistance induced by F and G proteins appears to be largely antibody mediated and is long-lasting whereas that induced by the M2 protein is almost exclusively mediated by CTLs (Connors 1992a) and appears to wane rapidly (Connors 1991).

CD4+ T Helper Lymphocytes

CD4+T lymphocytes play a central role in regulating the cell mediated immune response to infection. These cells are often known as "helper" T cells, as they act on other cells of the immune system to promote various aspects of the immune response, including immunoglobulin isotype switching and affinity maturation of the antibody response, macrophage activation, and enhanced activity of natural killer cells and CTLs.

CD4+ T cells recognise antigen that has been processed and is presented in association with a self class II MHC molecule. In initiating the immune response, this interaction takes place in the lymphoid tissue, and involves T cell interaction with so called "professional antigen presenting cells" -B cells, macrophages and dendritic cells - which take up, process and present the relevant antigen. Once a CD4+ T cell has been activated in this way, it is capable of recognising the antigen presented by any cell that expresses the appropriate class II MHC molecule. In this way, initial activation of the immune response is controlled within the lymphoid tissues, whilst primed CD4+ T cells are then able to respond to antigen presented at distant sites within the body.

There has been considerable recent interest in the role of CD4+ T cells in RSV disease. Much of this interest has arisen from the recognition of two distinct subgroups of CD4+ T cells that appear pivotal in determining the nature of the immune response.

The paradigm of type 1 and type 2 cytokine responses

CD4+ T cells act by releasing cytokines in response to antigenic stimulation. The cytokines produced have been used to differentiate these cells into the two major classes of CD4 effector T cell – T helper (Th) 1 or type 1 (producing type 1 cytokines) and Th2 or type 2 (producing type 2 cytokines) (Mosmann 1996). The differentiation of a naïve CD4

T cell into either a type 1 or type 2 cell has a critical impact on the outcome of an adaptive immune response, determining whether it will be dominated by macrophage activation or antibody production. Over recent years there has been growing recognition of a significant role for other cells in the production of type 1 and type 2 cytokines (Lucey 1996). Type 1 cytokines promote cell-mediated immunity and are required for effective responses to intracellular pathogens (such as viruses). Type 1 cytokines include IFN-γ (produced primarily by Type 1 T cells), IL-12 (from antigen presenting cells) and IL-18 (from activated macrophages). Type 2 cytokine production induces eosinophil differentiation, evokes a strong antibody response against extracellular pathogens and favours the development of allergic responses. Examples include IL-4, -5 and -10, all produced by Type 2 T cells. Cross regulation occurs between the two types of response, some type 1 cytokines decreasing type 2 cytokine levels and vice versa. .

There are many factors involved in determining the differentiation of CD4 T cells either along a predominantly type 1 or a predominantly type 2 route. All seem to be dependent, however, on a fundamental stepwise interaction between the antigen presenting cell and the naïve CD4 T cell. This interaction begins with the presentation of an antigen-MHC class II complex on the surface of an APC to the T Cell Receptor (TCR)/CD3/CD4 complex on naive T lymphocytes (Kapsenberg 1999). This interplay activates the naïve T cell, resulting in IL-2 receptor expression, IL-2 secretion, and CD40 ligand (CD40L) upregulation. IL-2 interacts with IL-2R in an autocrine manner, while the appearance of CD40L allows the T cell to bind to constitutively expressed CD40 on the surface of the APC. This interaction stimulates the APC to express membrane-bound ligands which interact with the CD28 molecule expressed on the T cell membrane. This interaction results in increased IL-2 secretion (and thus proliferation), induces the appearance of the anti-apoptotic molecule Bcl-x_L (promoting survival), and may contribute to future cytokine secretion (Walunas 1996, Lenschow 1996).

With ligation of CD28, the naive T cell may differentiate along more than one pathway, subject to a variety of inputs (Delespesse 1997). Both environmental and genetic factors appear to play a role. One important environmental factor that may influence CD4 T cell development is the MHC-TCR interaction itself. Very low and very high antigen doses have been suggested to promote a type 2 response, while moderate antigen levels predispose naive cells to become type 1 cells (Murray 1998). Alternatively, when dose and affinity of antigen are considered concurrently, exact opposite results are reported. Low and high doses of high affinity antigens yield type 1 T cells, while moderate doses of high affinity antigens yield type 2 cells (Rogers 1999). Other environmental factors that appear

to be play a role in determining CD4 T cell differentiation include the route of antigen entry, the physical form of immunogen and the type of adjuvant.

The time of availability plus the relative ratio(s) of cytokines systematically drives naïve T cells to one or more fundamental phenotypes (Delespesse 1997). Three cytokines appear to be of central importance in the differentiation of naïve T cells – IL-4, IL-12 and IFN-γ.

IL-12 and IL-4 have been considered the pivotal cytokines in influencing antigen-activated naive $CD4^+$ T cells to develop into type 1 and type 2 T cells, respectively (Murphy 1998, Mosmann 1996). IL-4 is secreted by type 2 T cells, mast cells and basophils and appears to be the dominant cytokine in early differentiation. If IL-4 levels reach a necessary threshold, differentiation of CD4 T cells to the type 2 phenotype occurs regardless of other cytokine production. IL-4 upregulates expression of it's own receptor, inhibits the secretion of IL-12 and downregulates expression of the IL-12 receptor. In addition, IL-4 is able to stimulate it's own production by both naïve and effector cells and can induce a type 1 to type 2 switch through activation of its own receptor on type 1 T cells (Paludan 1998, Coffman 1995). IL-12 is secreted primarily by antigen presenting cells following antigen presentation and ligation with T cells. IL-12 induces cytokine production, primarily of IFN-γ, from NK and T cells, acts as a growth factor for activated NK and T cells and enhances the cytotoxic activity of NK cells (Komastu 1998). IL-12 also induces type 1 specific immune responses by promoting the differentiation of type 1 T cells from naïve T cells at the expense of type 2 effector cells. IFN-γ is secreted by a variety of cells including NK cells, type 1 T helper cells and macrophages. IFN-γ produced by T and NK cells acts as a powerful feedback mechanism on the phagocytic cells producing IL-12, activating them and enhancing their ability to produce many proinflammatory cytokines, including IL-12 itself. IFN-γ also inhibits IL-4 receptor expression and interferes with IL-4 production (Paludan 1998), consequently inhibiting the development of a type 2 phenotype. There exists, therefore, a cross-regulatory balance between IL-4 (type 2 response) on the one hand and IL-12 / IFN-γ (type 1 response) on the other.

Another important factor determining the nature of the immune response is the timing of cytokine expression. IL-12 and IFN-γ are always synthesised first followed by a period of time before the appearance of IL-4. Thus the initial cytokine milieu favours a type 1 immune response. However, should IL-4 be produced in sufficient quantities to overcome any early type 1 cytokine production, then cells will be driven toward a type 2 phenotype (O'Garra 1998).

It is also clear that genetic background has a profound effect on T cell differentiation. Human studies have established linkage of atopy and IgE production to numerous loci. Significant linkage has been identified between serum total IgE levels and a region of chromosome 5q that contains numerous candidate genes for atopy including IL-4 (Marsh 1994). Similarly, chromosome 12q, an area encoding the IFN-γ gene, demonstrates significant association and linkage with elevated total serum IgE (Barnes 1996). Abnormal gene regulation may also influence the immune response. For example, one of the many allelic forms of the IL-4 promoter gene confers high transcriptional activity and results in over-expression of the IL-4 gene (Song 1996). However, the contribution of genetic factors to T cell differentiation is most easily demonstrated by studying inbred strains of mice. Mice infected with Leishmania major show marked genetic differences in disease manifestations: BALB/c mice are susceptible, exhibiting enlarging lesions that progress to systemic disease and death, whereas C57BL/6 are resistant, developing small, self-healing lesions. This marked difference appears related to the predominant host immune response to infection with BALB/c mice demonstrating a highly polarised type 2 response and C57BL/6 mice a highly polarised type 1 response. Linkage studies have identified multiple gene loci that are associated with control of T cell differentiation although the actual genes involved have yet to be established (Roberts 1997).

The discussion above is an oversimplification of a very complex system. For example, Transforming Growth Factor-β in the presence of IL-4 may drive naive T cells to a Th1 phenotype (Bird 1998) and IL-12 has been shown to amplify pre-established Th2 responses (Muraille 1998). Nevertheless, the type 1 / type 2 paradigm has provided a valuable framework for understanding the immune response in many diseases.

CD4+ T Helper Cells in RSV Disease

Studies in T cell depleted mice have identified a role for CD4+ T cells in viral clearance. RSV infection of mice depleted of either their CD4+ T cell or their CD8+ T cell subsets results in significant prolongation of viral shedding (Graham 1991b). However, passive transfer of RSV-specific CD4+ T cells to infected mice enhances viral clearance but simultaneously exacerbates disease severity and increases lung pathology (Alwan 1992). CD4+ lymphocyte responses in humans following RSV infection have been evaluated indirectly. In vitro lymphocyte transformation activity (LTF) is a marker of CD4+ activity and is increased following RSV infection in infants. The LTF response relates to disease severity, being significantly greater in those infants with bronchiolitis than in those with URTI alone (Welliver 1979). Thus, certain CD4+ T cell subsets have a possible role in RSV disease pathogenesis. This is discussed in detail below.

Role of Host Immunity in the Pathogenesis of RSV Disease

The first indication of a role for host immunity in the pathogenesis of RSV disease emerged as a result of field trials of formalin-inactivated RSV (FI-RSV) vaccine in the 1960s (Chin 1969, Kim 1969). The Bernett strain of RSV was initially propagated in human embryonic kidney cells and passaged in vervet monkey kidney cells. The infected cells were inactivated with formalin and concentrated by ultracentrifugation and alum precipitation. This preparation had a final concentration factor of 100 and was known as lot 100. Those children that were vaccinated with lot 100 developed a strong humoral response with high titres of complement-fixing and neutralising antibody detectable in the blood. However, vaccination failed to protect against RSV infection or disease with many vaccinees developing severe lower respiratory tract disease following infection with RSV. Post-mortem studies on two of the children that died following administration of this vaccine showed that they both had florid eosinophilic pulmonary infiltrates (Kim 1969). Subsequent examination of lymphocyte proliferation in the vaccinated group demonstrated a significantly increased LTF compared to control children. This lead to the hypothesis that the immunopathology observed following vaccination might be due to an exuberant cell-mediated immune response to RSV (Kim 1976). Since these initial observations, further evidence of immunopathological mechanisms in RSV disease has emerged through both animal and human studies.

Role of Humoral Immunity

The contribution of antibody responses to RSV disease remains hotly debated. Unlike other viral infections the most severe disease occurs in the first few months of life when maternally derived specific antibody is invariably present. It has previously been speculated that an immune complex reaction between maternally acquired IgG antibody and viral antigen occurs in the lungs of severely affected infants although direct evidence of this is lacking (McIntosh 1980).

A detailed analysis of serum antibodies following vaccination with FI-RSV highlighted the importance of a balanced humoral response for disease protection. Whilst FI-RSV produced very high levels of antibody to the F glycoprotein of the virus, this antibody was found to have a low level of neutralising activity. In addition, very low levels of antibody to the G glycoprotein were produced providing minimal protection against this important antigen (Murphy 1986c). Furthermore, FI-RSV was administered via the parenteral route and would have thus failed to produce a mucosal secretory antibody response. However, whilst these important humoral factors would render vaccinees susceptible to infection,

there is no evidence to suggest that this unbalanced response in anyway contributed to augmented disease.

In fact, a number of factors point to the probable protective rather than pathologic role of antibody in RSV disease. Firstly, RSV disease is rare in the first 6 weeks of life at a time when maternally acquired IgG is at it's highest (Holberg 1991). Secondly, the administration of prophylactic polyclonal or monoclonal antibody to infants is associated with a reduction in the severity of subsequent RSV disease (Robinson 2000) and, thirdly, high levels of RSV-specific antibody in umbilical cord blood are associated with less severe disease on subsequent infection (Kasel 1987).

On balance therefore it would appear that humoral immunity to RSV is essentially protective with only circumstantial evidence to suggest a role in immunopathogenesis.

Role of Cell-Mediated Immunity

RSV Vaccine-Enhanced Disease

As detailed above, children vaccinated with FI-RSV demonstrated increased RSV-specific lymphocyte proliferation following subsequent infection (Kim 1976). In the vaccinated group hospitalisation rates and illness severity were highest in the youngest group of children who were 2-7 months at the time of immunisation (Kim 1969). Children older than 24 months at the time of vaccination did not experience enhanced disease (Kapikian 1969). It therefore appears that the immune response to FI-RSV is more likely to result in enhanced disease if the individual's first exposure to RSV antigen is through vaccination. This observation has led to considerable investigation into the possible mechanisms involved in this aberrant immune response.

FI-RSV vaccination of mice or cotton rats also causes disease augmentation during subsequent RSV infection and these models have been used to further define the immune process. Following in vivo depletion of $CD4^+$ T cells from FI-RSV vaccinated mice there is a marked reduction in the severity of histological changes after RSV infection while $CD8^+$ T cell depletion has only a moderate effect (Connors 1992b). Immunisation of mice with FI-RSV results in a type 2 immune response as evidenced by the dominant expression of IL-4 mRNA relative to IFN-γ mRNA (Graham 1993). This contrasts with the predominantly type 1 immune response observed following live RSV infection (Graham 1993). The cytokine milieu at the time of vaccination also has a considerable influence on subsequent disease. Administration of anti-IL-4 to mice at the time of vaccination results in reduced clinical illness and increased viral clearance after live virus challenge. This is

associated with an augmented CD^+ T lymphocyte activity and increased expression of IFN-γ mRNA relative to IL-4 mRNA (Tang 1994). Similarly, mice immunised with FI-RSV and then simultaneously depleted of both IL-4 and IL-10 experience a dramatically milder clinical course on subsequent infection (Connors 1994). Whilst pulmonary histopathology is completely abrogated by depletion of both cytokines, depletion of IL-4 alone reduces bronchiolar, but not perivascular, changes and depletion of IL-10 alone has no effect.

Further insight into RSV induced immunity has been obtained from the study of mice immunised with recombinant vaccinia virus (rVV) expressing a variety of RSV surface proteins. Priming with rVV expressing the G protein induces a florid pulmonary eosinophilia on subsequent infection with histopathological changes reminiscent of those following vaccination with FI-RSV (Openshaw 1992). These changes are associated with a relative type 2 immune response whilst rVV expressing other RSV proteins induces lymphocytes with type 1 cytokine secretion profiles (Alwan 1993). Furthermore, T cell lines from mice primed with rVV-G induce more severe fatal illness when injected into RSV infected mice than when a similar transfer is made with T cells from mice primed with rVV-F (Alwan 1994).

These studies have led to the hypothesis that vaccine-augmented disease in the animal model is secondary to an unbalanced immune response with a predominance of type 2 over type 1 responses. Unfortunately, it is only possible to speculate as to the relevance of these findings to the human. Whilst lymphocyte proliferation studies hint at similar immune processes in those children vaccinated in the 1960s, a lack of suitably advanced immunological techniques at the time or appropriately stored specimens now precludes a more definitive understanding of these processes.

Primary RSV infection

There is considerable indirect evidence that cell mediated immunity has a significant role in RSV disease. Polymorphonuclear leucocytes dominate bronchial lavage fluid from infants ventilated for RSV bronchiolitis with CD4+ T cells constituting the largest lymphocyte subpopulation (Everard 1994). Moreover, lymphocyte proliferative responses following RSV infection suggest an association between T cell activity and clinical features of bronchiolitis. Welliver *et al* studied infants hospitalised with RSV infection and analysed lymphocyte transformation during the early convalescent period. He found a significantly increased stimulation index in those infants with a clinical diagnosis of bronchiolitis when compared to those with URTI or pneumonia (Welliver 1979).

Primary RSV Infection in the Mouse Model

Primary RSV infection in mice has been extensively studied. Following infection, RSV readily replicates in the nose and lung of BALB/c mice with peak viral titres reached between days 4 and 6 in the lung (Taylor 1984). There is considerable similarity between the histopathological changes observed in the lungs of these mice and that observed in postmortem specimens of infants with RSV infection (Taylor 1984). Using this model, T cell depletion studies have demonstrated the importance of both CD4+ and CD8+ T cells in terminating RSV replication after primary infection. Furthermore, depletion of CD4+ and CD8+ T cells results in prolonged RSV replication but absence of clinical disease implying a role for cell mediated immunity in disease pathogenesis (Graham 1991b). Primary infection appears to induce a predominantly type 1 cytokine profile in mice, with IFN-γ production increased in tracheobronchial lavage (Anderson 1990) and reduced IL-4 and IL-5 from stimulated lymph node mononuclear cells (Schwarze 1997). However, there is only circumstantial evidence to suggest a role for type 2 responses in more severe disease. Depletion of IL-5 abolishes increased airway hyperresponsiveness and eosinophilic infiltration following RSV infection with addition of IL-5 restoring these responses (Schwarze 1999) and, more recently, IL-5 mRNA expression has been shown to correlate with pulmonary pathology in RSV infected BALB/c mice (Stack 2000).

Primary RSV Infection in Infants

Specimen collection difficulties and ethical issues have hampered study of the cell-mediated immune response in infants. Early studies identified the presence of RSV-specific IgE in the nasopharyngeal secretions of those infected infants with wheeze as a major symptom (Welliver 1981). Since type 2 immune responses are involved with the switching of B-cells to IgE production, this provided indirect evidence for type 2 immune activation in bronchiolitis.

Cytokine Production by Stimulated Peripheral Blood Cells

Over recent years there has been an increased interest in the cytokine profile induced by human RSV infection. Much of the data relates to cytokine production from stimulated peripheral blood cells collected during RSV infection. Roman *et al* studied cytokine production following mitogen exposure of PBMCs taken from infants while hospitalized with RSV bronchiolitis and from age-matched controls. They found a significantly increased ratio of IL-4/IFN-γ production in infants with RSV infection compared to controls (Roman 1997). Similarly, Aberle *et al* identified lower levels of IFN-γ mRNA in

PBMCs from infants with severe RSV bronchiolitis than in those with a milder clinical course (Aberle 1999). In addition, a series of studies by Bont *et al* have analysed cytokine production from whole blood cultures obtained from hospitalised RSV-infected infants. They found no detectable difference in either IL-4 or IFN-γ production between hospitalised, non-ventilated infants and control subjects although ventilated patients had significantly depressed cytokine production with virtually undetectable IL-4 or IFN-γ levels (Bont 1999). In a further study, Bont *et al* analysed cytokine production from stimulated whole blood taken from infants requiring mechanical ventilation for respiratory failure secondary to RSV infection (Bont 2000b). IL-12 production was inversely correlated with duration of mechanical ventilation implicating a relative type 2 immune response in those with the most severe disease. Conversely, a recent study has found evidence of increased levels of the type 1 cytokine IFN-γ in those infants with severe disease compared to those with mild disease (Bendelja 2000). Following *in-vitro* stimulation of peripheral blood cells obtained from healthy controls and infants hospitalised with RSV infection, immunofluorescent staining of induced intracellular cytokines was assessed using flow cytometry. While RSV infected infants demonstrated a relative type 2 immune response (as evidenced by an increased IL-4 to IFN-γ ratio) compared to controls, IFN-γ production was increased and IL-4 reduced in those with bronchiolitis compared to those with URTI alone.

Cytokines in Respiratory Secretions

Few studies have analysed type 1 and type 2 cytokine levels in nasal and bronchial specimens from RSV-infected infants. Sheeran *et al* found elevated concentrations of IL-10 in both the nasopharyngeal and tracheal secretion of infants with RSV disease although the levels did not significantly correlate with disease severity (Sheeran 1999). In a similar study Van Schaik *et al* analysed cytokine levels in nasopharyngeal and tracheal secretions of infants hospitalised with acute respiratory infection (van Schaik 1999). IFN-γ, IL-4 and IL-10 concentrations were measured by enzyme linked immunosorbent assay (ELISA) in both nasal and tracheal secretions. Absolute IFN-γ levels and IFN-γ to IL-4 ratios were higher in subjects with bronchiolitis than in those with URTI alone and this observation held true when only RSV positive subjects were analysed. This led the authors to suggest that over-production of IFN-γ may be important in the pathogenesis of bronchiolitis. However, a recent study from Holland has questioned this conclusion. Bont *et al* identified higher levels of IFN-γ in the nasopharyngeal aspirates of infants with mild RSV disease (hospitalised, non-ventilated) compared to severe RSV disease (mechanically ventilated) (Bont 2001).

In summary, our current understanding of the cell-mediated immune response in primary RSV infection of infants is somewhat confused. Whereas some studies have identified a convincing link between RSV disease severity and a type 2 immune response, other studies have found the converse. There are many potential confounding factors that may explain such discordant findings. Firstly, control groups differ considerably between the studies with regard to symptoms (i.e. healthy, URTI etc), age, RSV status and need for hospitalisation. Secondly, the time of specimen collection varies significantly between studies with no study to date standardising collection times relative to symptom onset. Thirdly, due to the evolving nature of the host immune response in the first year of life, age differences between the control and study groups are extremely important. However, there has been no statistical control for these differences in the studies reported thus far. Finally, no study to date has analysed the relationship between local (respiratory secretions) and systemic (peripheral blood) immune responses to RSV in the same population.

Role of Eosinophils and Mast Cells

Whilst eosinophils have been traditionally regarded as mainly important in the innate immune response to helminthic infection, recent studies have suggested their possible contribution to RSV immunity. Products of eosinophil degradation are found in nasal lavage fluid and serum from children with severe RSV infection (Sigurs 1994) and eosinophilia has been observed in the peripheral blood of those infants that subsequently developed persistent wheeze (Ehlenfield 2000). As previously discussed, infected epithelial cells are capable of producing various eosinophil chemotactic factors including RANTES and MIP-1-α (Harrison 1999). Furthermore, RSV infected epithelial cells can induce degranulation of eosinophils *in vitro*, a process which appears to be mediated by the adhesion integrin Mac-1 (Olszewska-Pazdrak 1998b).

Whilst the recruitment and degranulation of eosinophils into the respiratory tract has been established, their exact role in protective immunity and/or pathogenesis has not been clearly defined. Following vaccination trials in the 1960s, those vaccinees with a severe response to subsequent natural RSV infection were found to have developed eosinophilia (Chin 1969). Moreover, post mortem studies of those children who died during the course of infection (2 children from the vaccinated group) demonstrated intense eosinophilic infiltration of the lungs (Kim 1969). These findings would suggest that eosinophils possibly played an important role in the pathogenesis of their severe disease. However, in contrast, eosinophils have been shown to have significant antiviral activity *in vitro* with

eosinophil derived secretory ribonucleases participating substantially in this process (Domachowske 1998b). This apparent dichotomy has led to the suggestion that whilst eosinophil recruitment to the lung may represent an important part of the normal immune response to RSV infection, an excessive response of this nature may contribute to severe disease.

Several studies have investigated the release of mast cell components in RSV disease. Histamine is found in significant concentrations in the nasal secretions of children with RSV infection and levels are substantially higher in children with associated wheeze (Welliver 1981). In vitro testing of mononuclear cells has shown that RSV can induce these cells to release histamine as well as factors known to induce mast cell degranulation (Chonmaitree 1991). Other mast cell products, including arachidonic acid-derived mediators, such as leukotrienes are elevated during the first few days of RSV infection in those children who develop bronchiolitis (van Schaik 2000). Elevated levels of leukotrienes C_4 persist for up to 28 days post-infection and may be one of the main effector agents involved in the prolonged wheeze associated with RSV infection (Volovitz 1988). However, another mast cell degranulation product, tryptase, has been found in only slightly elevated concentrations in respiratory specimens with levels considerably lower than those found in asthmatic patients (Everard 1995). Therefore, whilst mast cell degranulation clearly takes place in bronchiolitis, it is uncertain as to the importance of this process to the total inflammatory response.

Role of Immunoglobulin E

The primary biological function of IgE appears to be in the immune reaction and defence against parasitic diseases particularly helminthic infections. The IgE mediated immune reaction to helminths comprises both mast cell degranulation (through cross-linking of membrane IgE) and an antibody-dependent, cell-mediated cytotoxic response involving eosinophil recruitment and activation. In industrialized populations in which the frequency of helminthic parasitic infection is low, the immunological properties of IgE are manifested as a high frequency of allergy. It is hypothesised that the decreasing frequency of parasitism, which has occurred as a consequence of improved sanitation and hygiene, has left the immune system "unoccupied," thereby fostering immune responses to benign antigens that produce allergic responses. Individuals with atopy have higher IgE levels in the circulation than a normal individual, though the serum levels of IgE often do not reflect the severity of the atopic disease. A large proportion of IgE is surface bound to mast cells and basophils through a tight association with the high affinity IgE receptor. The type 2

cytokines IL-4 and IL-5 are important triggers for B cell production of IgE and for continued IgE production.

RSV infection is often accompanied by a specific IgE response. Welliver *et al* identified RSV-specific IgE in nasopharyngeal secretions collected from wheezy infants infected with RSV (Welliver 1981) and subsequent studies have also detected RSV-IgE in the serum of infected individuals albeit at much lower concentrations (Welliver 1985). Further investigation has revealed that the majority of the IgE produced in response to RSV infection is polyclonal and non-specific, but in a significant proportion of children there is a RSV-specific IgE response. Studies comparing the children who develop RSV-IgE with those who develop non-specific IgE responses, reveal specific responses in children at higher risk of developing recurrent wheezing episodes in the 48 months following initial RSV infection (70% versus 20%) (Welliver 1986).

Further insight into the importance of IgE production has recently emerged from long-term follow up studies of RSV-infected individuals. In a series of studies by Sigurs *et al*, children with a history of RSV positive bronchiolitis requiring hospitalisation have been monitored for the development of subsequent atopy. Using a combination of skin prick tests and specific IgE antibody assays, children with a history of RSV infection in infancy were found to have a significantly higher rate of sensitisation to inhalant allergens than controls at both 3 and 7 years of age (Sigurs 2000, Sigurs 1995). This apparent allergic sensitisation has also been investigated in the mouse model. Following RSV infection, BALB/c mice demonstrate significantly enhanced sensitisation to ovalbumin, with the development of both airway hyperresponsiveness and increased pulmonary inflammation (Schwarze 1997). Anti-IL-5 antibody negates this sensitising effect, suggesting the possible involvement of type 2 immune responses in this process.

RSV Bronchiolitis, Asthma and Atopy

The respiratory consequences of RSV bronchiolitis in early childhood have been debated for many years. The suspected link between bronchiolitis and subsequent asthma remains controversial despite a multitude of studies examining this relationship (Everard 1999, Wang 1998a). There is general agreement that after an episode of bronchiolitis a significant proportion of children subsequently have recurrent episodes of coughing and wheezing (Stein 1999, Noble 1997, Sigurs 1995). It is also generally accepted that the prevalence of these symptoms reduces with time (Stein 1999). However, it remains uncertain whether bronchiolitis causes asthma *per se* or merely represents a marker, acting as the first presenting respiratory illness in a child already prone to develop wheezing

lower respiratory illness. Several studies have prospectively followed children who have suffered RSV bronchiolitis monitoring respiratory symptoms, pulmonary function and the development of atopy. Unfortunately, there is little conformity between studies with regard to the diagnostic criteria used for bronchiolitis, atopy and asthma. This makes it difficult to relate the studies directly or to draw conclusions from the collective data.

The initial diagnosis of bronchiolitis is pivotal to studies of this nature and it is here that perhaps the greatest controversy lies. There are close clinical similarities between asthma and bronchiolitis with both having tachypnoea, subcostal / intercostal recession and wheeze as principal signs. When studying the relationship between bronchiolitis and the subsequent development of asthma, it is of paramount importance that children experiencing an asthma episode are not mistakenly diagnosed with bronchiolitis. The initial diagnosis of bronchiolitis must therefore be tightly defined. Bronchiolitis is generally regarded as an illness associated with cough and wheeze in infants under 12 months of age who display hyperinflation with wheeze and fine inspiratory crackles audible throughout the lung fields. Certain published studies have broadened these criteria to include all wheezing episodes associated with respiratory infection in children less than two years of age. Some of these episodes will undoubtedly be asthmatic in nature and this will reduce the conformity of the study group and hence the merit of the study findings.

Similarly important are the diagnostic criteria used for the primary outcome measures of atopy and/or asthma. The diagnosis of atopy or asthma has varied from confirmation of airway reactivity, serum IgE levels, skin prick tests to aeroallergens or IgE radioallergosorbent test (RAST) measurements for common aeroallergens, to a simple clinical diagnosis based on episodes of wheezing illness.

Lung Function Abnormalities Following Bronchiolitis

Many investigators have studied changes in lung function following an episode of bronchiolitis in infancy. Inclusion criteria have varied widely from parent reported episodes consistent with bronchiolitis to doctor diagnosed bronchiolitis with virological confirmation of RSV infection. Only studies where RSV infection has been confirmed will be discussed in this section.

In 1982, Pullan and Hey reported the results of a large prospective study of 180 children hospitalised in the first year of life with proven RSV lower respiratory tract infection (Pullan 1982). Of the 180 initially recruited, 130 were subsequently reviewed at 10 years of age with 107 able to complete a full pulmonary assessment including spirometry,

exercise testing and histamine challenge. 111 children with no history of respiratory problems were recruited as controls. The control cohort was of comparable age, sex and social class to the index group. Bronchial lability was increased to both histamine and exercise in the index group. Similarly, spirometry revealed generally reduced maximal flow rates with particularly marked decreases in FEV_1 and FEF_{25-75} (7.2% and 13.2% respectively). However, the control group had a significantly lower incidence of parental smoking than the index group that may have accounted for these differences.

Subsequent studies have followed a very similar framework with a variety of pulmonary function tests performed at variable periods following the initial RSV illness. Many of these studies have significant flaws including high drop-out rates (Hall 1984) and lack of appropriate controls (Sly 1989, Hall 1984). Moreover, the studies reported to date have investigated only infants hospitalised with RSV infection, thus selecting only those with the most severe disease and potentially biasing study outcomes. Despite these reservations, however, most of the studies provide remarkably similar results. Studies at 7 (Mok 1984), 8 (Hall 1984) and 10 (Noble 1997, Pullan 1982) years of age have shown both FEV_1 and FEF_{25-75} to be significantly reduced following RSV bronchiolitis as an infant. Changes in other lung function parameters have been less consistent. For example, bronchial reactivity is increased following RSV bronchiolitis in some studies (Sly 1989, Mok 1984), while other studies have not found this (Noble 1997).

Asthma and Asthma-like Symptoms Following Bronchiolitis

Asthma-like symptoms such as cough and wheeze appear to be significantly increased following an episode of bronchiolitis in childhood. With the passage of time there is a gradual reduction in the incidence of these symptoms often with complete resolution by adolescence. Whilst many studies have examined this sequence of events, few have addressed the methodological issues raised above. The definition of asthma, recruitment criteria, completeness of longitudinal follow up and the inclusion and characteristics of control populations have varied considerably between studies such that direct comparison is not possible. The largest, well controlled studies will be discussed further.

In their 10 year follow-up, Pullan and Hey found that 55 (42%) of 130 children with a history of RSV bronchiolitis in infancy had a history of wheeze compared with 21 (19%) of 111 children in the control group (Pullan 1982). Most of the excess wheeze in the index children occurred in the first four years of life with a markedly lower incidence in the 2 years prior to study. Similarly, a 7-year follow-up by Mok and Simpson found the lifetime occurrence of both cough and wheeze to be significantly increased following RSV

bronchiolitis in infancy (Mok 1982). However, these symptoms occurred with similar frequency in both index and control subjects in the year prior to study.

This pattern of progressive symptom resolution has recently been called into question by a series of studies from Sweden. Sigurs *et al* studied a group of 47 children following an episode of RSV bronchiolitis severe enough to require hospitalisation in infancy (Sigurs 2000, Sigurs 1995). For each index child, 2 matched controls were recruited from a local child health centre. All children were assessed at 3 and 7 ½ years of age using predetermined clinical criteria. Asthma was defined as at least three episodes of bronchial obstruction verified by a physician. Asthma and all other episodes of wheezing were grouped together under the umbrella term "any wheezing". Symptoms were regarded as current if they had occurred in the 12 months prior to review. At age 3, there was a significantly higher rate of current asthma (23% vs 1%) and current "any wheezing" (40% vs 9%) in the index group compared to controls. In contrast to earlier studies, the rate of current asthma (23%) was unchanged in the index group at 7 ½ years of age with only a small reduction in current "any wheezing" (38%). It remains to be seen whether these rates will remain elevated in this cohort or whether a gradual reduction will occur as observed in other studies.

As highlighted above there is a dearth of information regarding outcomes following RSV infection NOT requiring hospitalisation. However, some recent data from the Tuscon Children's respiratory study has gone some way to address this deficiency (Stein 1999). Children were enrolled at birth and their parents advised to take their children to the paediatrician with any signs or symptoms of lower respiratory tract illness. At each assessment, the paediatrician made a full examination and collected nasopharyngeal and throat swabs for viral culture. At 6, 8, 11 and 13 years of age parents were asked to complete a questionnaire about the child's history of wheeze. Current wheeze was defined as either infrequent wheeze (up to three episodes of wheezing in the past year) or frequent wheeze (more than three episodes in the past year). Those children with a history of a RSV lower respiratory tract illness were found to have an increased risk of infrequent wheeze and an increased risk of frequent wheeze at age 6. This risk reduced successively at 8 and 11 years of age and became non-significant by the age of 13.

Atopy Following Bronchiolitis

"Atopy" has previously been used as a poorly defined term to refer to allergic conditions that tend to cluster in families, including allergic rhinitis, asthma, eczema, and other specific and non-specific allergic states. More recently, atopy has been characterised by

the production of specific IgE in response to common environmental allergens (Jarvis 1998), and skin prick testing provides a convenient test for atopy in epidemiological studies. Studies that have used skin prick testing to determine the incidence of atopy following bronchiolitis have produced discordant results. Pullan and Hey's follow up of children 10 years post bronchiolitis and their matched controls, found an excess of positive skin prick tests in the control group (Pullan 1982). Meanwhile, a controlled study of 26 infants with a history of bronchiolitis, found no difference in sensitisation at age 8 to a wide range of aeroallergens and food allergens (Sims 1981). By contrast a series of studies from Nottingham, England found sensitisation was significantly increased in the bronchiolitis group at age 6 in 73 index children compared with 73 control subjects, although by age 10 this difference had disappeared in 61 index versus 47 control subjects (Noble 1997, Murray 1992). The recent studies by Sigurs *et al* have shown a significantly raised incidence of sensitisation in the bronchiolitis group at both 3 and 7 years of age (Sigurs 2000, Sigurs 1995). Interestingly, RSV bronchiolitis was found to be the second most important risk factor for atopic sensitisation behind an atopic family background.

Non-hospitalised RSV infection appears to have a less significant effect on subsequent atopy. Stein *et al* found no difference in ski-prick test positivity at either 6 or 11 years of age in their large community based study of childhood respiratory infections (Stein 1999).

An Overview

Our current knowledge of the relationship between RSV bronchiolitis, asthma and atopy is somewhat confused. The situation is not helped by the large number of heterogenous studies that have been reported. Whilst it is clear that children will encounter increased cough and wheeze following bronchiolitis, whether these symptoms constitute genuine asthma is not fully established. Certainly, the majority of the available data would suggest a transient increase in asthma in the first few years following bronchiolitis, with a similar pattern observed for subsequent atopy. However, recent Scandinavian studies have somewhat questioned the validity of this synopsis. Their observation of increasing asthma and atopy with age would appear to contradict many previous studies. This raises the possibility that ethnic differences may play an important part in determining the long-term outcome following bronchiolitis. The continued observation of this cohort will be of particular interest.

Prenatal Priming of the Immune System

An increasing body of evidence suggests that the newborn infant is not immunologically naïve as previously believed. Analysis of immune function both at birth and at various stages of foetal development has revealed evidence of significant immune maturation during foetal life.

Development of the Foetal Immune System

Human foetal haematopoiesis originates in the blood islands of the secondary yolk sac at approximately 3 weeks gestation with the generation of pluripotent myeloid and erythroid progenitors. From 4 weeks of gestation, there is migration of these primitive cells to the fetal liver, which then acts as the major haematopoietic organ. The liver enlarges considerably over the following 3-4 weeks as the number of nucleated cells rises. Subsequently, progenitor cells pass from the liver to the thymus, spleen and bone marrow where they undergo selective development.

Monocytes / Macrophages /Dendritic Cells

The cells of this lineage are important for the processing of antigen and its presentation to T cells. Macrophages are found in the primitive yolk sac from 4-6 weeks' gestation. The majority of these cells are MHC class II negative with only a small population that are MHC class II positive. Over the ensuing weeks, a similar distribution of macrophages is found in the lymph nodes, spleen, thymus and bone marrow.

MHC Class II positive macrophages are also found as Langerhans cells in the skin (from 7 week's gestation), in the lamina propria of the gastrointestinal tract (from 11 weeks' gestation) and as Kupffer cells in the liver (from 17 weeks gestation).

B Cells

Pre-B cells can be detected in the human liver by 8 weeks of gestation and in the fetal bone marrow by 13 weeks. Pre-B cells are distinguished by the presence of μ heavy chains in the cytoplasm. Surface IgM is expressed on liver B cells by 10-12 weeks with surface IgD found after 13 weeks' gestation. Following this, B cells increase rapidly in numbers and can be found in the spleen, bone marrow and circulation.

Early immunoglobulin production occurs primarily in the spleen from about 10 weeks' gestation with maximal production of both IgG and IgM at 17-18 weeks of gestation. From 20 weeks gestation serum IgG levels rise dramatically following upregulation of maternal IgG transfer across the placenta. IgE production is observed at 11 weeks' gestation in the fetal liver and lung with primarily splenic production from 21 weeks.

T Cells

T cell progenitors expressing the CD7 molecule (an early T-cell lineage marker) can first be identified in the fetal liver from 7 weeks of gestation. Subsequently, these cells seed the thymus at 8-9 weeks of gestation. At this stage, T-cell receptor genes have not begun to rearrange, and the cells lack the expression of CD3, CD4 and CD8 (triple-negative thymocytes). The thymic microenvironment triggers further thymocyte maturation by initiating gene segment rearrangements encoding the TCR chain. Over 95% of thymocytes become TCR$\alpha\beta^+$ with a minority becoming TCR$\gamma\delta^+$.

After successful TCR rearrangement, the thymocytes are found primarily in the thymic cortex and express TCR$\alpha\beta$-CD3 and both CD4 and CD8 (double-positive thymocytes). At this stage, thymocytes undergo vigorous positive and/or negative selection depending on their TCR$\alpha\beta$ specificity. During positive selection, T cells expressing TCR$\alpha\beta$ that recognise non-self peptides bound to MHC class I or II molecules are stimulated to continue differentiation. If this signal fails to develop, the cell undergoes apoptosis and is eliminated. Negative selection occurs when the TCR$\alpha\beta$ recognises self peptide-MHC complexes. This process results in the elimination of most thymic precursors. The remaining thymocytes are termed type III thymocytes and are found primarily in the thymic medulla. These cells express TCR$\alpha\beta$-CD3 and either CD4 or CD8 alone (single positive thymocytes).

$CD3^+$ T cells are detectable in the foetal circulation from about 15 weeks of gestation. The ability of these cells to proliferate in response to mitogenic stimuli such as phytohaemaglutinin is first seen at 17 weeks of gestation. The ontogeny of the proliferative response to specific antigens has not been studied in detail with only one study to date examining this area. Jones *et al* studied the proliferative response to various allergens of mononuclear cells obtained from terminated foetuses and premature babies (Jones 1996). A significantly greater proportion of those blood cells obtained after 22 weeks gestation were found to proliferate than those from an earlier gestation.

Cytokine production by neonatal T cells appears to be significantly impaired when compared to adult cells. This reduced capacity may contribute to the impaired responses of other neonatal cell populations. For example, low levels of IFN-γ production (Wilson 1986) may reduce the cellular cytotoxicity of natural killer cells.

Prenatal Priming of the Immune System to Allergens

Cell mediated immunity relies on the clonal expansion of antigen specific T lymphocytes for memory development. Therefore, a raised PBMC proliferative response to an antigen *in vitro* represents a previously exposed and primed T lymphocyte. Allergen-specific reactivity at birth has been demonstrated for a range of common allergens including hen's egg, cow's milk, house dust mite and pollens (Prescott 1997, Szepfalusi 1997, Piccinni 1993). Szepfalusi *et al* showed positive proliferative responses to the cow's milk allergens α-lactalbumin and β-lactoglobulin in 87% and 74% of cord blood samples respectively (Szepfalusi 1997). Similarly, Prescott *et al* identified positive proliferative responses to house dust mite and ovalbumin in 47% and 42% of cord blood samples respectively (Prescott 1997). Interestingly, Piccinni found that whereas proliferative responses to house dust mite were found in virtually all infants, responses to the major grass pollen allergen, Lol p1, were only found in those newborns studied during the spring when grass pollens were at their highest concentration in the environment (Piccinni 1993). A similar seasonal pattern of responsiveness was also observed in a study of birch pollen in Southampton (Jones 1996).

The significance of this priming process to the developing immune system has also been studied. Warner *et al* followed a group of infants through their first year of life having analysed the proliferative and cytokine response of their cord blood lymphocytes to stimulation with β-lactoglobulin and ovalbumin (Warner 1994). The development of atopic eczema with positive allergy skin-prick tests to cows' milk and egg at 1 year of age was significantly associated with raised proliferative responses and defective IFN-γ production to stimulation with betalactoglobulin with a trend for similar responses to ovalbumin. In similar studies, Prescott *et al* assessed the cytokine profile produced by allergen-stimulated cord blood cells using a combination of protein and mRNA analysis (Prescott 1998). Their results suggest a generalised skewing of the allergen-specific immune responses analysed toward a type 2 cytokine phenotype. The subsequent development of these responses is extremely important for the expression of atopy later in life. Those infants who subsequently develop atopic disease continue to express a type 2

cytokine phenotype whereas there is rapid suppression of type 2 responses during the first year of life in non-atopic children (Prescott 1999).

Prenatal Priming of the Immune System to Infectious Agents

Parasites

The influence of chronic parasitic infection during pregnancy on the foetal immune system has long been recognised (Lewert 1969). In areas where parasitic infection is endemic, women are frequently infected through pregnancy resulting in potential priming of the foetus through leakage of parasite-specific antigens and even the migration of entire parasites across the placenta (Loke 1982).

The consequences of this exposure have been studied in a number of parasitic diseases with somewhat inconsistent findings. Children born to mothers with chronic filiarisis in the Cook Islands have reduced T-cell responses *in vitro* to parasite-specific antigens when studied in adolescence (Steel 1994). This observation has led to the suggestion that exposure to filarial antigens *in utero* may induce deletion or paralysis of antigen-specific precursors of functional T-cell clones by a mechanism similar to that of self-tolerance (Clark 1994).

However, other studies have shown that prenatal exposure is associated with sensitisation of the foetus as opposed to tolerance. Cord blood lymphocytes (CBL) from the offspring of shistosome-infected mothers proliferate specifically in response to both parasite antigens (Novato-Silva 1992) and antiidiotypic antibodies (Eloi-Santos 1989). Similar parasite-specific proliferative responses have been observed in the infants of mothers chronically infected with *Trypanosoma cruzi* (Neves 1999), *Onchocerca volvulus* (Soboslay 1999) and *Necator americanus* (Pit 2000).

Increasing evidence has highlighted the importance of the host immune response to disease pathogenesis in parasitic infections. Type 2 immune responses, for example, are associated with host protection and expulsion of intestinal nematodes. In this context, the nature of the immune response produced by prenatal priming may be important in determining the clinical severity of subsequent infection. Malhotra *et al* studied the cytokine profile produced by CBLs stimulated with antigens from the helminths *Schistosoma haematobium* and *Brugia malay* (Malhotra 1999). No cytokine production was detectable from CBLs of North American offspring. However, CBLs of infants from Kenya, where helminthic infection is endemic, demonstrated cytokine production similar to that observed in adults. A similar study by Pit *et al* analysed cytokine production by antigen-stimulated CBLs

from infants of mothers with chronic helminthic infection (Pit 2000). Helminth-specific antigens of *Necator americanus* and *Onchocerca volvulus* induced lymphocyte proliferation and the production of a balanced cytokine profile with no apparent type 1 or type 2 cytokine dominance. This led the authors to suggest that whilst prenatal exposure may prime the immune system, it is postnatal events that ultimately determine the nature of the immune response to subsequent infection.

Bacteria

Evidence for the possibility of prenatal priming to bacterial antigens has largely come from studies of infants born to mothers who were immunised during pregnancy. Gill *et al* studied the specific immune response of infants born to 42 pregnant women following immunisation with tetanus toxoid in the fifth and eight months of pregnancy (Gill, III 1983). Levels of tetanus specific IgM were quantified and tetanus toxoid lymphocyte reactivity assessed in blood at birth, 7 and 13 months of age. Infants of immunised mothers were found to have cord blood levels of IgM antibody comparable to maternal levels whereas babies of control mothers had no detectable IgM. IgM levels remained higher in the immunised group following subsequent immunisation with diphtheria, pertussis and tetanus vaccine; with significantly raised levels still evident 13 months after birth. Lymphocyte reactivity was also increased in the immunised group throughout the first year of life. These data suggest that transplacental immunisation of the foetus occurs following immunisation of the mother to tetanus.

Viruses

There have been sporadic reports in the literature of viral infections in the pregnant mother resulting in sensitisation of the infant.

Following an epidemic of influenza, six of 46 CBL specimens collected between 2 and 8 months later were found to have proliferative responses to antigens from the causative virus strain (Ruben 1981).

Clerici *et al* studied 23 cord blood specimens of infants born to mothers seropositive for Human Immunodeficiency Virus (HIV) (Clerici 1993b). Vertical transmission of HIV infection was found in three of the 23 infants using polymerase chain reaction (PCR) and viral culture assays. CBLs were stimulated with five different synthetic peptides corresponding to the envelope region of HIV-1 and their proliferative response measured. Eight out of the 23 CBL specimens responded to two or more of the synthetic peptides.

This suggests that sensitisation to HIV can occur *in utero* without the transmission of whole virus (and hence foetal infection) across the placenta.

Respiratory Syncytial Virus

Two small studies have suggested the possibility of prenatal priming to RSV. Scott *et al* studied the response of 18 CBL specimens to stimulation with RSV antigen prepared by heat inactivation of virus and demonstrated significant proliferation in one of the specimens only (Scott 1981). An earlier study by Sieber *et al* studied the response of cord blood lymphocytes to stimulation with a crude RSV antigen and RSV purified by sucrose density ultracentrifugation. The incorporation of tritiated thymidine was used to quantitate the degree of stimulation compared to controls. Significant stimulation was found in 11 out of 12 cord blood lymphocyte specimens (Sieber 1976).

In conclusion, foetal studies have shown that the cellular immune system is capable of responding to mitogenic stimuli *in vitro* from 17 weeks of gestation. Despite this, the concept of prenatal priming to specific antigens has not been widely accepted, since it challenges traditional immunological concepts that the cellular immune system is naïve at birth. However, over the last 2 decades, an increasing body of evidence has emerged to support such a process for certain allergens and various infectious agents including viruses.

Conclusion and Presentation of the General Hypothesis

The studies described within this thesis were designed to test the following overall hypothesis:

RSV bronchiolitis is associated with a deficient type 1 / excess type 2 immune response.

In addition, this thesis aimed to study the potential occurrence of prenatal sensitisation to RSV and it's immunological consequences. Antigen-specific priming of foetal T-cells can occur *in utero* from 22 weeks gestation and there is some previous evidence to suggest such a process for RSV. The study described in Chapter 3 investigated whether evidence of *in utero* priming to RSV could be observed in cord blood mononuclear cells in relation to exposure of the mother to RSV from 22 weeks gestation. Since prior sensitisation to

RSV has been shown in experimental models to be associated with both protective type 1 and harmful type 2 responses to subsequent RSV infection, the study also investigated whether antenatal priming induced protective type 1 or harmful type 2 cytokine responses.

The immune response to RSV infection appears to have a significant influence over disease severity. Murine studies indicate that prior sensitisation to individual RSV surface proteins followed 3 weeks later by RSV infection can induce polarised cytokine responses which follow broad type 1 and type 2 repertoires. Mice with type 2 cytokine responses developed enhanced disease with pulmonary haemorrhage and eosinophilia while those with type 1 responses had reduced immunopathology and enhanced viral clearance. Similar immune mechanisms have been suggested in human studies of RSV bronchiolitis (Roman 1997, Rabatic 1997), while other similar studies reach directly conflicting conclusions. These studies are conflicting and inconclusive as a result of a failure to control for numerous confounding factors. Part of this thesis (Chapters 4 & 5) describes a carefully designed prospective birth cohort study to control for these factors to investigate whether RSV bronchiolitis is associated with excess type 2/deficient type 1 cytokine responses. The *in-vivo* immune responses of infants to their first natural proven RSV infection was examined through analysis of cytokine production in both nasal lavage fluid and stimulated blood mononuclear cells taken at identical times from onset of symptoms of infection. Subjects were prospectively monitored to determine if infection resulted in bronchiolitis or upper respiratory signs and symptoms alone. Cytokine responses were then analysed in relation to age and disease outcome and virus load and virus clearance were assessed.

The quantity of RSV within the respiratory tract has previously been correlated with disease severity (Buckingham 2000). However, no study to date has analysed the RSV load using a molecular-based quantitation assay or during the early stages of infection in infants within the community. A secondary aim of this thesis was to analyse the relationship between disease severity and changes in viral load during early RSV infection. The study described in Chapter 6 investigates this relationship using a unique real-time PCR assay to quantitate RSV at two defined time points during early infection.

Finally, as another secondary aim, this thesis sought to examine the pathogenic aetiology of respiratory infections during infancy. Previous studies have examined respiratory pathogens using culture-based techniques in the community (Kellner 1988) or using molecular techniques in hospitalised infants (Weigl 2000). However, no previous study has used molecular techniques to detect pathogens in the respiratory tract of infants with signs of respiratory infection in the community. As part of this thesis (Chapter 7) infants

were prospectively followed through their first winter and respiratory secretions collected with each episode of respiratory infection. To establish the incidence of infection during infancy, specific primer pairs for eight separate pathogens were used to detect pathogens within the secretions by RT-PCR.

Chapter 2:

Materials and Methods

Study of Cord Blood Responses to RSV

Over a 5-month period between June 1997 and October 1997, 38 blood specimens were collected from the umbilical cord of randomly selected newly born infants. All infants were delivered at term (>37 weeks gestation) in the delivery suite of Princess Ann Hospital, Southampton. The study was approved by the Joint Ethics Committee of Southampton University Hospitals. Informed consent was obtained from the parents of all recruited infants.

Upon delivery of the infant, the placenta was delivered into a sterile kidney dish. Blood was collected within minutes of delivery by cannulation of the umbilical veins using a 19-gauge needle. The cord blood specimens were collected into glass tubes containing lithium heparin. The amount of blood collected varied between 5 and 13 ml. Blood specimens were transferred to the laboratory for analysis within 12 hours.

Cell Separation

Cord blood mononuclear cells (CBMCs) were isolated from heparinised blood samples by Histopaque (Sigma, UK) gradient centrifugation (1350 x g for 30 minutes). These cells were then resuspended at 1 x 10^6 cells/ml in RPMI-1640 medium supplemented with 10% autologous plasma (filtered through 0.22μm filter Flowpore, ICN)), 2mM glutamine, 100μg/ml streptomycin and 100 IU/ml penicillin.

Total IgA Levels in Cord Blood Specimens – Exclusion of Maternal Blood Contamination.

Contamination by maternal blood was excluded in all samples by ELISA quantitation of total serum IgA. The assays were performed by the immunology department at Southampton General Hospital using an in-house sandwich ELISA method. The ELISA utilised an optimised capture and detection (HRP-conjugated) Rabbit anti-human IgA antibody pair supplied by DAKO (Ely, UK). IgA standards were supplied by the United Kingdom National External Quality Assessment Service (Sheffield, UK).

RSV Culture and UV-inactivation

The A2 strain of RSV was cultured to provide virus stocks in Clone 16 cells (MRC Common Cold Unit, Salisbury, UK). Titration of stored aliquots was carried out by inoculating serial dilutions of virus stock onto Clone 16 cells and observing the cell cultures daily for the development of cytopathic effect (CPE). The viral titer was established by determining the dilution that produced CPE in one-half of the cumulative number of cell cultures (the 50% tissue culture infective dose or $TCID_{50}$) by the Reed-Muench method (Red 1938). RSV culture, titration and $TCID_{50}$ determination were performed by Mrs Gwen Sanderson (Research Technician, University Medicine, Southampton University).

Virus samples were rendered non-infectious by exposure to a 254-nm ultraviolet (UV-) light source at 10cm distance for 10 minutes on ice. The lack of virus infectivity of this preparation was confirmed by culturing them in C16 monolayers and observing for viral cytopathic effect for 5 days. Control uninfected C16 cells were exposed to the same UV-light conditions. The same stock preparations were used throughout each phase of the study to minimise any variability.

To optimise the experimental conditions, time and dose response curves were defined for UV-inactivated RSV from a subgroup of 4 babies, using lymphoproliferation assays. Optimal conditions were identified as UV-inactivated RSV at a multiplicity of infection (MOI) of 1 with an incubation period of 6 days (Appendix 1)

Proliferation Assays

A total of 1×10^5 CBMC per 100µl culture medium were plated onto 96 round-bottom-well microtitre plates (Costar, Northumbria Biologicals, UK). Uninfected negative control C16 cells and cells containing UV-inactivated RSV (MOI 1) were added in triplicate wells. The microtitre plate was incubated for 6 days in a humidified incubator at 37°C with 5%CO_2. As a positive control, cells were also cultured under the same conditions for 3 days with phytohaemaglutinin (PHA, Sigma, Poole ,UK) at a final concentration of 1µg/ml. Samples were labelled with 1µCi of ^{3}H- thymidine per well 16 hours before harvesting, as a measure of DNA synthesis. Cells were then harvested onto glass fibre mats and the thymidine incorporation was measured as disintegrations per minute (dpm) by liquid scintillation counting. The mean of each triplicate was used as a measure of proliferation.

The results of proliferation assays may be expressed in two main ways:

(1) the difference between stimulated cultures and the control cultures (delta dpm)

(2) the ratio of total dpm in the stimulated wells to the control wells (stimulation index).

Whilst the stimulation index provides a good estimation of the stimulation response, it can be misleadingly elevated if the control wells are of low magnitude. Various cut-offs have been used in previous studies with stimulation index values of ≥2 generally regarded as consistent with a positive proliferative response (Jones 1996, Piccinni 1993). More recent studies have also used the delta dpm in the definition of a positive proliferative response, thus avoiding the confounding effects of low control well dpm (Prescott 1999, Prescott 1997). In the current study a positive proliferative response was defined as both:

(1) a delta dpm ≥ 1000

(2) a stimulation index of ≥ 2

CBMC Supernatant Preparation

CBMC suspensions of 1 x 10^6 cells in 1 ml supplemented culture medium were placed in 24 well microtitre plates (Costar, Northumbria Biologicals, UK). Cells were stimulated with uninfected negative control C16 cells or cells containing UV-inactivated RSV (MOI 1) for 24 hours in a humidified incubator at 37°C with 5%CO_2. The supernatants were subsequently collected and frozen at –70°C for later analysis.

CBMC Supernatant Analysis

Measurements of IFN-γ (lower detection limit 5pg/ml), IL-10 (lower detection limit 6pg/ml) and IL-12 (lower detection limit 6pg/ml) levels were measured using matched antibody pairs using the protocol detailed below (Nasal Lavage - Cytokine Measurements in Stored Supernatant). IL-4 (lower detection limit 0.32pg/ml) was measured with a high sensitivity ELISA kit commercially available from CLB (Netherlands). This assay was performed according to the manufacturer's instructions.

Study of Respiratory Infections during Infancy

Study Design (Figure 2.1)

The study was approved by the Joint Ethics Committee of Southampton University Hospitals.

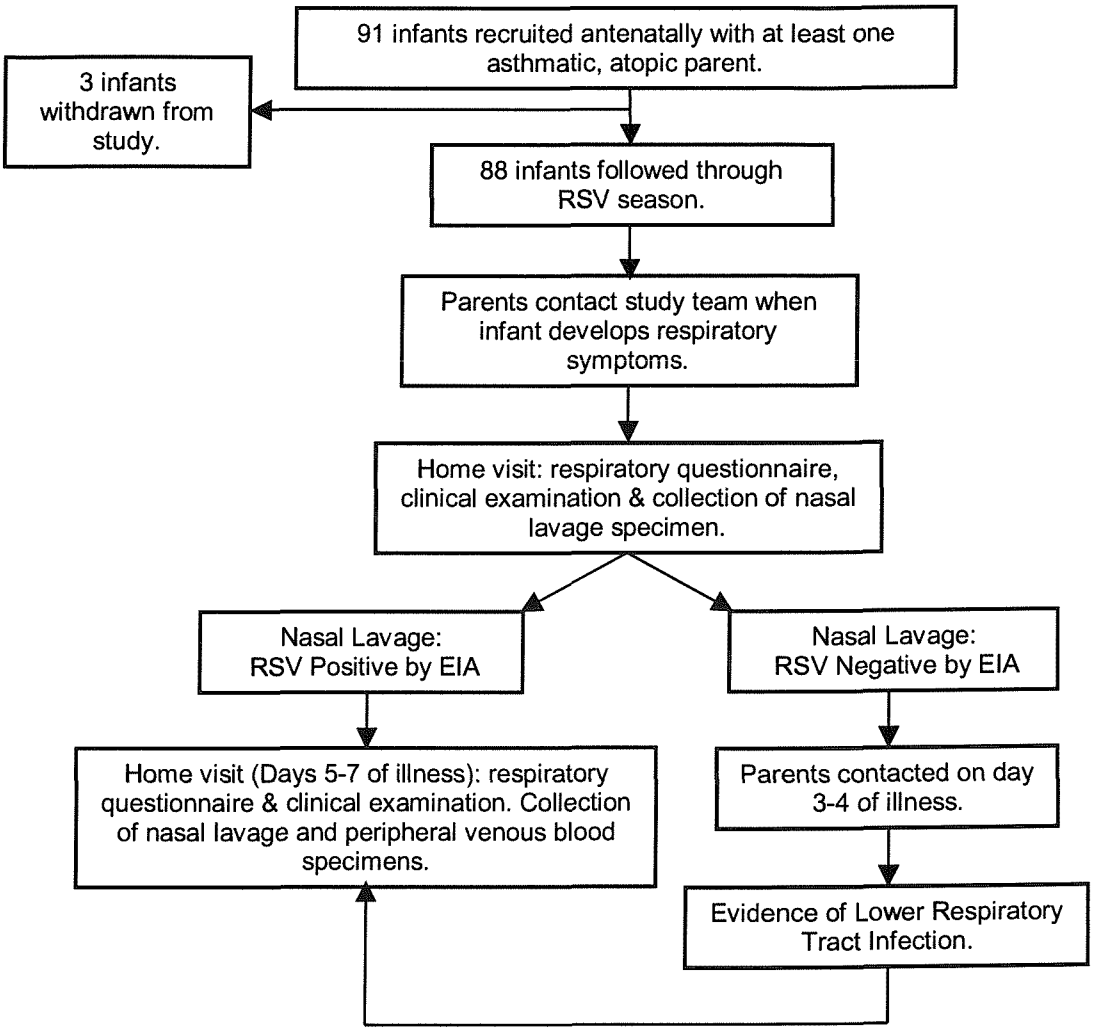

Figure 2.1 Study Protocol

Ninety-one babies were recruited antenatally on the grounds of having at least one atopic, asthmatic parent. Parents attended an initial recruitment interview during the second trimester of pregnancy where they were asked to complete a questionnaire concerning symptoms of allergy (Appendix 2). In addition, details of current medications and past medical history were established by direct questioning.

Each parent then underwent skin prick testing (SPT) to a panel of common aero-allergens.

The allergens tested were selected because they include the most common allergies encountered in the Southampton area. Selected allergens standardised for use in the skin prick test, were all manufactured by ALK-Abelló, UK:

i) house dust mite (*Dermatophagoides* pteronyssinus)

ii) cat dander

iii) dog dander

iv) mixed grasses

v) mixed trees

vi) mould spores *Alternaria, Cladosporidium* and *Aspergillus*

Allergens were kept refrigerated at all times between use to maintain their potency. SPTs were performed according to standardised techniques (Dregborg 1987). Droplets of the test allergen were placed on the skin. The tip of a lancet was inserted into the skin at an angle of 20^{o} to the surface through the droplet of fluid. The tip was gently lifted and removed, only penetrating very superficially. After 15 minutes the wheal size was recorded. Only the wheal size was recorded as an index of response (not the flare). A test was regarded positive if the wheal diameter was greater than 3mm. The test validity was determined by a negative control and a positive histamine control (Dregborg 1987).

Parents were recruited if at least one parent had been diagnosed as asthmatic by a physician, had suffered symptoms of asthma in the previous 12 months and had a positive skin prick test to at least one of the aero-allergens.

The recruited infants were subsequently monitored through their first winter - 1st November 1997 to 31st March 1998. This study period was used to coincide with the peak rate of infantile RSV infections as determined from Southampton General Hospital Public Health Laboratory virological data for the 1996-1997 winter.

Monitoring of Respiratory Disease

Whenever a baby developed respiratory symptoms, parents were asked to telephone the study team within 24 hours. One of the research team (JPL) then visited the family at home. During this visit a respiratory questionnaire was completed to establish the exact nature of the respiratory symptoms and their impact on feeding and sleep (Appendix 3). A clinical examination was then performed to elicit any signs of respiratory disease. Following this a

nasal lavage specimen was collected. Parents were given a daily diary sheet (Appendix 4) to complete for 7 days from the visit date and asked to send the sheets in a prepaid envelope to the research team on completion.

If the nasal lavage specimen tested positive for RSV by enzyme immunoassay (EIA), a further home visit was made 5 to 7 days after the initial onset of respiratory symptoms. At this visit, a further respiratory questionnaire and clinical examination were performed. In addition, another nasal lavage was carried out and a 5-10 ml specimen of peripheral venous blood collected into a lithium heparin blood tube.

If the nasal lavage specimen tested negative for RSV, the family was contacted by telephone 3 to 4 days later to ascertain the infant's progress. If the child had developed symptoms and signs of lower respiratory tract involvement, a further home visit was made 5-7 days after the initial visit as detailed above.

To ensure compliance with the study protocol, study families were contacted by telephone or mail every 3 weeks to enquire as to the health of their infant and as a reminder of the indications for contacting the research team.

Clinical Diagnosis

A clinical diagnosis was made according to predetermined diagnostic criteria:

(1) *Upper Respiratory Tract Infection (URTI).*

Acute URTI was defined as new-onset rhinorrhea with or without fever or cough but without signs of bronchiolitis.

(2) *Acute bronchiolitis (AB).*

AB was diagnosed if the infant had all 3 of tachypnoea (>60 breaths per minute), subcostal recession at rest and inspiratory crackles on chest auscultation.

(3) *Croup*

Croup was defined as a clinical syndrome consisting of inspiratory stridor and barking cough.

Exclusion Criteria

Exclusion criteria were determined prior to the commencement of the study. Infants were excluded if they had one of the following: known hepatic or renal dysfunction,

immunodeficiency, congenital heart disease or chronic respiratory disease (including congenital lung abnormalities, bronchopulmonary dysplasia and asthma).

Two infants were excluded on the basis of these criteria. Two infants (twins) developed symptoms and signs of asthma and required treatment with bronchodilators prior to the start of the study period. One child was withdrawn by the parents for personal reasons.

Venous Blood – Processing and Analysis

PBMC Isolation and Culture

PBMCs were isolated from the collected venous blood by density gradient centrifugation on Histopaque lymphocyte separation media (Sigma, Poole, UK). PBMCs were washed in culture medium (RPMI 1640 medium supplemented with 2mM glutamine, 100μg/ml streptomycin and 100U/ml penicillin) and suspended at 1 x 10^6 cells/ml in culture medium supplemented with 10% filtered autologous plasma in 24 well microtitre plates (Costar, Northumberland Biologicals, UK). Cells were stimulated with phytohaemaglutinin (PHA, final concentration 1μg/ml) or lipopolysaccharide (LPS, final concentration 5μg/ml) for 24 hours at 37°C with 5%CO_2.

Total RNA Isolation

Following culture, cells were pelleted by centrifugation 400g for 5 mins. Cells were lysed by adding 0.25 ml Trizol to each 1 x 10^6 cell pellet. The mixture was vortexed and incubated for 5 minutes at room temperature following which 50μl of chloroform were added. After shaking vigorously by hand for 15 seconds, the samples were incubated at room temperature for 3 mins and then centrifuged at 10000 x *g* for 15 mins. The colourless aqueous phase was transferred to a fresh tube containing 200μl isopropanol at –20°C. The samples were incubated for 10 mins at –20°C before centrifugation at 10000 x *g* for 5 mins at 4°C. Following removal of the supernatant, the RNA pellet was washed with 75% ethanol, vacuum-dried for 1-2 mins and then resuspended in 9μl RNase-free water placed at 55°C for 10 mins. DNA was then removed by adding 1μl of DNase and 1μl DNase buffer and incubating firstly for 15 mins at room temperature followed by 10 minutes at 65°C.

cDNA synthesis

2μl Random Hexamer Primers (Promega, Southampton, UK) and 7μl UHQ water were added to each RNA sample. Total RNA was annealed with random hexamer primers by

incubation at 70°C, then cooled quickly on ice. The reverse transcription cocktail was prepared in bulk. First strand synthesis of cDNA was performed at 37°C for 1 hour in the presence of 8µl 5X RT buffer (Gibco BRL, Paisley, UK), 4µl 0.1M DTT (Promega), 2µl dNTP mixture (10mM), 2µl (200u /µl) Superscript Reverse Transcriptase enzyme (Gibco BRL), 0.8µl RNase-Inhibitor (Rnasin, 40u/µl, Promega) and 4µl Bovine Serum Albumin (Acetylated, 1µg/µl, Promega). Reverse Transcriptase was then inactivated by incubation at 95°C for 5 mins. The resultant cDNA samples were stored at -70°C.

Polymerase Chain Reaction

cDNA was amplified for IFN-γ, IL-4, IL-12, IL-18 and glyceraldehyde phosphate dehydrogenase (GAPDH) using specific primer pairs (Table 2.1). The reaction mixture contained 5µl of cDNA, 1µM of the specific sense and antisense primers, 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100, 1.5 – 2.0 mM of $MgCl_2$, 0.25 mM of each dNTPs, and 0.75 units of *Taq* DNA polymerase (all Promega, Southampton, UK). The specific quantities of primers and $MgCl_2$ varied with each PCR reaction as detailed in Table 2.1.

For all PCR amplifications, negative controls (water only) and appropriate positive controls were included. These controls were processed the same way as the samples throughout the PCR process. Standard precautions were taken to avoid carryover contamination. Pipetting was performed with aerosol-resistant tips, and different biosafety cabinets were used for sample extraction and first amplification or nested amplification. Amplicon detection was performed in a different room.

PCR products were electrophoresed on 2% agarose gels containing VISTRAGreen. The resultant image was analysed by direct fluorescence on a Storm 850 imager (Molecular Dynamics, Sunnyvale, California, USA) using ImageQuant software. Levels of cytokine mRNA expression were determined relative to GAPDH.

Primer	Sense/Anti-sense Sequence	PCR product size (bp)	Primer Concentration	$MgCl_2$ Concentration	Cycle Profile
GAPDH	GGGAAGGTGAAGGTCGGAGT TGGAAGATGGTGATGGGATTTC	661	1μM	1.5 mM	94°C / 15 secs; 50°C / 20 secs; 72°C / 30secs
IL-4	AACACAACTGAGAAGGAAACCTTC GCTCGAACACTTTGAATATTTCTC	276	1μM	1.5 mM	94°C / 15 secs; 50°C / 20 secs; 60°C / 30 secs; 72°C / 30secs
IL-12	ATGTCGTAGAATTGGATTGGTATCCG GTACTGATTGTCGTCAGCCACCAGC	358	1μM	1.5 mM	94°C / 15 secs; 50°C / 20 secs; 72°C / 30secs
IL-18	ATGGCTGCTGAACCAGTAGAAGACA ACTTTTTGTATCCTTGATGTTATCAGGAG	571	1μM	2 mM	94°C / 15 secs; 55°C / 20 secs; 72°C / 30secs
IFN-γ	ATGAAATATACAAGTTATATCTTGGCT GATGCTCTTCGACCTCGAAACAGCAT	501	1μM	2 mM	94°C / 15 secs; 50°C / 20 secs; 72°C / 30secs

Table 2.1 Primer sequences, reaction conditions and cycle profiles for cytokine mRNA amplification

Nasal Lavage – Collection and Processing

The infant was held in the supine position and a soft latex/rubber 8 FG (Vygon, UK) catheter introduced into one nostril with the tip positioned in the nasopharynx. 2.5 ml of phosphate buffered saline (PBS) at room temperature was then instilled into the nostril using a needle-less syringe with simultaneous suction through the catheter (Figure 2.2). The catheter was attached to a standard mucus extractor (Vygon UK Ltd, Gloucester, UK) connected to a portable suction device and was gradually withdrawn through the nasal cavity as the PBS was infused slowly over 8-10 seconds. This procedure was then repeated in the other nostril. After collection, the nasal lavage fluid was immediately placed on ice and returned to the laboratory for analysis within 3 hours.

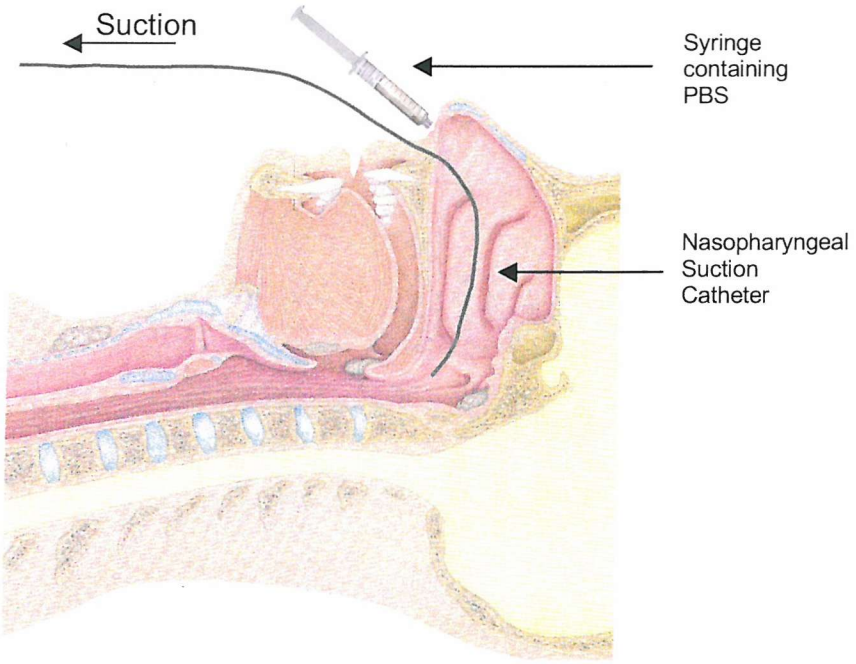

Figure 2.2 Nasal Lavage

The infant is held in the supine position and a soft catheter is introduced into one nostril with the tip in the nasopharynx. 2.5 mls of room temperature phosphate buffered saline is then instilled into the same nostril with simultaneous suction through the catheter.

The total unprocessed specimen was measured and vortexed vigorously for 1 minute. An aliquot of the vortexed sample was then tested for the presence of RSV using a commercially available enzyme immunoassay according to the manufacturer's instructions (RSV Testpack, Abbott Diagnostics). A further 500µl aliquot was mixed with 2 mls of virus transport medium (Hanks balance salt solution 10% (Flow labs, Irvine, UK), bovine serum albumin 0.5%, sodium bicarbonate 0.015M, amphotericin 20mg/ml, ciprofloxacin 40 m g/ml) for storage at -70°C. The remaining specimen was filtered through a

100μm cell strainer to remove mucus and cell debris and centrifuged at 400g for 10 minutes. The supernatants were separated, put into aliquots and stored at -70°C until analysis.

Nasal Lavage - Cytokine Measurements in Stored Supernatant

IFN-γ, IL-10 and IL-12 levels in nasal lavage supernatant were measured using commercially available matched antibody pairs optimised for sandwich ELISA (Pharmingen, San Diego, CA). These assays were performed using an enhanced protein-binding 96 well ELISA plate (Nunc Maxisorp, Pharmingen, San Diego, CA). 50 μl of the coating antibody for each cytokine was added to each well and incubated overnight at 4°C. The plate was then brought to room temperature and washed with PBS. All subsequent washes were with PBS-Tween 20 (0.005%). 200μl of blocking buffer (10% fetal bovine serum in PBS) were then added to each well and the plate was incubated at room temperature for 2 hours to prevent non-specific binding. Following washing, standards (Pharmingen, San Diego, CA) and samples were added to the plate (100μl per well) and incubated at room temperature for 3 hours to allow binding to the coating antibodies. Non-specific binding was found to be a problem with initial assays and this was subsequently resolved by a 50% dilution of all specimens (using standards diluent). After washing, a second biotinylated anti-cytokine antibody was then added (100μl per well) to respective cytokine plates, and incubated for 1 hour at room temperature to allow binding to adherent cytokine. After washing to remove excess antibody, horseradish peroxidase (HRP) conjugated avidin (Pharmingen, San Diego, CA) was added for 30 minutes to bind to the biotinylated site on the conjugated antibody. Following removal of non-bound avidin-HRP conjugate, a colour product was formed with the addition of orthophenylenediamine dihydrochloride substrate solution and was terminated by stop solution (1M H_2SO_4). The optical density, read at 450nm by a microtitre plate reader, was proportional to the cytokine level in the original samples and standards.

IL-4, IL-5 and IFN-α were measured using commercially available high sensitivity ELISA kits. The IL-4 ELISA was purchased from CLB (Netherlands), the IL-5 ELISA from Biosource (California, USA) and the IFN-α ELISA from Amersham Biosciences (Little Chalfont, UK). All assays were performed according to the specific manufacturer's instructions provided with each kit.

Assays for IFN-γ, IL-4, IL-10 and IL-12 were performed in duplicate. Only assays with an inter- and intra-assay variation coefficient of less than 10% were accepted. Single estimations of IL-5 and IFN-α were performed due to limited sample quantities.

Standard curves were constructed using serial dilutions of recombinant human cytokines of known concentration. Computer software (Excel 97, Microsoft, USA) was used for standard curve fitting according to manufacturers' guidelines. The sensitivity of each assay was defined as the mean background signal plus 3 standard deviations (Davies 1994).

The ELISA sensitivities for each cytokine were as follows:

IFN-γ	5 pg/ml
IL-10	6 pg/ml
IL-12	6 pg/ml
IL-4	0.32 pg/ml
IL-5	0.35 pg/ml
IFN-γ	0.48 pg/ml

Nasal Lavage – cDNA Synthesis

RNA Isolation

A 50μl aliquot of the stored specimen was diluted in 200μl of ultra high quality (UHQ) water and 250μl of Trizol reagent (GibcoBRL) added. The mixture was vortexed and placed on ice for 15 minutes following which 50μl of chloroform were added. After shaking vigorously by hand for 15 secs, the samples were placed on ice for 3 mins and then centrifuged at 10000 x *g* for 15 mins. The colourless aqueous phase was transferred to a fresh tube and 800μl isopropanol added. The samples were incubated overnight at –20°C before centrifugation at 10000 x *g* for 20 mins. Following removal of the supernatant, the RNA pellet was washed with 70% ethanol, vacuum-dried for 5-10 mins and then resuspended in 20μl RNase-free water and 1μl RNA guard (Pharmacia Biotech, Little Chalfont, UK) at 50°C.

Reverse Transcription

2.5μl Random Hexamer Primers (Promega, Southampton, UK) and 4μl UHQ water were added to each RNA sample. Total RNA was annealed with random hexamer primers by incubation at 70°C, and then cooled quickly on ice. The reverse transcription cocktail was prepared in bulk. First strand synthesis of cDNA was performed at 37°C for 1 hour in the presence of 10μl 5X RT buffer (Promega), 5μl 0.1M DTT, 1.25μl dNTP mixture (10mM), 2μl (10u /μl) superscript reverse Transcriptase (Life Technologies, Paisley, UK) and 6μl UHQ water. The resultant cDNA samples were stored at -70°C.

Nasal Lavage – Respiratory Pathogen Detection

cDNA was amplified for the presence of respiratory pathogen DNA using individual PCR reactions. Amplification was performed on a model 9600 DNA Thermal Cycler (Perkin-Elmer) programmed specifically for each PCR reaction. The PCR products were sized by gel electrophoresis on 2% agarose gels containing VISTRAGreen (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the image analysed using a Storm 850 imager (Molecular Dynamics, California, USA) using ImageQuant software.

For all PCR amplifications, negative controls (water only) and appropriate positive controls were included. These controls were processed the same way as the samples throughout the PCR process. Standard precautions were taken to avoid carryover contamination. Pipetting was performed with aerosol-resistant tips, and different biosafety cabinets were used for sample extraction and first amplification or nested amplification. Amplicon detection was performed in a different room.

Respiratory Syncytial Virus

RSV nucleic acid was amplified using a nested PCR. The primers used were specific for the 22K membrane protein (O'Donnell 1998). For the first amplification, 3µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 2.5mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK). For the second amplification, 3µl of PCR product was made up to a volume of 50µl with the same reaction mixture as for the first amplification.

RSV PCR - First Amplification Reaction	
Sense Primer (length)	ATGTCACGAAGAATCCTTGC (21)
AntiSense Primer (length)	TAGCTCTTCATTGTCCCTCAG (21)
Cycle Profile	94°C for 2 mins
	then 30 cycles - 94°C / 20 ''; 50°C / 20''; 72°C / 30''
Product Size	360 bp
Second Amplification Reaction	
Sense Primer (length)	GAGGTCATTGCTTAAATGG (19)
AntiSense Primer (length)	GCAACACATGCTGATTGT (18)
Cycle Profile	94°C for 2 mins
	then 25 cycles - 94°C / 20 ''; 50°C / 20''; 72°C / 30''
Product Size	259 bp

Adenoviruses

The adenovirus PCR utilised primers specific for the to the hexon gene (Hierholzer 1993). 5µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 3.5mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK).

Adenovirus PCR	
Sense Primer (length)	GCCGAGAAGGGCGTGCGCAGGTA (23)
AntiSense Primer (length)	TACGCCAACTCCGCCCACGCGCT (23)
Cycle Profile	94°C for 2 mins
	then 40 cycles - 94°C / 20 sec; 60°C / 20 sec; 72°C / 30secs
Product Size	161 bp

Parainfluenza Viruses

The three main parainfluenza types – parainfluenza 1 (PIV-1), PIV-2 and PIV-3 – were detected using a multiplex PCR. Primer pairs were specific for the haemagluttinin-neuraminidase coding region of PIV-1, PIV-2 and PIV-3 as previously described (Corne 1999). 5µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 2mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK).

Parainfluenza Viruses PCR	
PIV-1 Sense Primer (length)	CAGAATTAATCAGACAAGAAGT (22)
PIV-1 AntiSense Primer (length)	AGGATACATATCTGAATTTAAG (22)
Product Size	430 bp
PIV-2 Sense Primer (length)	GGATAATACAACAATCTGCTG (21)
PIV-2 AntiSense Primer (length)	CACAGGTTATGTTGGGATG (19)
Product Size	370 bp
PIV-3 Sense Primer (length)	CTCGAGGTTGTCAGGATATAG (21)
PIV-3 AntiSense Primer (length)	CTTTGGGAGTTGAACACAGTT (21)
Product Size	180bp
Cycle Profile	94°C for 2 mins
	40 cycles - 94°C / 20 s; 60°C / 20 s; 72°C / 30 s

Picornaviruses

Both human rhinoviruses and enteroviruses were detected using a previously validated primer pair (Johnston 1993). The primer pair was specific for the 5' noncoding region of picornaviruses (Gama 1989). 10µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 1.5mM of $MgCl_2$, 1.5mM mM of each dNTPs, 1.5µM of the specific sense and antisense primers and 4.25 units of *Taq* DNA polymerase (all Promega, Southampton, UK).

Picornaviruses PCR	
Sense Primer (length)	GCACTTCTGTTTCCCC (16)
AntiSense Primer (length)	CGGACACCCAAAGTAG (16)
Product Size	380bp
Cycle Profile	40 cycles - 94°C / 30 s; 50°C / 30 s; 72°C / 2 mins 72°C / 4 mins

Mycoplasma Pneumoniae

The primers used for the detection of *Mycoplasma Pneumoniae* were designed to amplify a conserved sequence within the 16S rRNA gene (van Kuppeveld 1994). 3µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 3mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK).

***Mycoplasma Pneumoniae* PCR**	
Sense Primer (length)	AAGGACCTGCAAGGGTTCGT (20)
AntiSense Primer (length)	CTCTAGCCATTACCTGCTAA (20)
Product Size	277 bp
Cycle Profile	94°C for 2 mins then 40 cycles - 94°C / 20 sec; 60°C / 20 sec; 72°C / 30sec

Coronaviruses

The two major serotypic groups of coronavirus were detected using a multiplex nested PCR. The primers were specific for the nucleocapsid coding region of the LP strain of human coronavirus 229E and of OC43 as previously described (Myint 1994). For the first amplification, 3µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 2mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK). For the second amplification, 3µl of PCR product was made up to a volume of 50µl with the same reaction mixture as for the first amplification.

Coronaviruses PCR - First Amplification Reaction	
229E Sense Primer (length)	GGTACTCCTAAGCCTTCTCG (20)
229E AntiSense Primer (length)	TGCACTAGGGTTAATGAAGAGG (22)
Product Size	370 bp
OC43 Sense Primer (length)	AGGAAGGTCTGCTCCTAATTCC (22)
OC43 AntiSense Primer (length)	TGCAAAGATGGGGAACTGTGGG (22)
Product Size	450 bp
Cycle Profile	94°C for 2 mins 30 cycles - 94°C / 20 ''; 60°C / 20''; 72°C / 30''
Second Amplification Reaction	
229E Sense Primer (size)	TTTGGAAGTGCAGTGTTGTGG (22)
229E AntiSense Primer (size)	GACTATCAAACAGCATAGCAGC (22)
Product Size	116 bp
OC43 Sense Primer (length)	GTTCTGGCAAAACTTGGCAAGG (22)
OC43 AntiSense Primer (length)	TTATTGGGGCTCCTCTTCTGGC (22)
Product Size	100 bp
Cycle Profile	As for First Amplification

Influenza Viruses

The primer pairs used for detection of the influenza viruses have been previously validated in field studies (Ellis 1997). The primer pairs were designed to amplify the haemagglutinin gene of the two circulating influenza A viruses (H1N1 and H3 N2) and influenza B virus. For the first amplification, 3µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 2.5mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers for Influenza AH1 and Influenza B, 0.125µM of the specific sense and antisense primers for Influenza AH3, and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK). For the second amplification, 3µl of PCR product was made up to a volume of 50µl with the same reaction mixture as for the first amplification.

Influenza Viruses PCR - First Amplification Reaction	
Influenza AH1 Sense Primer (length)	CAGATGCAGACACAATATG (20)
Influenza AH1 AntiSense Primer (length)	AAACCGGCAATGGCTCCAAA (20)
Product Size	1015 bp
Influenza AH3 Sense Primer (length)	CAGATTGAAGTGACTAATGC (20)
Influenza AH3 AntiSense Primer (length)	GTTTCTCTGGTACATTCCGC (20)
Product Size	883 bp
Influenza B Sense Primer (length)	GTGACTGGTATACCACT (20)
Influenza B Antisense Primer (length)	TGTTTTCACCCATATTGGGC (20)
Product Size	900 bp
Cycle Profile	94°C for 2 mins 30 cycles- 94°C/20 ‘’; 50°C/20’’; 72°C/1min 72°C for 10 mins
Second Amplification Reaction	
Influenza AH1 Sense Primer (length)	ATAGGCTACCATGCGAACAA (20)
Influenza AH1 AntiSense Primer (length)	CTTAGTCCTGTAACCATCCT (20)
Product Size	944 bp
Influenza AH3 Sense Primer (length)	AGCAAAGCTTTCAGCAACTG (20)
Influenza AH3 AntiSense Primer (length)	GCTTCCATTTGGAGTGATGC (20)
Product Size	591 bp
Influenza B Sense Primer (length)	CATTTTGCAAATCTCAAAGG (20)
Influenza B Antisense Primer (length)	TGGAGGCAATCTGCTTCACC (20)
Product Size	767 bp
Cycle Profile	As for First Amplification

Chlamydia Pneumoniae

Chlamydia Pneumoniae infection was investigated using a nested PCR. The nested primers were selected from published sequences of the ompl gene that encode for the major outer membrane protein (Cunningham 1998). For the first amplification, 3µl cDNA was made up to a reaction volume of 50µl containing final concentrations of 1 x PCR buffer (50mM KCl, 10Mm tris HCl (pH 9 at 25°C), 0.1% Triton x100), 2.5mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.25µM of the specific sense and antisense primers and 2.5 units of *Taq* DNA polymerase (all Promega, Southampton, UK). For the second amplification, 3µl of PCR product was made up to a volume of 50µl with the same reaction mixture as for the first amplification.

***Chlamydia Pneumoniae* PCR**	
First Amplification Reaction	
Sense Primer (length)	AATTCTCTGTAAACAAACCC (20)
AntiSense Primer (length)	ATTAAGAAGCTCTGAGCATA (20)
Product Size	571 bp
Cycle Profile	94°C for 2 mins then 30 cycles - 94°C / 20 ''; 52°C / 20''; 72°C / 30''
Second Amplification Reaction	
Sense Primer (length)	AGCCTAACATGTAGACTCTGAT (22)
AntiSense Primer (length)	CTCTAGCCATTACCTGCTAA (20)
Product Size	487 bp
Cycle Profile	As for First Amplification

Nasal Lavage - Quantitative PCR for RSV F protein

Real-Time Quantitative PCR

Principles of Real-Time Quantitative Fluorescent PCR

Real-time quantitative PCR for RSV F-protein was performed using the TaqMan fluorogenic detection system (TAQMAN™ , Applied Biosystems, USA). The principles of this system are schematically summarized in Figure 2.3. Specific oligonucleotide probes are designed to anneal to the target between the two PCR primers. The probe contains 6-carboxy-fluorescein as the fluorescent reporter dye covalently linked to the 5' end. The quencher dye, 6-carboxy-tetramethyl-rhodamine, is usually covalently linked close to the 3' end. In addition, the probe

contains a 3'-blocking phosphate group to prevent probe extension during PCR cycling. The closeness of the quencher to the reporter emitter means that the reporter fluorescence is suppressed, mainly by Förster-type energy transfer (Livak 1995). During PCR cycling, the probe specifically hybridises to the corresponding template and then is cleaved via the 5'→3' exonuclease activity of *Taq* DNA polymerase. This cleavage results in an increase of fluorescence emission of the 6-carboxy-fluorescein reporter dye, without affecting the emission of quencher dye. This sequence of events occurs in each PCR cycle, in the enzymatic reaction, and in the PCR product accumulation. Because the exonuclease activity of the *Taq* polymerase acts only if the fluorogenic probe is annealed to the target (but cannot hydrolyse the probe free in solution), the increase of fluorescence is proportional to the amount of the specific PCR product.

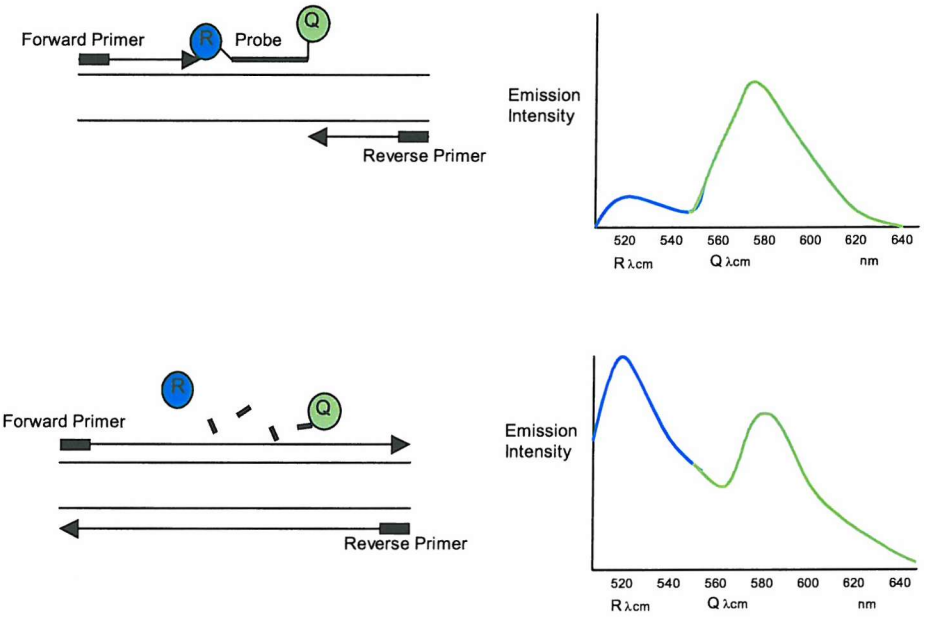

Figure 2.3 Fluorescence emission by Taqman probe reaction

The fluorogenic probe aligns itself along the genomic sequence which is to be amplified. When both dyes are attached to the probe the reporter dye emission is prevented by the quencher dye. The sense and anti-sense primers attach to the relevant sequences of the genome and the extension phase of PCR starts under the influence of DNA polymerase. The extension process causes cleavage of the probe and separates the reporter dye from the quencher dye thus increasing the reporter dye specific fluorescent emission at 518nm.Cleavage removes the probe from the target strand allowing primer extension to continue unimpeded to the end of the template strand.

Reagent and Reaction Conditions

cDNA was prepared as described above and subsequently amplified for RSV F-protein using real-time quantitative fluorescent PCR (TAQMAN^{TM} , Applied Biosystems, USA) with the ABI 7700 sequence detector.

The reagent used for each 25 µl reaction were:

cDNA	5µl
TAQMAN buffer A	2.5µl
$MgCl_2$	1µM
Probe	500nM
Primers	300nM
dATP / dCTP / dGTP	200nM
dUTP	400nM
AmpErase UNG	0.01 u/ml
AmpliTaq Gold	0.05 u/ml
UHQ water	6.125µl

The reaction conditions used for all the reactions were as follows: an initial hold at 50°C for 2 minutes to activate the AmpErase- uracil-N-glycosylase (UNG)®, followed by 10 minutes at 95°C to inactivate the enzyme and activate the AmpliTaq Gold ™. The PCR process consisted of a 2-stage process: firstly 95°C for 15 seconds followed by annealing and extension at 60°C for 60 seconds. This process was repeated for 50 cycles. The probe and primers were designed using Primer Express® Oligo Design software (Applied Biosystems, USA). Sequences were chosen to enable PCR amplification of an area in the 3' conserved region of the F gene (Figure 2.4). The PCR was thus able to detect both A and B strains of RSV and the common A strain sub-types.

Probes were designed according to the manufacturer's guidelines (Perkin-Elmer. 1997). Briefly, the TaqMan probe should *(a)* be 20–40 bases long, to ensure good hybridisation and specificity of binding; *(b)* have a GC content of 40–60% and avoid long runs of a single nucleotide; and *(c)* neither hybridise nor overlap with the forward and reverse primers. It is important to design a probe that forms a more stable hybrid than do the PCR primers; thus, the melting temperature of the probe should be at least 5 °C higher than that of the PCR primers. The primer and probe sequences were:

Sense Primer	TGCAGTCACATTTTGTTTTGCTT
AntiSense Primer	TTCTCAGAGCACTAAGATAGCCTTTG
Probe	TCAAAACATCACTGAAGAATTTTATCAATCAACATGC

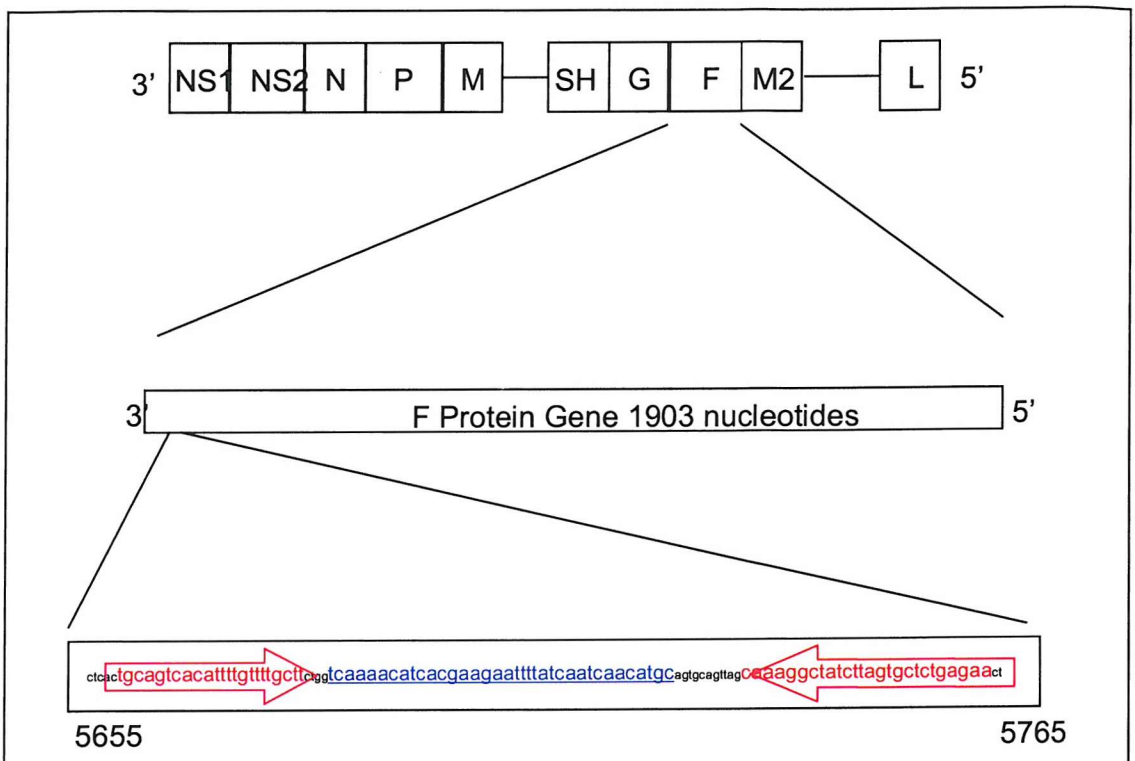

Figure 2.4 Graphical depiction of RSV genome

Figure shows area of F protein genome amplified by quantitative PCR. The nucleotide sequence from nucleotide 5655 to 5765 of the RSV genome is depicted. Forward red arrow represents target sequence for the sense primer. Backward red arrow represents target sequence for the anti-sense primer. The target region for the probe is represented by the blue nucleotide sequence.

The use of AmpErase-UNG® eliminated the risk of contaminating samples. The recombinant enzyme acts on single and double-stranded dUTP containing DNA, releasing uracil from the complex. The enzyme's actions are specific, with no cross-reaction with RNA or dT-containing DNA. The inclusion of this enzyme, coupled to the use of dUTP in the PCR process ensured that contamination of samples by previously amplified reactions was prevented.

Construction of DNA Standards for Taqman

Plasmid DNA standards were constructed by Dr Imran Hussain (Department of Molecular Biology, Southampton University). A 586-bp DNA fragment encompassing the F gene was obtained by PCR from the A2 strain of RSV. The amplified cDNA was digested with *Bam*

H1 and *Xho* 1 (Promega, Southampton, UK), purified, and inserted into the multiple cloning region of the pCI vector (Promega, Southampton, UK).

The plasmid DNA was introduced into competent Escherichia Coli (*E.Coli*) cells using a calcium chloride based transformation procedure. After transformation the *E.Coli* cells were plated on Luria-Bertani (LB)/ ampicillin (5μg/ml) plates and then isolated bacterial colonies were incubated in LB broth.

The plasmid DNA was purified using a modified alkaline lysis procedure according to manufacturer's instructions (Quiagen Plasmid Giga Kit, Quiagen, Crawley, UK). The concentrations of the plasmid preparations were determined by measuring the Optical Density (OD) at 260 nm. Conversion to genome equivalents was performed using the equations 1 OD260 unit equals 50 mcg/ml and one base pair equals 660 g/mol, resulting in a molecular weight of (4589x660) for the plasmid. A standard curve was constructed using 10-fold serial dilutions of the plasmid preparation, representing 1-10^7 copies of DNA/μl

Preliminary experiments used these plasmid standards to determine the lower limit of detection for the Taqman assay. 5 gene copies was the lowest concentration that could be consistently measured.

Statistical Methods and Data Presentation

Specific statistical methods are detailed in each chapter. Unless otherwise stated continuous variables were analysed using the unpaired Student's *t* test and categorical data was analysed by means of χ^2 or Fisher's exact test where appropriate. Correlations were determined using the non-parametric Spearman test. The methods used for analysis of data in this thesis, were chosen in consultation with experienced senior biostatisticians (primarily Dr J. Lorraine Low, Department of Medical Statistics, Southampton University). A p value of less than 0.05 was considered to be significant. Data processing and statistical analyses used SPSS 9.0 (SPSS Inc., Chicago, IL).

Graphics were performed using either SPSS or Powerpoint (Microsoft Corp., Redmond,WA). The final presentation of figures and diagrams was done using Adobe Illustrator 7.0 (AdobeSystems Inc, San Jose, CA).

Chapter 3:

Prenatal Sensitisation to RSV

Introduction

Maternal-Foetal Immune Interactions

Pregnancy is an immunological balancing act in which the mother's immune system has to remain tolerant of foeto-paternal MHC antigens expressed by embryonic tissues and yet maintain normal immune competence for defence against microorganisms. Complex mechanisms have evolved to allow two independent immune systems to coexist in pregnancy, without harming or rejecting each other. Maternal alloantibodies to paternally derived Human Leukocyte Antigen (HLA) are present in up to 15% of normal term deliveries (Johnson 1989). Various mechanisms have evolved to protect the foetus from these potentially harmful antibodies. Maternal alloantibodies specific for foetal HLA alleles will bind to these antigens on macrophages, fibroblasts and endothelial cells thus preventing their entry into the foetal circulation. In certain conditions such as Rhesus Disease this "blocking" system fails with well-recognised consequences. Maternal autoantibodies to leucocytes are also present in pregnancy. These include anti-idiotypic antibodies to foetal HLA-specific T cell receptors, antibodies that block Fc receptors on B-cells and antibodies that inhibit other T-cell functions (Johnson 1989).

The placenta separates foetal and maternal blood and lymphatic systems and it is the foetal trophoblast that plays the major role in evading recognition by the maternal immune system. Trophoblast cells fail to express classical MHC class I or class II molecules and the extravillous cytotrophoblast cells strongly express the nonclassic MHC gene encoding HLA-G, which might downregulate NK cell function (Schmidt 1993). In addition, the trophoblast expresses Fas ligand - a membrane protein that mediates apoptosis through its interaction with Fas. This confers immune privilege since maternal immune cells that express Fas will undergo apoptosis at the placenta/decidua interface (Guller 1999). A third protective mechanism exploited by the trophoblast is the expression of the complement regulatory proteins CD46, CD55, and CD59 which appear to play an important role throughout gestation in protecting the foetus from maternal complement (Holmes 1992).

Pregnancy is associated with decreased cell mediated immunity and a reciprocal increase in humoral immunity, without compromising general maternal immune function. This is mediated by a complicated cytokine network in the utero-placental tissues, under hormonal influences. There is a dramatic increase in both oestrogens and progesterones during pregnancy. Oestrogen enhances humoral immunity (Lin 1993) via a complex mechanism that is at least partly mediated by increased IL-10 production from monocytes (Kanda 1999). Progesterone functions as a potent inducer of type 2 cytokine production by T cells (Piccinni 1995) and enhances T suppressor function (Holdstock 1982). These hormonally-mediated effects promote foetal growth and enable a symbiotic relationship to develop between foetal and maternal immunity. The utero-placental tissues contain many activated IL-1 producing macrophages and cytokines such as GM-CSF and IL-3 promote growth and differentiation of the trophoblast. (Armstrong 1989). Recent studies have identified a cytokine milieu at the materno-foetal interface that is deviated toward a type 2 immune response. Type 1 cytokines appear to interfere with the delicate immunological balance that is essential for a successful pregnancy. The type 1 cytokines IL-2 and IFN-γ are known to activate NK cells. Activated NK cells have been shown to attack trophoblasts and to play an important role in spontaneous abortion (Gendron 1988). IFN-γ also decreases HLA-G expression and increases expression of the immunogenic HLA II proteins that enhance cell mediated immune responses (Rodriguez 1997). These potentially deleterious effects are countered by the production of type 2 cytokines such as IL-4, IL-5 and IL-10 within the utero-placental unit (Wegmann 1993).

The Placental Barrier

The placenta is traditionally regarded as a barrier between the mother and foetus, excluding harmful substances and organisms from the developing foetus. Macromolecules including foreign proteins, such as drugs, can be exchanged between the mother and foetus. Most protein in the foetus is synthesised *de novo* using the amino acids transferred by different transport mechanisms in the microvillous membrane (Battaglia 2001). However, recent evidence has highlighted the ability of both peptides and proteins to be transferred across the placenta. The foetal response to the arrival of maternally derived proteins into the circulation is determined primarily by the stage of development. Should the immune system be capable of mounting a response, then the foetus may develop specific immunity and hence become primed to the protein.

Mechanisms of Prenatal Sensitisation

Prenatal sensitisation of the fetal cellular immune response requires at least three crucial factors to be present. Firstly, the fetal immune system must be developed sufficiently to mount a specific T-cell response. Secondly, the priming antigen / protein must be present in the maternal bloodstream in a suitable form to enable transfer across the placenta and into the foetal circulation. Thirdly, a mechanism must exist which enables transfer of the priming antigen across the placenta in an immunogenic form.

Ontogeny of the Fetal Cellular Immune Response

Lymphocytes can be first identified in the primitive yolk sac from approximately 3 weeks gestation and subsequently in the thymus from 8-9 weeks gestation. At 10 weeks gestation, some lymphocytes have matured sufficiently to demonstrate proliferative responses to the mitogen PHA and histocompatibility antigens (Toivanen 1981). Proliferative responses are dependent on the production of IL-2 by lymphocytes and the cell surface expression of its receptor CD25. CD25+ cells increase with gestational age and are found at 50% of adult levels at 22 weeks gestation (Warner 1996). This progressive phenotypic maturity is paralleled by functional maturity with a gradual increase in mitogenic responsiveness throughout pregnancy (Jones 1996). Antigen-specific lymphocyte responses develop somewhat later in gestation. Between 20 and 22 weeks gestation, the fetal thymus contains larger numbers of antigen-specific binding thymocytes than in neonatal or adult thymic tissue (Dwyer 1970). The ability of T cells to proliferate in response to environmental allergens has been studied in cord blood samples from prematurely born infants and aborted foetuses (Jones 1996). A positive proliferative response to β-lactoglobulin, ovalbumin and beech pollen is first observed at approximately 22 weeks gestation. This would suggest that prenatal exposure to an antigen from around 22 weeks gestation may result in primary sensitisation to that antigen.

Simultaneous Presence of Antigens in Maternal and Foetal Circulations

Animal studies have shown the presence of circulating food antigens such as β-lactoglobulin and ovalbumin in the serum of pregnant rats after feeding (Dahl 1984). These proteins are also found in the fetal circulation often at higher levels than in the maternal blood (Dahl 1984). Recent evidence in humans has shown a similar relationship for both tetanus antigen (TT-Ag) (Malek 1998) and the house dust mite antigen Der p 1 (Holloway

2000). Malek *et al* found a high ratio of fetal to maternal TT-Ag in blood samples collected at birth (Malek 1998). Similarly, Holloway *et al* found that Der p 1 was detectable simultaneously in the maternal blood and cord blood of 25% of paired samples analysed with significantly higher levels identified in the cord blood specimens (Holloway 2000). The presence of TT-AG and Der p 1 in the umbilical-cord plasma infers that antigen from the maternal circulation can cross the placenta under certain conditions.

Mechanisms of Maternofoetal Exchange Across the Placenta

Two main cellular layers separate the maternal and foetal blood streams during pregnancy – the syncytiotrophoblast and the foetal capillary endothelium. There appear to be four primary mechanisms of transfer across these layers:

(1) *Flow-Limited Diffusion* – this is the mechanism by which small hydrophobic molecules such as the respiratory gases move across the placenta. This mode of transfer is limited only by the rate at which molecules arrive at the placenta in the maternal blood and then are taken from the maternofoetal interface by the foetal bloodstream (Schroder 1995).

(2) *Paracellular / Transcellular Diffusion* - Both in vivo and in vitro studies have shown that the permeability of the human placenta to inert, hydrophilic molecules (e.g. inulin, mannitol) is proportional to their coefficients of free diffusion in water. These data would therefore suggest that transfer by paracellular diffusion can occur for such molecules. However, the anatomical correlate for the paracellular route of diffusion in the human placental villus is controversial since the syncytiotrophoblast is a true syncytium. The situation is further confused by evidence for transcellular diffusion of certain hydrophilic molecules such as mannitol. Various experimental findings have gone some way to explain this apparent dichotomy. For example, the syncytiotrophoblast is crossed by a network of tubular structures that may provide a conduit for diffusion (Kertschanska 1997).

(3) *Transport Protein-Mediated Transfer*- The transfer of nutrients from the mother to the foetus and the elimination of metabolic waste products from the foetus are also facilitated by various transporters that are expressed in the maternal-facing brush-border membrane and the fetal-facing basal membrane of the syncytiotrophoblast. An ever-increasing array of specific transport proteins has been identified in the syncytiotrophoblast including transporters for amino acids, organic cations and anions, prostaglandins and vitamins.

(4) *Endocytosis / Exocytosis*- Large molecules such as immunoglobulin G appear to be transferred across the syncytiotrophoblast by endocytosis at the maternal-facing plasma

membrane, vesicular diffusion across the cytosol and exocytosis at the foetal-facing plasma membrane (Malek 1995).

The exact mechanisms by which proteins are transferred from the maternal to foetal circulations have not been defined. Studies using radiolabelled proteins show that the rate of increase in protein concentrations entering the foetal circulation, varies inversely with the square root of the molecular weight (Gitlin 1974). These studies included proteins of increasing molecular weight including transferrin (MW 90000) and fibrinogen (MW 341000). Recent evidence has suggested that this transfer may occur by passive diffusion in many cases (Malek 1998). Interestingly, some proteins of relatively low molecular weight (e.g. growth hormone MW 29000) are excluded from passage across the placenta (Gitlin 1974).

Many other potential mechanisms by which protein or peptide epitopes may enter the foetal circulation have been proposed. At term, there is a close correlation between foeto-maternal ratios of serum tetanus antigen and anti-tetanus IgG (Malek 1998). This observation would suggest the possibility of simultaneous transport of antigen together with IgG antibody using the endocytosis/exocytosis mechanism detailed above. Another possibility is the transfer of anti-idiotypic IgG across the placenta. Anti-idiotypic antibodies bear epitopes of the original antigen and may therefore act to present these epitopes to the foetal immune system.

Foetal Origin of Proliferative Response

There are many potential confounding factors that may explain the proliferative responses of cord blood mononuclear cells to specific antigens. Non-specific cross reactivity, maternal contamination and an exaggerated primary response have all been proposed as possible explanations for the observed proliferation (Piastra 1994). However, maternal contamination is an unlikely explanation given that many cord blood samples that demonstrate specific reactivity to common allergens, have no such response to vaccine antigens such as tetanus (Holt 1995). Moreover, a recent study has confirmed the fetal origin of the proliferating cells using DNA typing (Prescott 1998). T cell clones specific for house dust mite and ovalbumin were derived from cord blood specimens, expanded by polyclonal stimulation and their specificities confirmed by subsequent restimulation. DNA was extracted from the clones and compared with maternal cells and cells from the original infant at 6 months of age using microsatellite genotyping at two gene loci. The clones were

found to be genotypically identical to the samples from the same donor at 6 months of age and distinct from maternal cells

Prenatal Sensitisation to Infectious Agents

Parasites

The influence of chronic parasitic infection during pregnancy on the foetal immune system has long been recognised (Lewert 1969). In areas where parasitic infection is endemic, women are frequently infected through pregnancy resulting in potential sensitisation of the foetus through leakage of parasite-specific antigens and even the migration of entire parasites across the placenta (Loke 1982).

The consequences of this exposure have been studied in a number of parasitic diseases with somewhat inconsistent findings. Children born to mothers with chronic filiarisis in the Cook Islands have reduced T-cell responses *in vitro* to parasite-specific antigens when studied in adolescence (Steel 1994). This observation has led to the suggestion that exposure to filarial antigens *in utero* may induce deletion or anergy of antigen-specific precursors of functional T-cell clones by a mechanism similar to that of self-tolerance (Clark 1994).

However, other studies have shown that prenatal exposure is associated with sensitisation of the foetus as opposed to tolerance. Cord blood mononuclear cells (CBMCs) from the offspring of shistosome-infected mothers proliferate specifically in response to both parasite antigens (Novato-Silva 1992) and antiidiotypic antibodies (Eloi-Santos 1989). Similar parasite-specific proliferative responses have been observed in the infants of mothers chronically infected with *Trypanosoma cruzi* (Neves 1999), *Onchocereca volvulus* (Soboslay 1999) and *Necator Americanus* (Pit 2000).

Increasing evidence has highlighted the importance of the host immune response to disease pathogenesis in parasitic infections. Type 2 immune responses, for example, are associated with host protection and expulsion of intestinal nematodes. In this context, the nature of the immune response produced by prenatal sensitisation may be important in determining the clinical severity of subsequent infection. Malhotra *et al* studied the cytokine profile produced by CBLs stimulated with antigens from the helminths *Schistosoma haematobium* and *Brugia malay* (Malhotra 1999). No cytokine production was detectable from CBMCs of North American offspring. However, CBMCs of infants from Kenya, where helminthic infection is endemic, demonstrated cytokine production similar to that observed in adults. A

similar study by Pit *et al* analysed cytokine production by antigen-stimulated CBMCs from infants of mothers with chronic helminthic infection (Pit 2000). Helminth-specific antigens of *Necator americanus* and *Onchocerca volvulus* induced lymphocyte proliferation and the production of a balanced cytokine profile with no apparent type 1 or type 2 cytokine dominance. This led the authors to suggest that whilst prenatal exposure may prime the immune system, it is postnatal events that ultimately determine the nature of the immune response to subsequent infection.

Bacteria

Evidence for the possibility of prenatal sensitisation to bacterial antigens has largely come from studies of infants born to mothers who were immunised during pregnancy. Gill *et al* studied the specific immune response of infants born to 42 pregnant women following immunisation with tetanus toxoid in the fifth and eight months of pregnancy (Gill, III 1983). Levels of tetanus specific IgM were quantified and tetanus toxoid lymphocyte reactivity assessed in blood at birth, 7 and 13 months of age. Infants of immunised mothers were found to have cord blood levels of IgM antibody comparable to maternal levels whereas babies of control mothers had no detectable IgM. IgM levels remained higher in the immunised group following subsequent immunisation with diphtheria, pertussis and tetanus vaccine; with significantly raised levels still evident 13 months after birth. Lymphocyte reactivity was also increased in the immunised group throughout the first year of life. These data suggest that transplacental immunisation of the foetus occurs following immunisation of the mother to tetanus.

Viruses

There have been sporadic reports in the literature of viral infections in the pregnant mother resulting in sensitisation of the infant.

Following an epidemic of influenza, six of 46 CBMC specimens collected between 2 and 8 months later were found to have proliferative responses to antigens from the causative virus strain (Ruben 1981).

Clerici *et al* studied 23 cord blood specimens of infants born to mothers seropositive for HIV (Clerici 1993b). Vertical transmission of HIV infection was found in three of the 23 infants using polymerase chain reaction and viral culture assays. CBMCs were stimulated with five different synthetic peptides corresponding to the envelope region of HIV-1 and

their proliferative response measured. Eight out of the 23 CBMC specimens responded to two or more of the synthetic peptides. This suggests that sensitisation to HIV can occur *in utero* without the transmission of whole virus (and hence foetal infection) across the placenta.

Respiratory Syncytial Virus

Two small studies have suggested the possibility of prenatal sensitisation to RSV. Scott *et al* studied the response of 18 CBL specimens to stimulation with RSV antigen prepared by heat inactivation of virus and demonstrated significant proliferation in one of the specimens only (Scott 1981). An earlier study by Sieber *et al* found significant stimulation in 11 out of 12 cord blood mononuclear cell specimens on culturing with a crude RSV antigen.

Aims

The aims of this part of the study were:

(1) to establish the prevalence of prenatal sensitisation to RSV,

(2) to investigate the relationship between RSV prenatal sensitisation and the timing of the yearly RSV epidemic,

(3) to analyse the nature of the immune response generated by the sensitisation process.

Results

Prevalence of Prenatal Sensitisation to RSV

Mononuclear cells were isolated from 36 random umbilical cord blood samples as detailed in Chapter 2. The separated cells were cultured for 6 days in the presence of either uninfected negative control C16 cells or cells containing UV-inactivated RSV. As a positive control, cells were also cultured for 3 days with PHA. DNA synthesis was measured by measuring tritiated thymidine uptake. A positive proliferative response was defined as delta dpm (counts in stimulated wells – negative controls) >1000 with a stimulation ratio (stimulated well counts / negative controls) >2. Of the 36 samples analysed, 7 were found to have a positive proliferative response to UV-inactivated RSV. All samples tested demonstrated a positive proliferative response to PHA.

Relationship between Sensitisation and Timing of RSV Epidemic

Hospital virological data obtained from the Public Health Laboratory Service diagnostic laboratory at Southampton General Hospital was used to establish the timing of the local RSV epidemic. The epidemic was defined as the date of the first (27th October 1996) and last (10th April 1997) detection of RSV in clinical samples sent in to the laboratory by the hospital or General Practitioners in the surrounding area. Using this data the infants were divided into two groups according to whether they were potentially exposed antenatally to RSV after 22 weeks gestation (exposed infants were those born between 27th October and 20th August, n=18), or were not exposed to the RSV epidemic at any time antenatally from 22 weeks gestation (n=18). This division is graphically represented in Figure 3.1.

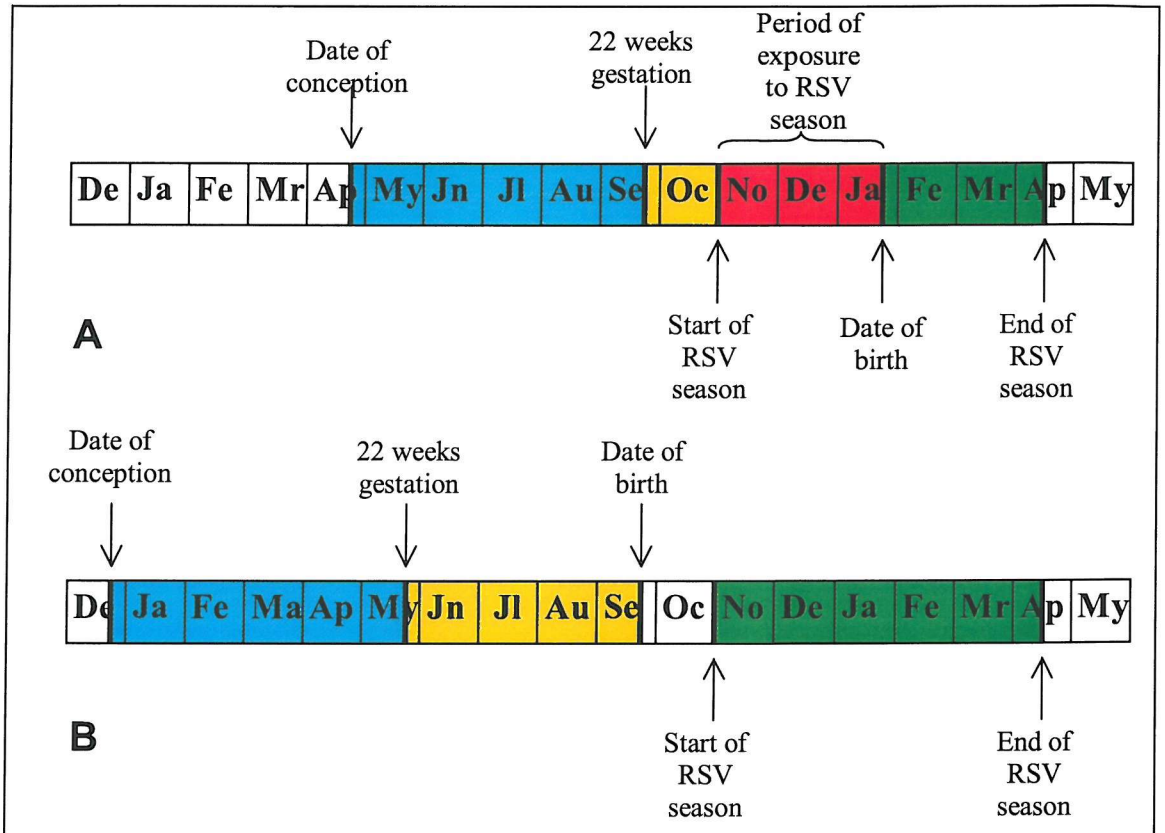

Figure 3.1 **Pictorial Representation of Prenatal Exposure to RSV Epidemic**
(RSV Season: 27th October to 10th April)

(A) A baby born on 20th January reaches 22 weeks gestation on the 26th September and is exposed to the RSV season *in utero* after 22 weeks gestation from 27th October to birthdate. This exposure occurs for all babies born between 27th October and 20th August (i.e. encompassing the majority of those infants who are greater than 4 months of age during the RSV season).

(B) A baby born on 27th September reaches 22 weeks gestation on 24th May and is therefore not exposed to the RSV season *in utero* after 22 weeks gestation. This lack of exposure occurs for all babies born between 21st August and 26th October (i.e. encompassing those babies who are youngest during the RSV season).

Of those not exposed only 1 of 18 had a positive proliferative response to UV-inactivated RSV while, 6 of 18 the potentially exposed group had positive proliferative responses (P=0.035). These data confirmed that antenatal sensitisation to RSV can occur in infants potentially exposed to RSV via the mother from 22 weeks gestation.

Cytokine Production by Cord Blood Mononuclear Cells Stimulated with RSV – Relationship to Prenatal Sensitisation

To investigate the nature of the immune response generated by prenatal sensitisation to RSV, type 1 and type 2 cytokine production was compared in those infants sensitised and not sensitised to RSV antenatally.

IL-4, -10 and –12 were undetectable in the supernatants of cord blood mononuclear cells cultured with UV-inactivated RSV. Following stimulation with UV-inactivated RSV, IFN-γ production from specimens with positive proliferative responses to RSV (sensitised) was 3 fold higher than from those with negative proliferative responses to RSV (not sensitised) (*P* = 0.018, Figure 3.2).

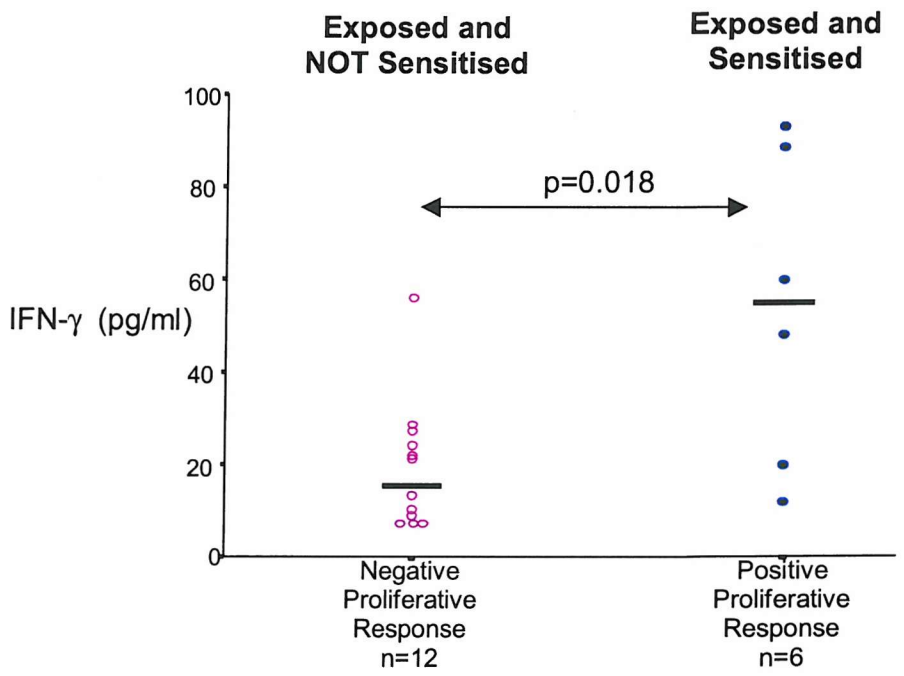

Figure 3.2 **IFN-γ (pg/ml) production by cord blood mononuclear cells stimulated for 24 hours with UV-inactivated RSV.**

Infants were divided into two groups: primed to RSV antenatally (*n*=6, filled circles), or not primed (*n*=12, open circles) according to whether their cord blood mononuclear cells proliferated in response to UV-RSV. IL-4 and IL-10 were not produced in response to RSV. RSV-induced IFN-γ production was significantly augmented in infants with antenatal sensitisation, compared to those not sensitised (*P*=0.018). Figure shows individual values and mean values.

The specificity of the cord blood mononuclear cell response to stimulation by UV-inactivated RSV was demonstrated by the absence of detectable levels of IFN-γ in supernatants of cord blood mononuclear cells cultured with lysates from uninfected C16 cells and the presence of a strong and significant correlation (r = 0.806, P = 0.029) between UV-RSV-induced IFN-γ production and the UV-RSV-stimulation index (Figure 3.3).

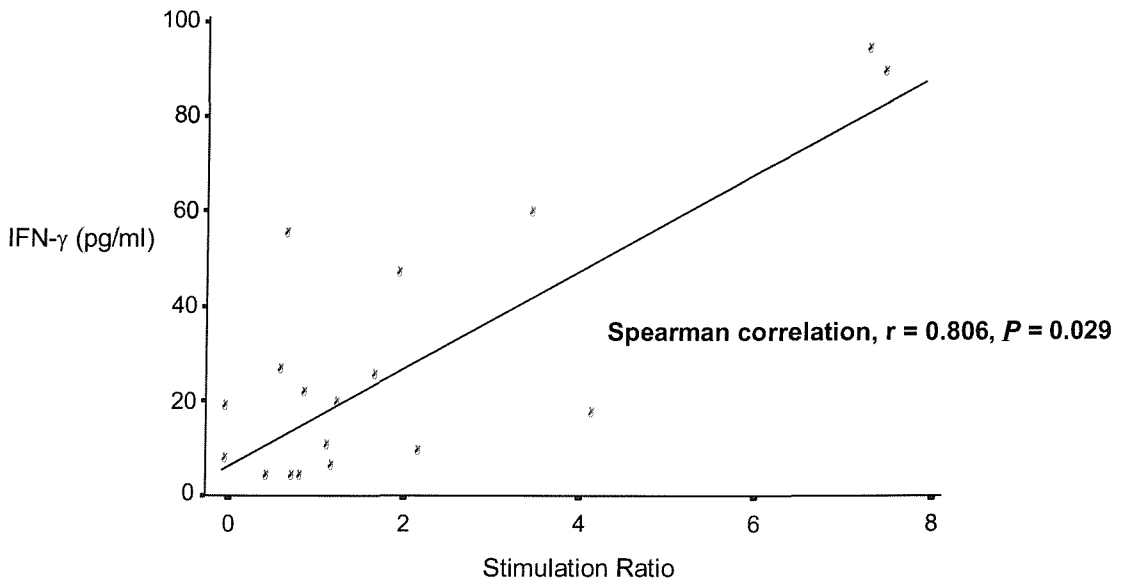

Figure 3.3 Relationship between IFN-g (pg/ml) production and stimulation ratio

Cord blood mononuclear cells were stimulated for 24 hours with UV-inactivated RSV and IFN-γ production measured by ELISA. This is plotted against the stimulation ratio for cord blood mononuclear cells stimulated for 6 days with UV-inactivated RSV. A significant correlation was observed between RSV-specific proliferation (stimulation index) and IFN-γ production (r=0.806, P=0.029).

Total IgA Levels in Cord Blood Specimens – Exclusion of Maternal Blood Contamination.

Contamination by maternal blood was excluded in all samples by ELISA quantitation of total serum IgA. IgA levels were low in all samples (range 1.04 – 8.3 μg/ml) with a maximal concentration of 8.32 μg/ml - i.e. all below the level considered to represent maternal blood contamination (Ownby 1996).

Discussion

The data presented demonstrate that prenatal sensitisation to RSV does occur in one third of infants exposed to a RSV epidemic at the appropriate time of gestation. Furthermore, sensitisation is associated with a type 1 response as evidenced by greater IFN-γ production in those infants with a positive proliferative response to UV-inactivated RSV, compared to those exposed but without such a response.

The proliferative and cytokine responses observed could possibly be explained by maternal contamination of the collected cord blood samples. The foetus does not normally produce IgA *in utero* and IgA does not pass across the placenta from the maternal circulation. The presence of IgA in a cord blood specimen is therefore generally regarded as a sign of maternal contamination. There is, however, considerable debate as to which level of IgA denotes significant admixture of maternal and cord blood. Ownby *et al* used the IgA and IgE levels in 847 cord blood samples to calculate values of IgA consistent with significant maternal contamination (Ownby 1996). Low, but measurable, concentrations of IgA were found in all specimens indicating low-level production of IgA by the normal infant prior to birth. They concluded that cord blood IgA values of greater than 10 μg/ml were predictive of maternal blood contamination of cord blood samples. Other methods for excluding maternal contamination have been suggested including the analysis of the relationship between allergen specific IgE in cord and maternal blood and the simultaneous measurement of maternal and cord blood total IgE and IgA levels (Lilja 1990). However, the disadvantages of these techniques include the necessity of obtaining a maternal sample as well as a considerable increase in the number of assays to be performed. Cord blood IgA remains the most convenient and well established method of excluding maternal contamination provided appropriate limits are applied (Ownby 1996).

The cord blood cytokine responses observed could also represent an exaggerated primary response to RSV. Two aspects of the study were intended to minimise this possibility. Firstly, CBMCs were stimulated with UV-inactivated RSV in order to analyse the secondary immune response to RSV antigens and not the primary immune response resulting from infection with live RSV. Secondly, cytokine production was quantified within the first 24 hours of UV-inactivated RSV exposure thus minimising the opportunity for *de novo* sensitisation of T cells in culture.

It is now well recognised that cord blood lymphocytes are more naïve than adult lymphocytes. The analysis of cell surface markers has provided the most convincing evidence of this immaturity. CD45 (lymphocyte common antigen) is a receptor-linked

protein tyrosine phosphatase that is expressed on all leucocytes, and which plays a crucial role in the function of these cells. On T cells the extracellular domain of CD45 is expressed in several different isoforms, and the particular isoforms expressed depend on the particular subpopulation of cell, their state of maturation, and whether or not they have previously been exposed to antigen. The high molecular weight isoform CD45RA antigen is expressed on naive T lymphocytes, and is present on approximately 50% of CD4+ and 75% of CD8+ T cells. By contrast, the low molecular weight isoform CD45RO appears to represent a primed population of memory T lymphocytes. The majority of cord blood lymphocytes have been found to be CD45RA positive whereas the majority of adult lymphocytes bear the CD45RO antigen (Bofill 1994). In addition, it appears that purified $CD45RA^+$ cells from cord blood produce only small quantities of IL-2 on stimulation compared to purified adult $CD45RA^+$ cells (Hassan 1997). Thus cord blood $CD45RA^+$ cells may be more naïve than adult $CD45RA^+$ cells. The apparent immaturity of cord blood T cells is also evident when comparing cytokine responses of cord blood and adult lymphocytes. *In vitro* studies of lymphocyte cytokine production following mitogenic stimulation have found low levels of IFN-γ, IL-2, IL-4 and IL-12 production from cord blood, although certain cytokines, such as IL-6, are secreted at adult levels (Sautois 1997, Liao 1996). Similar results have been obtained using intracellular cytokine staining which enables quantitation of individual cytokine-producing cells (Chalmers 1998). In the current study, RSV stimulation of CBMCs induced only IFN-γ production whilst the other measured cytokines were undetectable. The specificity of this response was confirmed by the absence of detectable levels of IFN-γ in supernatants of PBMCs cultured with uninfected C16 cells and the presence of a significant correlation between IFN-γ production and the proliferative response to RSV. The cytokine profile observed in the current study is somewhat surprising given the relatively poor IFN-γ response of cord blood lymphocytes to mitogenic stimuli (Sautois 1997). However, antigen-specific responses often differ considerably from those induced by mitogenic stimuli. Although stimulation with mitogens is an acceptable means of assessing lymphocyte function, it is not always the best *in vitro* correlate of cellular immune responsiveness. Non-specific polyclonal stimulation of the lymphocyte subpopulations forms the basis of such an assay system and is not an accurate measure of specific immune functions. By contrast, antigen-induced proliferation assays provide a valuable insight into the nature of the specific immune response. The specific IFN-γ response observed in the current study, together with the absence of IL-4 production implies a relative type 1 immune response of CBMCs to RSV.

The nature of the immune response produced by prenatal sensitisation to an antigen has important implications for the immunological outcome to subsequent exposures. The consequences of prenatal sensitisation have been most extensively investigated for food and aero- allergens. For example, CBMCs from infants with evidence of prenatal exposure to the milk protein β-lactoglobulin demonstrate a deficient type 1 immune response to this protein (Warner 1994). It is these same infants that subsequently develop atopic eczema and a positive skin prick test to milk – both outcomes of a relative type 2 immune response to milk allergens. Similarly, house dust mite and ovalbumin have been shown to produce a generalized skewing of allergen-specific immune responses toward the atopy-associated type 2 cytokine phenotype (Prescott 1998). The immunological consequences of prenatal sensitisation to infectious agents have received far less attention. However, the limited data available do not imply a similarly polarised type 2 response as observed to allergens. As discussed above, two studies of the CBMC cytokine response to stimulation with various helminthic antigens have identified balanced type 1/type 2 cytokine profiles with significant production of IFN-γ in the majority of cases (Pit 2000, Malhotra 1999). Interestingly, CBMCs from Kenyan infants (where tuberculosis and BCG vaccination rates are high) were found to produce significant levels of IFN-γ in response to purified protein derivative (PPD) but little or no IL-4 or IL-5 (Malhotra 1999). The findings of the current study appear to concur with this data, further highlighting the apparent differences between the immunological outcomes of prenatal sensitisation to allergens and infectious agents.

Whilst the current study has identified evidence of prenatal sensitisation to RSV, it is only possible to postulate the possible mechanisms by which the foetus is exposed to RSV antigens. As has been detailed above, three important factors are required for prenatal sensitisation to RSV:

(1) Firstly, the foetal immune system must be adequately developed to enable a cellular memory response to occur. The data presented provide evidence of a significant proliferative response to RSV antigen after 22 weeks of gestation. This is in keeping with previous work that has identified evidence of a detectable cell mediated immune response after this time point (Jones 1996).

(2) The second crucial component of sensitisation is the presence of the priming antigen / protein in the maternal bloodstream in a suitable form to enable transfer across the placenta. RSV infection of the respiratory tract does not appear to be associated with viraemia in humans and it has not been possible to recover replicating virus from peripheral blood by culture (O'Donnell 1998). However, *in vitro* studies have previously demonstrated the

ability of RSV to successfully infect and replicate in human mononuclear cells and macrophages (Panuska 1990, Midulla 1989). Furthermore, the expression of RSV antigens on the surface of circulating mononuclear lymphocytes during RSV infection has been demonstrated suggesting *in vivo* infection of these cells (Domurat 1985). Two recent studies have further supported the concept of RSV spread to tissues outside the respiratory tract using PCR technology. The first study investigated two groups of infants – a group of neonates requiring intensive care and a group of infants hospitalised with suspected RSV lower respiratory tract infection (Rohwedder 1998). All infants were followed prospectively for RSV infection with weekly nasopharyngeal and blood sampling. The blood was subsequently analysed for the presence of RSV-RNA using an RT-PCR protocol that incorporated a nested PCR system. RSV-RNA was detected in the blood from over 52% of the infants although no correlation was found between blood RNA and the severity of RSV disease. In a similar study, RSV-RNA was identified by RT-PCR in both whole arterial blood and separated peripheral blood mononuclear cells from infants with RSV infection (O'Donnell 1998). Both genomic and messenger RSV-RNA were detected suggesting the presence of ongoing viral replication within the studied specimens. In summary, whilst RSV cannot be directly cultured from the circulation during infection of the respiratory tract, there is indirect molecular evidence of ongoing RSV replication in the blood stream within mononuclear cells.

(3) The mechanisms by which priming RSV antigens can cross the placenta have not been studied. As noted above, the latest studies have identified the transfer of immunogenic peptides across the placenta coupled to the specific transport of IgG antibody (Malek 1998). Whilst this is a possibility, no evidence exists to support the presence of suitably coupled RSV proteins or peptides within the circulation. Recent molecular evidence suggesting the presence of RSV in blood and mononuclear cells has, however, raised another possible avenue of placental transfer. It has traditionally been believed that the placenta acts as a barrier to maternal blood cells preventing their passage into the foetal circulation. However, recent studies have shown that maternofoetal cellular transfer occurs in a significant number of pregnancies. Through the use of real-time quantitative PCR, maternal nucleated cells have been detected in 24% of cord blood specimens (Lo 2000). Using similar techniques, maternal blood cells have also been identified in foetal blood specimens collected during the second and third trimesters of pregnancy with evidence of both mononuclear and polymorphonuclear cell transfer (Petit 1997). The transfer of RSV-infected maternal cells across the placenta therefore represents a potential mechanism by

which RSV antigens may enter the foetal circulation and subsequently evoke an immune response.

Conclusions

- RSV-specific proliferative responses are observed in one third of cord blood samples taken from infants whose mothers have been potentially exposed to RSV during the local RSV epidemic from 22 weeks gestation. These data indicate that sensitisation to RSV can occur antenatally if the mother is exposed to natural RSV infection at the appropriate stage of gestation.
- In all infants primed to RSV antenatally, stimulation of cord blood mononuclear cells with RSV induces augmented IFN-γ production with no induction of IL-4, IL-10 or IL-12. These data indicate that sensitisation is associated with induction of a relative type 1 cytokine response on stimulation with RSV.

Chapter 4:

The Immune Response of the Upper Respiratory Tract to RSV infection.

Introduction

Anatomy and Physiology of the Nasal Cavity and Nasopharynx

In comparison with the lower respiratory tract, the nose and nasopharynx are relatively accessible and provide an ideal site for the collection of respiratory secretions and/or epithelium. A variety of sampling methods have been used with varying degrees of success. A thorough understanding of the anatomy and physiology of the upper airway is an important prerequisite for the satisfactory execution of many of these sampling techniques.

RSV infection is restricted to the nasal cavity and nasopharynx in the majority of cases. The nasal cavity extends from the external nostrils (anterior nares) to the choanae (posterior nares). The palatine bones, which separate it from the mouth cavity, form the floor of the nasal cavity and its roof is comprised of the ethmoid bone. A bony midline partition, the septum, divides the nasal cavity into a right and left cavity. The lateral wall of each cavity is roughly semicircular in shape and is covered with a highly vascular mucous membrane that adheres firmly to the periosteum of the underlying bone. Three nasal conchae (turbinate bones) project downwards like scrolls from the lateral wall, controlling the calibre of the nasal cavity through erectile tissue located in the overlying mucosa.

The pharynx is a tubelike structure that extends from the base of the skull to the oesophagus and lies anterior to the cervical vertebrae. The nasopharynx is located behind the nasal cavity and extends from the posterior nares to the level of the soft palate. Its walls are highly muscular and its patency is ensured by the rigidity of the surrounding pharyngo-basilar fascia. The adenoids or pharyngeal tonsils are located on its posterior wall and may occlude the nasopharyngeal space if enlarged significantly.

The primary functions of the nasal cavity and nasopharynx are to filter, moisten and warm air as it passes to the lungs. Both are lined with a mucous membrane that has a pseudostratified ciliated columnar epithelium rich in goblet cells and containing many

mucous glands. The epithelium plays a complex role in the defence system against inhaled infective agents. Classically, the continuous cell layer of the epithelial mucosa acts as a protective barrier, but its physiological role is much more complex and varied. Secretions are transported by a collaborative effect of the ciliary movements and the rheological properties of the mucosa. The epithelium also participates in inflammatory responses through interaction with inflammatory cells in the mucosa itself and by its capacity to produce inflammatory mediators. Furthermore, the epithelium contributes to anti-infectious mechanisms via its microorganism receptors, its relationship with immune and inflammatory cells, its participation in mucociliary clearance and finally by the secretion of anti-infectious molecules (lysozymes, IFN-α) by the mucosa.

Upper Airway Sampling

The mucosal lining of the nose and nasopharynx is the most accessible area of respiratory epithelium in the human body. Many sampling techniques have been used to collect nasal/nasopharyngeal secretions but no single technique has gained universal acceptance. An ideal collection technique would (i) enable collection of respiratory samples from children without nasal symptoms, (ii) be well tolerated by all age groups, (iii) not require lavage fluid for specimen collection and (iv) cause no mucosal irritation.

Blowing the nose, nasopharyngeal aspiration and other direct suction techniques produce samples that are undiluted and thus reflect actual *in vivo* concentrations of mediators or inflammatory cells. However, the sample size produced by these methods is often too small for laboratory analyses particularly if there are no nasal signs or symptoms (Klimek 1999).

Various absorption methods have been described based on the use of materials such as cotton strips, filter paper, gauze and rubber foam. The material is introduced into the nasal cavity where secretions are collected by capillary action over a set period of time. Centrifugation is subsequently used to extract the absorbed fluid. The collected specimen accurately reflects "true" nasal concentrations with no confounding dilutional factor. The technique does, however, produce varying degrees of mucosal irritation and is unlikely to be well tolerated by young children.

Methods based on nasal/nasopharyngeal washing involve the introduction of a known volume of Ringer lactate or saline into the nasal cavity either with a syringe or a spray pump. Fluid is then recollected either passively into a collection dish by appropriate

positioning of the patient or actively by suction through a catheter. This technique is well tolerated and produces good sample volumes with minimal mucosal irritation from healthy as well as symptomatic children. The main concern when using lavage techniques is the unknown dilution of the secretions produced by the washing fluid.

Nasal Lavage Techniques

The technique used to collect nasal lavage fluid depends largely on the age and compliance of the subject. In children of school age and above, saline can be instilled into the nasal cavity with the nasal tip pointing toward the ceiling, held for 10 seconds and the specimen then evacuated into a collecting dish by tilting the head forward. This technique has been used in many studies and appears to be safe and well tolerated (Wojnarowski 1998). In young children specimens can be successfully collected with the child in the supine position. Lavage fluid is introduced into each nostril in turn with simultaneous suction through a catheter located in the nasal cavity/nasopharynx.

Both techniques produce ample specimen volumes with over 50% of the lavage fluid generally retrieved. The collected samples contain mucus in varying quantities and require appropriate filtration or the addition of a suitable mucolytic agent such as dithioerythritol.

Dilution Factors

Dilution of respiratory secretions is an inevitable consequence of all nasal lavage techniques. Heikkinen *et al* analysed dilution factors by comparing cytokine concentrations in simultaneously obtained nasopharyngeal aspirates and nasal lavage specimens from 52 children with upper respiratory tract infection (Heikkinen 1999b). The dilution factors of the nasal lavage specimens were found to vary widely between 1.8 and 432 (median 11.2). This large variability constitutes a considerable problem for interpretation of measured concentrations of various substances in the specimens.

Various methods of controlling for dilution factors have been proposed:

(1) *Endogenous Proteins.* A variety of endogenous proteins have been used in an attempt to correct for the unknown dilution of the specimens. Plasma-derived proteins, such as albumin and total protein, can be measured readily in nasal lavage samples but their value is limited by the considerable variation of levels associated with altered mucosal vascular permeability as observed in many inflammatory

processes (Noah 1995). Recent interest has therefore focussed on locally produced proteins which are unaffected by changes in vascular permeability (Heikkinen 1999a). To date, however, no single protein has been found that has minimal variation between different individuals as well as during the course of a pathological process in the upper airway.

(2) *Exogenous Compounds.* Dilution can also be established by the addition of an inert, non-irritant compound to the lavage fluid. Inulin (Cohen 1970), tritiated water, lithium chloride (Virolainen 1993) and others have been described for this purpose. The concentration of the compound is measured in both the nasal wash fluid and the collected nasal lavage specimen. This provides a ratio of dilution that can be used to determine what proportion of the collected specimen consists of nasal secretions. Consequently, the measured substance can be expressed per volume of nasal secretions. The main concern with using exogenous compounds to assess dilution is the potential interactions that they may have with the substance to be measured or with the assay employed for this measurement (Balfour-Lynn 1999).

If dilution factors are ignored there is a consequent increase in intra-individual variability and a reduction in the power of the study to detect statistically significant differences between groups. Therefore differences observed without correction for dilution are likely to become more significant if an appropriate dilution control is applied to the data.

Relationship between Upper and Lower Airway Samples

Our understanding of respiratory disease is greatly enhanced by knowledge of local immune/inflammatory processes in the lung. However, the collection of suitable specimens from the lung is fraught with difficulty, requiring time-consuming and potentially hazardous procedures. Upper respiratory samples are easy to collect using safe, well-tolerated techniques that are relatively quick to perform. A close association between immune/inflammatory processes in the upper and lower respiratory tract would enable valuable insights into lung pathology from the analysis of upper airway samples.

The relationship between upper and lower respiratory tract specimens has been little studied. Healthy volunteers exposed to swine dust demonstrate similar cytokine and cellular changes in both nasal lavage and bronchoalveolar lavage specimens (Cormier 1997). In asthmatic patients, nasal mucosal biopsy specimens demonstrate histological changes that are similar to those found in the bronchial mucosa and it has been suggested that nasal

biopsy might be useful in the treatment of asthma (Poulter 1991). Only one study to date has compared nasal and pulmonary specimens in RSV disease. Joshi *et al* analysed IL-2 levels in nasopharyngeal and tracheal aspirates collected from nine infants requiring mechanical ventilation for bronchiolitis (Joshi 1998). IL-2 levels correlated well between the two sites with comparable absolute values. Whilst these studies suggest a useful role for nasal specimens in the study of lung disease, more research is required to further elucidate the relationship between nasal and pulmonary changes in respiratory disease. Only following such studies will it be clear whether nasal sampling can be used to accurately reflect conditions in the lower airway.

RSV Infection of the Upper Airway

RSV is primarily spread by large droplets or through fomite contamination. Unlike other respiratory viruses such as the rhinoviruses, spread by small-particle aerosol generation is extremely rare (Hall 1981a). As a result, direct spread between individuals requires close contact. More frequently, infection occurs following physical contact (usually the hand) with fomites from surfaces contaminated with infected respiratory secretions. RSV is then subsequently transferred to the nares by rubbing of the nose.

The epithelial lining of the nasopharynx is extremely susceptible to RSV infection. The virus initially attaches to the surface of the epithelium through a process involving both F- and G- surface proteins. The surface proteins appear to interact with cellular heparan sulfate to enhance the avidity of virus binding and thus facilitate the subsequent interactions required for infection (Feldman 2000). Following attachment, the virus penetrates the epithelial cell wall and its envelope becomes incorporated into the host cellular membrane. Viral transcription and translation occur entirely within the cytoplasm with no input from the host genetic apparatus. The resultant proteins and progeny RNA assemble beneath the cell membrane where they become associated with transmembrane viral glycoproteins. Viral components become clustered together within circumscribed regions of the cell surface. From these areas progeny viruses are seen to bud from the cell surface, acquiring an envelope of cell membrane during the process.

The virus spreads readily between epithelial cells and is able to infect neighbouring cells directly without the need for an extracellular phase (Shigeta 1968). Experimental infection of adult volunteers has demonstrated an incubation period of 5-6 days following inoculation with low titres of nasal RSV detectable during this period. (Noah 2000). The onset of symptoms is associated with a dramatic increase in viral shedding. Viral titres of up to 10^7

$TCID_{50}$ per ml of nasal secretion have been identified in young infants on admission to hospital (Hall 1975a) with somewhat lower titres in adult volunteers (Mills 1971). Virus shedding continues for a variable period with high titres usually maintained throughout the first week before falling rapidly (Hall 1976a). RSV is generally undetectable 7 days after hospital admission although certain individuals exhibit delayed viral clearance of up to 3 weeks duration (Hall 1976a).

Immune Response of the Upper Airway to RSV Infection

Eosinophils and Eosinophil Cationic Protein

Whilst traditionally associated with the inflammatory response to infection with parasitic worms, eosinophils are now recognised as having a critical role in many other diseases including allergic disorders such as asthma and allergic rhinitis (Martin 1996). Activation of eosinophils results in their degranulation and the release of preformed granular constituents such as eosinophil cationic protein (ECP), major basic protein and eosinophil peroxidase. The constituents are cytotoxic and cause desquamation and destruction of epithelium.

Cytological evaluation of nasal specimens collected during RSV infection reveals an overwhelming predominance of neutrophils with only small numbers of lymphocytes and mononuclear cells (Everard 1994). Eosinophils do not appear to be present in collected secretions (Everard 1994). Despite this, many studies have identified high levels of the inflammatory mediator ECP in nasal secretions. High nasal ECP levels are not exclusive to RSV with evidence of similar (Colocho 1994), if not considerably higher (Reijonen 1997), levels in many other respiratory viral infections such as parainfluenza. During RSV bronchiolitis, nasal ECP levels are 20-50x higher than serum levels (Colocho 1994). The clinical significance of these elevated levels is not clear. Garofalo *et al* found a strong correlation between nasopharyngeal ECP and the severity of RSV disease as assessed by the initial PaO_2 concentration (Garofalo 1992). However, further studies have not verified this association (Reijonen 1997, Sigurs 1994). Great interest has also been shown in the possibility of predicting subsequent wheezers from nasal ECP levels at the time of RSV infection. Reijonen *et al* studied 87 children aged 1-23 months admitted with a diagnosis of acute bronchiolitis (Reijonen 1997). Those children that subsequently required hospital admission for wheezing had significantly higher concentrations of nasal ECP at entry than those without subsequent hospital admissions. However, the diagnostic criteria used for bronchiolitis would undoubtedly have included children with a first episode of asthma and

only a small proportion (27%) of the studied group were RSV positive. A smaller study using tighter enrolment criteria for initial bronchiolitis, found no evidence of an association between nasal ECP and subsequent bronchial obstructive episodes over the following 2 years (Sigurs 1994). Hence, the potential use of nasal ECP to predict subsequent wheeze requires further clarification.

Antibody Response to RSV Infection

The local antibody response to RSV infection has been thoroughly investigated. Early studies were limited by the use of relatively insensitive assays such as complement fixation or fluorescence antibody staining and the inability of these assays to detect surface protein specific responses. Using these techniques, it was possible to detect RSV-specific IgG, IgA and IgM in the nasal secretions of RSV-infected children (Kaul 1981, McIntosh 1979). An age dependent response was noted with diminished antibody levels in infants less than 6 months of age (Kaul 1981).

With the development of immunoaffinity-purified virus antigens came the ability to develop sensitive ELISA assays. This has enabled the investigation of protein-specific antibody responses as well as the analysis of antibody subclass production. Using an ELISA assay, Murphy *et al* demonstrated the production of nasal IgG and IgA production to both F- and G- surface proteins by the majority of children aged 9-21 months at the time of RSV infection (Murphy 1986b). In keeping with earlier studies, a significant proportion of infants under 8 months failed to mount a response to either protein.

Further investigation has also shown a qualitative difference in the antibody response at different ages. Infants under 6 months of age produce nasopharyngeal IgA against the G protein predominantly. This contrasts with the predominant IgA F antibody response in older children. Furthermore, maternally derived serum IgG antibodies to the F protein are inversely correlated to nasopharyngeal IgA, a fact that suggests a suppressive role of passively acquired immunoglobulin in the generation of local antibody responses to RSV (Yamazaki 1994a).

Interestingly, analysis of infants infected with distinct strains of RSV has demonstrated significant differences in protein specific antibody responses. Antibodies produced to the F protein show a great degree of cross reactivity between RSV strains, whereas antibodies to the G protein appear to be highly strain specific (Yamazaki 1994b).

RSV-specific IgE

IgE is a vital component of the allergic response. The type 2 cytokines IL-4 and IL-13 are important triggers for B cell production of IgE and for continued IgE production. The presence of IgE is therefore regarded as an indirect indicator of a type 2 cytokine response.

The possible role of IgE in the immunopathogenesis of RSV disease has been highly debated for many years. Initial investigations by Welliver *et al* in the early 1980s demonstrated IgE antibodies bound to RSV infected cells in the nasal secretions of infected infants (Welliver 1980) and later free virus specific IgE in the nasal secretions (Welliver 1981) using an antigen capture ELISA. IgE was detected more frequently in infants with lower respiratory tract disease and peak IgE levels were found to correlate with both disease severity (Welliver 1981) and the incidence of subsequent symptoms (Welliver 1986). The results of these initial studies have been difficult to reproduce. Russi *et al* also found evidence of specific IgE to RSV surface proteins in nasal samples from RSV infected children, with levels correlating significantly with disease severity (Russi 1993). However, other investigators have not been able to detect the presence of RSV-specific IgE in nasal specimens, despite similar study populations and the use of similar assay techniques (De Alarcon 2001, Toms 1996). The reasons for this apparent discrepancy are not clear and the role/significance of IgE in RSV disease remains in question.

Local Cytokine Production During RSV Infection of the URT

Interferon-α

Interferon-α is produced by many cell types following viral infection and makes several important contributions to host defence (Bogdan 2000). The binding of IFN-α to its receptor results in a cascade of events that ultimately induce the synthesis of host-cell proteins that inhibit viral replication. IFN-α also increases expression of MHC class I molecules, transporter proteins and various components of the proteasome. This enhances the ability of host cells to present viral peptides to $CD8^+$ T cells should infection occur. Furthermore, IFN-α induces a 20- to 100-fold increase in the cytotoxic activity of natural killer cells.

The local production of IFN-α during the course of RSV infection has received little recent attention. In 1978, two studies examining nasal IFN-α production in RSV infection were published in the same issue of the *Journal of Pediatrics* (McIntosh 1978a, Hall 1978a). Both studies employed a plaque reduction assay to quantify total interferon activity and

both used an international interferon standard to construct a standard curve. Interferon levels were low or undetectable in all collected specimens. IFN production was not found to correlate with RSV shedding or disease severity. By contrast, nasal IFN levels were found to be consistently raised in children infected with influenza or parainfluenza. These findings were subsequently corroborated in infected adults (Hall 1981c).

Two further studies have quantified IFN-α in nasopharyngeal secretions of RSV infected infants using specific immunoradiometric assays (Taylor 1989, Isaacs 1989b). Using this technique, IFN-α was detectable at higher levels and in a greater proportion of subjects than with plaque reduction. However, IFN-α levels were still found to correlate poorly with either disease severity or viral clearance.

Thus, it can be concluded that RSV infection induces a poor host IFN-α response. Furthermore, the lack of correlation of IFN-α levels with either disease severity or viral clearance, would suggest a limited role for IFN-α in recovery from RSV infection.

Chemokines

Chemokines constitute a superfamily of small, inducible, secreted, proinflammatory cytokines involved in a variety of immune responses, acting primarily as chemoattractants and activators of specific types of leucocytes. The two principle chemokine subfamilies are divided according to their arrangement of the first two cysteines that are either separated by one amino acid (CXC chemokines) or are adjacent (CC chemokines). CXC chemokines act preferentially on neutrophils inducing a shape change, chemotaxis, the release of granule contents and the upregulation of adhesion proteins and the respiratory burst (Baggiolini 1992). Interleukin (IL-) 8 is the prototypic CXC chemokine and is produced by a wide range of cells in response to various stimuli including cytokines (e.g. IL-1), tissue growth factors and viral infection. The spectrum of activity of CC chemokines is broader. RANTES and MIP-1-α mediate the trafficking and homing of classical lymphoid cells such as T cells and monocytes as well as being chemoattractant for a range of other cells including eosinophils and basophils (Saito 1997). MCP-1 has similar properties although its activities appear limited to monocytes, lymphocytes and basophils (Baggiolini 1995).

A number of studies have analysed the local production of chemokines during RSV infection. Noah *et al* studied changes in nasal levels of chemokines following artificial infection of adult volunteers with RSV (Noah 2000). They noted significant increases in IL-8, RANTES and MIP-1-α in the nasal secretions of those adults that developed symptoms

of an URTI when compared to asymptomatic volunteers. Similar increases have been found in childhood following natural RSV infection. Compared to uninfected controls, children with RSV related disease have higher levels of nasal IL-8 (Sheeran 1999, Abu-Harb 1999, Noah 1995), RANTES (Sheeran 1999)and MIP-1-α (Sheeran 1999). Interestingly, in one study nasal wash RANTES concentrations correlated significantly with both RSV concentrations and disease severity while nasal wash white cell counts correlated with IL-8 and RANTES (Sheeran 1999). These observations suggest a probable role for chemokines in mediating the respiratory tract inflammation induced by RSV.

Type 1 and Type 2 Cytokines

The nature of an immune response can be classified according to the profile of the cytokines produced. While something of a simplification, the division into type 1 and type 2 cytokines has provided a valuable framework for understanding the immune response in many diseases. Type 1 cytokines include IL-2, TNF-α and IFN-γ and are important for the eradication of intracellular pathogens, including bacteria, yeasts and viruses. Type 1 cytokines activate macrophages and promote a predominantly cell-mediated immune response. In contrast, type 2 cytokines are generally anti-inflammatory and promote humoral immune responses against extracellular pathogens. Type 2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13.

Nasal samples have been analysed during RSV disease for the majority of these cytokines. Noah *et al* found increased levels of IL 1-β, TNF-α and IL-6 in nasal lavage fluid from children during an acute URTI compared to baseline (Noah 1995). Similarly, Sheeran and colleagues found significantly increased levels of IL-6 and IL-10 in nasal wash specimens from children with RSV disease compared to controls (Sheeran 1999). Increased levels of TNF-α (Matsuda 1995), IFN-γ (van Schaik 1999), IL-4 (van Schaik 1999), IL-6 (Hornsleth 1998) and IL-10 (van Schaik 1999) have also been reported in the nasal secretions of children infected with RSV.

The Balance between Type 1 and Type 2 Cytokines

The type 1-type 2 cytokine balance plays a pivotal role in determining the outcome of the immune response to an infectious organism. Initial observations were made during experimental infection of inbred strains of mice with the intracellular protozoan parasite Leishmania major (Reiner 1995). In this model system, a predominant type 1 response

characterized by IL-2 and IFN-γ secretion leads to self-curing disease, whereas a predominant type 2 response (IL-4, IL-10) leads to non-healing disease. The importance of the type 1-type 2 cytokine balance to disease outcome has now been highlighted in many other viral, bacterial and parasitic diseases (Lucey 1996).

One study to date has analysed the relationship between type 1 and type 2 cytokines in the nasopharynx during natural RSV infection. Van Schaik *et al* measured the concentrations of IFN-γ, IL-4 and IL-10 in the respiratory secretions of 82 young children (55 RSV positive by culture) during an acute episode of virus induced wheezing (van Schaik 1999). 47 children with an upper respiratory tract infection (26 RSV Positive) were sampled as controls. Cytokine concentrations did not differ in groups of subjects with and without RSV infection and all data was therefore pooled for analysis. IFN-γ to IL-4 ratios were higher in children with bronchiolitis than in children with URTI with the increased values primarily due to an increase in IFN-γ. IL-10 levels did not differ significantly between the groups. These data therefore suggest an association between bronchiolitis and a relative overproduction of IFN-γ. The implications of this study and its findings are further debated within the discussion section of this chapter.

Aims

The aims of this part of the study were:

(1) to analyse the relationship between type 1 and type 2 cytokines in the URT during natural RSV infection

(2) to analyse the production of IFN-α in the URT using ELISA

(3) to study the relationship between cytokine responses in the URT and RSV disease severity

(4) to study the relationship between cytokine responses in the URT and viral load

Results

Patient Characteristics and Outcome

Of the 28 children with RSV detected by EIA, nine developed signs and symptoms consistent with acute bronchiolitis. The remaining 19 infants developed signs of an URTI alone. Table 4.1 represents demographic data for the 2 groups of patients (AB and URTI). No statistically significant differences were observed.

Characteristic	WholeGroup (n=28)	Acute Bronchiolitis (n=9)	Upper Respiratory Tract Infection (n=19)	p (AB vs URTI)
Sex				
Male	15 (54%)	3 (33%)	12 (63%)	0.228
Female	13 (46%)	6 (66%)	7 (37%)	
Mean (SD) age at RSV infection (days)	195 (116)	147 (84)	219 (125)	0.132
Breast Fed >3 months	15 (54%)	5 (56%)	10 (53%)	0.604
Nasal lavage collection - mean (SD) time from start of illness (hours)				
Days 1-2	50 (17)	47 (16)	51 (18)	0.616
Days 5-7	138 (29)	129 (32)	143 (26)	0.293
Mean (SD) Gestational age (weeks)	39.54 (1.0)	39.15 (1.2)	39.37 (0.9)	0.324
Mean (SD) Birth Weight (kg)	3.52 (0.60)	3.55 (0.77)	3.5 (0.52)	0.872

Table 4.1 Patient Characteristics

Cytokine Levels in Nasal Lavage Fluid (Table 4.2)

Cytokine and time point	Acute Bronchiolitis (n=9)	Upper respiratory Tract Infection (n=19)	Geometric Mean Ratio (95% Confidence Intervals)	p-value (AB v URTI)
IFN-γ (Geometric mean)				
Day 1-2	15.74	56.89	3.61 (1.15, 11.35)*	0.029
Day 5-7	9.18	22.44	2.44 (0.93, 6.40)*	0.068
IL-4 (Geometric mean)				
Day 1-2	1.19	0.71	1.66 (0.89, 3.10)	0.106
Day 5-7	1.04	0.51	2.03 (1.19, 3.44)	0.011
Day 1-2	464.52	202.74	2.29 (0.86, 6.10)	0.094
Day 5-7	79.98	111.17	0.72 (0.27, 1.89)	0.488
IL-12 (Geometric mean)				
Day 1-2	20.04	89.54	4.47 (0.78, 25.59)*	0.090
Day 5-7	23.71	90.36	3.82 (0.58, 25.23)*	0.157
IFN-α (Geometric mean)				
Day 1-2	4.07	3.03	4.47 (0.78, 25.59)	0.661
Day 5-7	5.32	2.22	3.82 (0.58, 25.23)	0.159
IL-5 (Geometric mean)				
Day 1-2	0.84	2.59	4.47 (0.78, 25.59)*	0.093
Day 5-7	1.54	2.16	3.82 (0.58, 25.23)*	0.676

*Geometric mean ratio (URTI/AB)

Table 4.2 Cytokine Levels In Nasal Lavage Fluid

To investigate whether the nature of the host immune response differed between those who developed AB and those with URTI alone, nasal lavage fluid from both groups of patients

was analysed by ELISA for cytokine production. As the distributions of the nasal lavage cytokine values and the derived cytokine ratios were skewed to the right, the data was logarithmically transformed to obtain approximate normality before analysis. To account for age differences between groups, the transformed cytokine levels and cytokine ratios were compared between AB and URTI groups using analysis of covariance (ANCOVA), in which age (in days) was incorporated as a covariate. The magnitude of difference between groups for this data is summarised using the geometric mean ratio (gmr) together with a 95% confidence interval. In the figures the centre of location for each variable is represented by the geometric mean.

In those RSV positive infants who developed AB, IFN-γ levels were significantly lower on days 1-2 than in those RSV-positive infants with signs of an URTI alone [p=0.029; gmr 3.61 (1.15, 11.35), Figure 4.1].

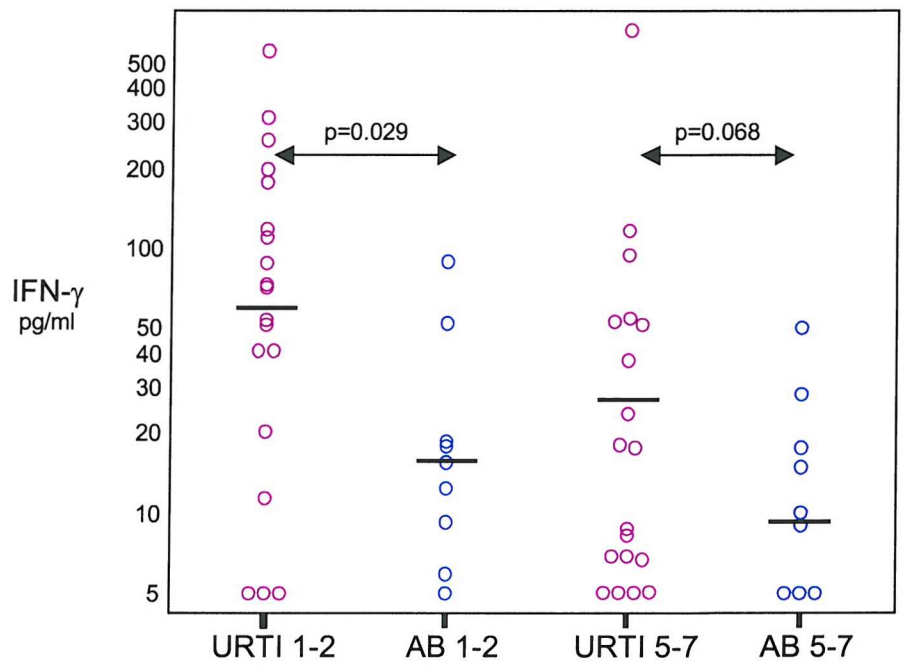

Figure 4.1 IFN-γ levels in nasal lavage.

Nasal lavage specimens were collected on days 1-2 and days 5-7 of each illness. Specimens were vortexed, filtered and then stored at -70°C. IFN-γ levels were determined by specific ELISA. Levels are shown on days 1-2 and days 5-7 of the illness in infants with Upper Respiratory Tract Infection (URTI) and Acute Bronchiolitis (AB). Figure shows individual values and the geometric mean. Significantly increased IFN-γ levels were detected on days 1-2 in the URTI group.

Conversely, IL-4 was significantly higher on days 5-7 in the bronchiolitis group [p=0.011; gmr 2.03 (1.19, 3.44)] with a trend toward higher levels on days 1-2 (Figure 4.2).

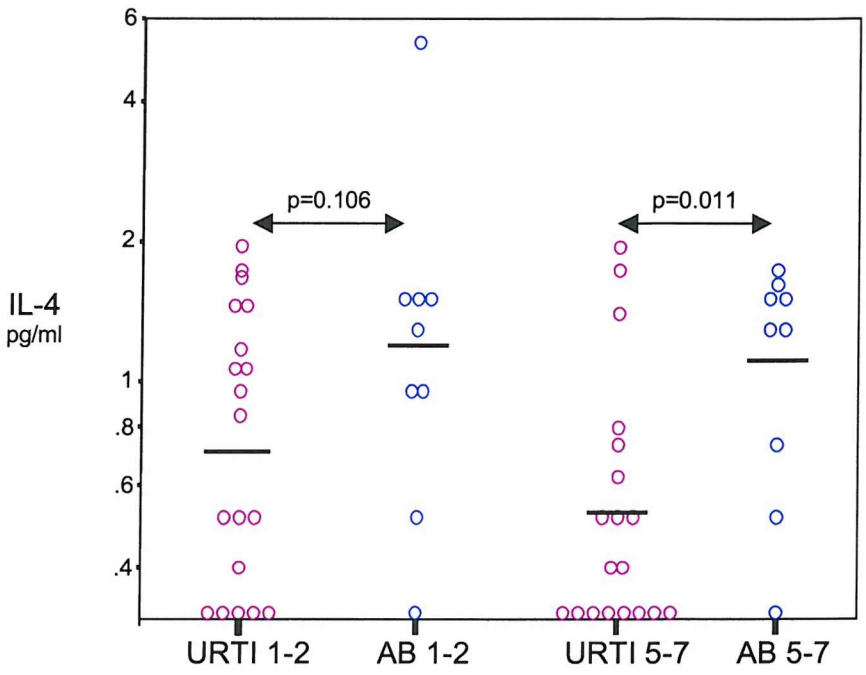

Figure 4.2 IL-4 levels in nasal lavage

Nasal lavage specimens were collected on days 1-2 and days 5-7 of each illness. Specimens were vortexed, filtered and then stored at -70°C. IL-4 levels were determined by a high sensitivity ELISA kit. Levels are shown on days 1-2 and days 5-7 of the illness in infants with Upper Respiratory Tract Infection (URTI) and Acute Bronchiolitis (AB). Figure shows individual values and the geometric mean. Significantly increased IL-4 levels were detected on days 5-7 in the AB group.

There were no significant differences in IL-5, IL-10, IL-12 or IFN-α levels observed between the two groups (Table 4.2).

Type 1: Type 2 Cytokine Ratios in Nasal Lavage Fluid (Figures 4.3 & 4.4)

In order to examine the balance between type 1 and type 2 cytokine responses, the results were also expressed as ratios (IL-4/IFN-γ and IL-10/IL-12). By representing the data in this way, it is not necessary to control for dilutional differences between specimens.

The ratio of IL-4/IFN-γ from infants with AB was markedly higher on both days 1-2 [p=0.014; gmr 6.00 (1.48,24.32)] and days 5-7 [p=0.001; gmr 4.94 (2.06, 11.89)] than from infants with an URTI (Figure 4.3).

In addition, those with AB demonstrated a significantly higher IL-10/IL-12 ratio [p=0.015; gmr 10.23 (1.64,63.68)] on days 1-2 of the illness (Figure 4.4).

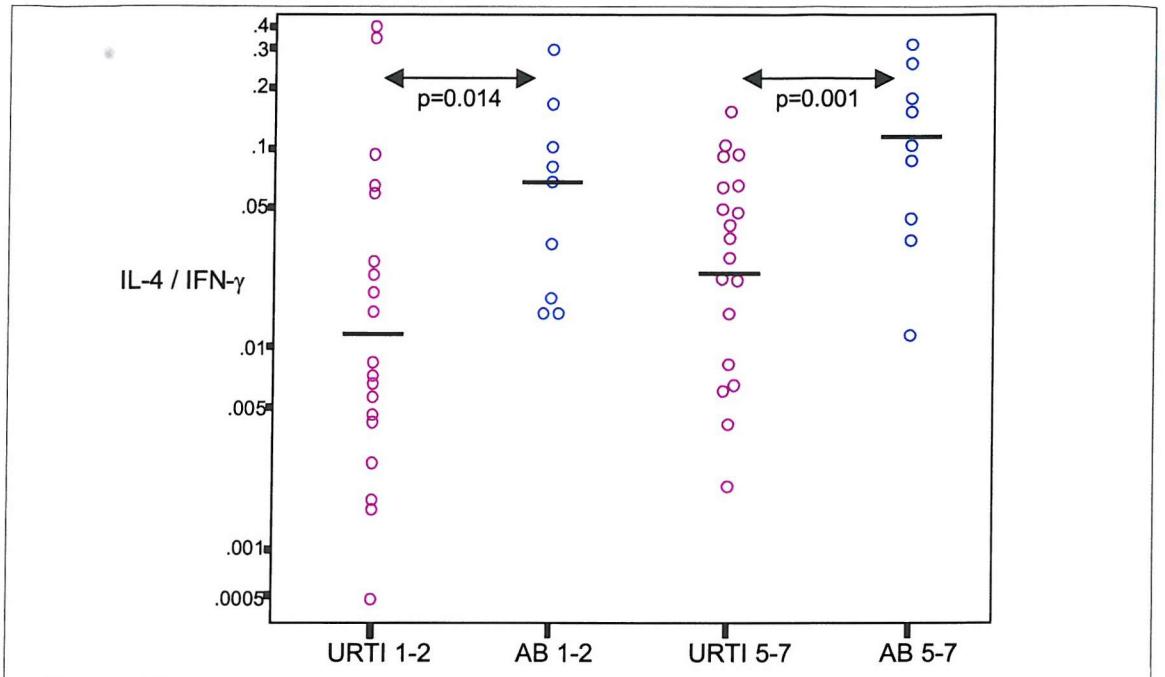

Figure 4.3 Ratio of IL-4/IFN-γ in nasal lavage

Nasal lavage specimens were collected on days 1-2 and days 5-7 of each illness. Specimens were vortexed, filtered and then stored at -70°C. Cytokine levels were determined by specific ELISA kits. Ratios are shown on days 1-2 and days 5-7 of the illness in infants with Upper Respiratory Tract Infection (URTI) and Acute Bronchiolitis (AB). Figure shows individual values and the geometric mean. An increased IL-4/IFN-γ ratio was detected at both time points in the AB group.

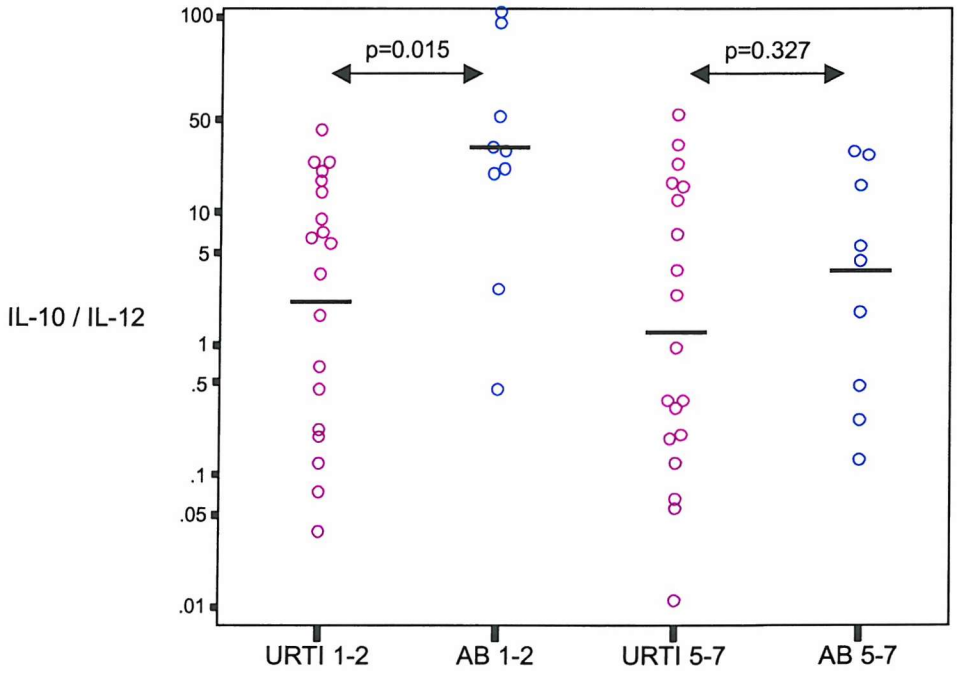

Figure 4.4 Ratio of IL-10/IL-12 in nasal lavage

Nasal lavage specimens were collected on days 1-2 and days 5-7 of each illness. Specimens were vortexed, filtered and then stored at -70°C. Cytokine levels were determined by specific ELISA kits. Ratios are shown on days 1-2 and days 5-7 of the illness in infants with Upper Respiratory Tract Infection (URTI) and Acute Bronchiolitis (AB). Figure shows individual values and the geometric mean. The IL-10/IL-12 ratio was increased on days 1-2 in the AB group.

Discussion

The data presented demonstrate that RSV bronchiolitis is associated with a profound imbalance in type 1/type 2 cytokines with deficient type 1 and excess type 2 responses. The study design permitted controlling for age and dilutional factors in nasal lavage and sampling of the two groups concurrently in relation to onset of signs of infection. The results were therefore not confounded by the effects of age or sampling error and reflect the immune response to RSV during early infection.

IFN-γ and IL-4 represent the archetypal cytokines of the type 1/type 2 paradigm and are the most substantiated example of a pair of mutually counter-regulatory cytokines. Whereas IFN-γ inhibits the development of a type 2 immune response and promotes type 1 responses, IL-4 induces type 2 cytokine production and suppresses type 1 responses (Maggi 1992, Scott 1991). The data demonstrate a significantly higher IL-4 to IFN-γ ratio in the upper respiratory tract in RSV bronchiolitis. This provides strong evidence for an excess type 2 or a deficient type 1 immune response in infants developing bronchiolitis.

IL-10 is a product of various cell types including T-cells and monocytes and possesses a wide range of activities including suppression of type 1 cytokine secretion (Hsu 1992). Conversely, IL-12 is rapidly released by antigen presenting cells in response to infection and is essential for the development of a type 1 immune response (Hsieh 1993). IL-10 and IL-12 represent a further counter-regulatory balance between type 1 and type 2 responses (Meyaard 1996, D'Andrea 1993). The significantly increased IL-10 to -12 ratio in nasal lavage fluid on days 1-2 in those with bronchiolitis also demonstrates the development of an excessive type 2 or deficient type 1 immune response in these infants.

During RSV infection, viral replication occurs in the respiratory epithelium and inflammatory processes in the nasal air passages have been shown to reflect those in the lower airways (Joshi 1998, Everard 1994). Nasal lavage therefore represents a minimally invasive, reproducible method of obtaining specimens with immunological relevance to the respiratory tract as a whole. No reliable method exists for calculating the volume of actual epithelial lining fluid collected during nasal lavage. This raises the possibility that the differences in nasal lavage cytokine levels between our two groups (AB and URTI) may merely represent differences in dilution of nasal secretions. By expressing results as a ratio, we were not only able to examine the balance between type 1 and type 2 cytokines but also to eliminate the need to control for dilutional differences between specimens. The fact that the results were similar whether expressed as cytokine levels, or as a ratio confirms that

dilutional factors were not important in the conclusions drawn from the data.

The only other published study to analyse the local type 1 and type 2 cytokine balance during RSV infection found higher ratios of IFN-γ to IL-4 in the respiratory secretions of infants with bronchiolitis (van Schaik 1999). These data therefore contradict the findings of the current study. There are many possible explanations for this disparity:

(1) The definitions of upper respiratory tract infection and acute bronchiolitis were different. The current study required the presence of inspiratory crackles, tachypnoea and intercostal / subcostal retractions for the diagnosis of acute bronchiolitis. Van Schaik *et al* defined bronchiolitis as a first episode of wheezing on auscultation or hypoxia and radiographic evidence of hyperinflation of the chest in the absence of wheezing.

(2) All infants in the current study were RSV positive by enzyme immunoassay. Less than two thirds of the infants in Van Schaik's study had documented RSV infection. Whilst it is stated that the cytokine concentrations in groups of subjects with or without RSV infection were similar, the effects of including infants without RSV infection in the statistical analyses are difficult to predict.

(3) Both studies differed significantly in their design. The current study prospectively identified infants with RSV infection from the same birth cohort and geographical area that were infected in the same season with the same virus. This study design enabled the results to be controlled for age and ensured simultaneous timing of sampling relative to onset of infection. The exact design of Van Schaik's study is not clear from the published article, although it is likely that the absence of one or more of the above design features would impact considerably on the results obtained.

(4) The recruited infant populations differed between the two studies. All infants recruited to the current study had at least one parent with atopic asthma and were therefore at increased genetic risk of atopy. By contrast, the majority of the infants in the study by Van Schaik and colleagues had no family history of asthma. Furthermore, there was no attempt to define the atopic status of the parents and the definition used for a family history of asthma was extremely broad (member of the immediate family had received β-adrenergic agents or corticosteroids for asthma). It is therefore likely that the infants recruited to the current study would have a greater tendency to a type 2 cytokine response than those in Van Schaik's study.

Previous studies of IFN-α production in RSV disease have found absent or low levels in respiratory secretions with no correlation between IFN-α concentration and disease severity

or viral clearance (Taylor 1989, Isaacs 1989b, McIntosh 1978a, Hall 1978a). In the current study, IFN-α was undetectable using a high sensitivity ELISA kit in 18 of the 56 nasal lavage samples assayed with low levels (median = 3.09 pg/ml) identified in the other samples. In keeping with previous reports, the low levels of measured IFN-α did not correlate significantly with disease severity (URTI vs AB). These data provide further evidence of a poor IFN-α response to RSV infection in humans.

In the current study, RSV clearance was reduced in those infants that developed signs and symptoms of acute bronchiolitis (Chapter 6). The mechanism of impaired clearance is unclear. Many cells are known to contribute to the clearance of RSV from the respiratory tract. $CD8^+$ T cells are critical for the control of viral infections via direct cytolysis as well as secretion of the antiviral cytokines IFN-γ and TNF-α (Zinkernagel 1996). Recent murine studies have suggested that a relative type 2 immune response diminishes CD8+ CTL activity and delays viral clearance. Transgenic mice that over express IL-4 relative to IFN-γ have delayed clearance of RSV from the lungs together with a markedly diminished primary CTL response (Fischer 1997). The suppression of RSV-specific cytotoxicity in this model appears to be the direct result of local IL-4 expression at the time of antigen presentation (Aung 1999). Whilst the presence of such a process in humans is merely speculative, it is intriguing to note our finding of increased nasal IL-4 levels in infants with RSV bronchiolitis and evidence of impaired viral clearance.

Conclusions

- Infants that develop signs and symptoms of bronchiolitis following RSV infection demonstrate a relative type 2 local cytokine response compared to infants with an upper respiratory tract infection alone.
- Low levels of IFN-α are detectable in nasal lavage specimens from infants with early RSV infection. There is no association between IFN-α concentrations and disease severity.

Chapter 5:

The Systemic Immune Response to RSV infection.

Introduction

Relationship between Local and Systemic Immune Responses

The mucosal surfaces represent a very large surface area of contact to exogenous agents including microorganisms and provide an important protective anatomical, mechanical and chemical barrier to these agents. In addition, the mucosal surfaces also possess a complex system of immunological defence mechanisms to further contribute to the integrity of not only the local mucosae themselves but also to the body as a whole. The local immune response often has important consequences for systemic immunity. These systemic consequences can be most clearly observed following the local release of cytokines into the circulation. For example, influenza virus infection and replication in the respiratory tract directly injures the nasal and tracheobronchial epithelium, resulting in the typical respiratory symptoms of cough, reduced tracheobronchial clearance and depressed pulmonary function. This local infection also results in a substantial local immune response with the influx of inflammatory cells and the subsequent production of inflammatory mediators. Whilst these mediators are important for local protection against influenza, their release into the circulation produces many of the systemic symptoms of influenza infection such as fever, myalgia and headache (Hayden 1998).

The introduction of this thesis discussed the current understanding of many aspects of the systemic response to RSV infection including innate immune responses to infection, humoral responses and the role of eosinophils, mast cells and IgE. This chapter concentrates on systemic cell-mediated immunity during RSV infection primarily focusing on the type 1/type 2 cytokine response to RSV.

Systemic Cell-Mediated Immune Response to RSV Infection in Infancy

The systemic cell-mediated response to RSV has previously been investigated in two main ways:

(1) Analysis of cytokine levels in peripheral blood collected at the time of infection.

(2) Stimulation of peripheral blood cells (specifically or non-specifically) collected at the time of infection followed by evaluation of cell mRNA and supernatant protein levels.

(1) The analysis of peripheral blood for cytokines enables an assessment of the *in vivo* immune response at a particular time point without the need for extrinsic stimulation. This technique is, however, limited by the extremely low (and often undetectable) cytokine levels that are present in such specimens. Furthermore, such analyses do not allow the evaluation of individual components of the immune response.

A number of studies have attempted to assess type 1 and type 2 cytokine levels in peripheral blood during RSV infection. Smyth *et al* found extremely low levels of the type 2 cytokine IL-4 in both the acute and convalescent phase of RSV bronchiolitis (Smyth 1997). They concluded that it was not possible to accurately assess the type 1 / type 2 cytokine balance based on their data. In a similar study, Oymar *et al* measured serum IL-5 levels in 25 children needing hospitalisation for their first episode of bronchiolitis (Oymar 1996). IL-5 was detected in the sera of only 2 children with no apparent association with disease severity. However, the ELISA used for IL-5 measurement was insensitive (1 ng/l) making it difficult to draw significant conclusions from this study. Somewhat more meaningful results were obtained during a Spanish study which found significantly raised levels of both serum IL-10 and IL-12 in infants with acute bronchiolitis when compared to healthy controls (Blanco-Quiros 1999). However, no correlations were observed between the serum cytokine levels and either disease severity or subsequent wheeze. Interestingly, the type 1 cytokine IL-12 was reduced in the cord blood of newborns who later developed bronchiolitis suggesting the possibility that these infants had a type 1/type 2 immune imbalance present at birth that may have contributed to their clinical response to RSV.

Blood serum levels of type 1 and type 2 cytokines have proved equally difficult to interpret in other childhood respiratory infections. During adenovirus infection, for example, serum levels of the inflammatory cytokines IL-1, IL-6, IL-8 and tumour necrosis factor-α are low or undetectable in all but the most severe of cases whilst the immunomodulatory cytokine

IL-4 is undetectable regardless of severity (Mistchenko 1994). Similarly during influenza infection, IL-2, IFN-γ and TNF-α are rarely detected in serum collected at the time of infection (Kawasaki 2002).

(2) The measurement of cytokine production by stimulated blood cells has certain important advantages over serum analysis. Stimulation is technically undemanding, requires few cells and produces readily quantifiable levels of cytokines. In addition, this technique enables the analysis of specific components of the immune system such as T cell or monocyte responses.

Ex vivo stimulation does, however, have certain limitations including the potential to deviate the immune response according to the mode of stimulation. Polyclonal activators such as phytohaemaglutinin and lipopolysaccharide enable a broad stimulation of the immune system and, in short term use, reduce selective pressure on antigen-reactive cells. However, the particular receptor/ligand system used to activate a cell population can influence the immunological response with differing cytokine profiles produced as a consequence. It is of paramount importance, therefore, that comparisons between groups are based on stimulation assays involving identical experimental conditions.

There have been numerous previous attempts to characterise the systemic immune response to RSV during infancy (Table 5.1). Some studies have identified an association between RSV disease severity and a type 2 cytokine response (Bendelja 2000, Bont 2000b, Aberle 1999, Roman 1997) whereas others have found no such association (Bont 1999) or have produced contrary findings (Brandenburg 2000a). However, the various studies differ significantly in many aspects of their design, which may explain these inconsistent findings:

(i) There are significant demographic and clinical differences between recruited subjects. The ages of the recruited children differ significantly between studies, some only including infants less than 6 months of age (Brandenburg 2000a) whilst others use 12 months (Bont 2000b, Aberle 1999, Renzi 1999, Bont 1999) and even 24 months (Bendelja 2000) as the upper age limit for recruitment. Clinical recruitment criteria also vary considerably. Many studies attempt to stratify the severity of RSV disease using a variety of criteria including need for ventilation (Brandenburg 2000a, Bont 1999), supplemental oxygen requirement (Brandenburg 2000a, Aberle 1999) and clinical features (Bendelja 2000). Other studies attempt to recruit a more homogeneous group by defining precise clinical and demographic parameters for inclusion (Bont 2000b, Renzi 1999, Roman 1997) although these parameters also vary considerably between studies.

Reference	Study Site	Specimen Type	Stimulation Method Used	Symptoms	Controls	Total Number of Infants Studied (Index & Control)	Cytokine Analysis Technique and assay sensitivities (pg/ml)	Main Findings
Bendelja *et al* 2000 (Bendelja 2000)	University Hospital, Zagreb, Croatia	Peripheral Whole Blood Cultures	PMA & Ionomycin for 6 hours	URTI, Bronchiolitis or Pneumonia	Normal Healthy Controls	30 Index (7 URTI, 17 Bronchiolitis, 6 Pneumonia) 10 Control	Intracellular Flow Cytometry Staining for IL-4 and IFN-γ	1)RSV-infected infants had significantly higher percentage of IL-4 producing lymphocytes
Brandenburg *et al* 2000 (Brandenburg 2000a)	Sophia's Children's Hospital, Rotterdam, Netherlands	Bulk stimulation of PBMCs with UV irradiated RSV infected autologous B-LCL.	UV irradiated RSV infected autologous B-LCL for 16 days IL-2 added Days 3, 7 and 11 PHA for 14 days. Then restimulated for 48 hours with UV irradiated RSV infected autologous B-LCL	URTI, Bronchiolitis	Mild RSV Disease Vs Severe RSV Disease Vs RSV Negative	111 infants (95 RSV positive; 50 severe RSV disease, 45 mild RSV disease) 15 selected infants for T cell studies	ELISA for IL-2 (300), IL-4 (7), IL-5(20), IFN-γ (25) , IL-10 (20) Intracellular Flow Cytometry Staining for IFN-γ and IL-4	1)Low levels of cytokines were secreted using this stimulation method (IFN-γ detected in 6 of 13 RSV +ve patients, IL-4 1 of 13, IL-10 2 of 13) 2) No association between cytokine levels and disease severity
Bont *et al* 2000 (Bont 2000a)	Wilhelmena Children's Hospital, Utrecht, Netherlands	Peripheral Whole Blood Cultures	LPS and IFN-γ for 48 hours	Requiring Mechanical Ventilation for RSV Infection	None	30 infants	ELISA for IL-10 (7.8) and IL-12 (7.8)	1)IL-12 production inversely related to duration of mechanical ventilation
Bont *et al* 1999 (Bont 1999)	Wilhelmena Children's Hospital, Utrecht, Netherlands	Peripheral Whole Blood Cultures	PHA for 48 hours	URTI, Bronchiolitis	Healthy Controls	50 Index infants with RSV disease (14 needing ventilation) 27 Controls	ELISA for IFN-γ (25), IL-4 (4.7)	1)IFN-γ and IL-4 levels significantly lower in ventilated infants compared to non-ventilated infants 2)IFN-γ, IL-4 and IFN-γ/IL-4 ratios not significantly different between non-ventilated infants and controls
Renzi *et al* 1999 (Renzi 1999)	Ste-Justine and Notre Dame Hospitals, Montreal, Canada	Peripheral Blood Mononuclear Cells (Monocytes Removed by Adherence)	IL-2 for 3 days	Bronchiolitis	None	32 infants	ELISA for IFN-γ(20), IL-4(25)	1)IFN-γ production significantly lower at time of bronchiolitis in infants subsequently developing asthma compared to infants with no asthma 2)IL-4 production not significantly different between asthma and no asthma infants
Aberle *et al* 1999 (Aberle 1999)	St Anna Kinderspital, Vienna, Austria	Peripheral Blood Mononuclear Cells isolated by density centrifugation	None	LRTI due to RSV (hospitalised)	Healthy Infants undergoing minor surgery	20 Index (11 Moderate Severity (No O_2 required); 9 Severe (requiring O_2)) 6 Control	RT-PCR (Semi-nested) for IFN-γ relative to β-actin	1)Moderate Illness associated with significantly higher levels of IFN-γ than severe disease and controls
Roman *et al* 1997 (Roman 1997)	Hospital Roberto del Rio, Santiago, Chile	Peripheral Blood Mononuclear Cells separated by density centrifugation	PHA or PWM for 24 and 48 hours	Severe LRTI (bronchial obstruction)	Healthy Infants	15 Index 17 Controls	ELISA for IFN-γ(2) and IL-4(2)	1)IL-4/IFN-γ ratio for PHA stimulated PBMCs significantly increased in index compared to controls
Renzi *et al* 1997 (Renzi 1997)	Ste-Justine and Notre Dame Hospitals, Montreal, Canada	Peripheral Blood Mononuclear Cells (Monocytes Removed by Adherence)	IL-2 or *D.Farinae* antigen for 3 days	Bronchiolitis	None	28 Index	ELISA for IFN-γ(25) and IL-4(20)	1)IFN-γ and IL-4 levels unchanged 5 months following bronchiolitis

Table 5.1 Previous studies examining the peripheral blood cytokine response to RSV infection during infancy

Key: PMA – Phorbol 12-myristate 13-acetate; URTI – Upper Respiratory Tract Infection; B-LCL – B lymphoblastic cell lines; LPS – Lipopolysaccharide; PHA-Phytohaemaglutinin; PWM-Pokeweed Mitogen; D. Farinae- Deramtophagoides farinae

(ii) The control group used varies between studies. Some studies use healthy children admitted for minor surgery (Bendelja 2000, Aberle 1999, Bont 1999, Roman 1997) whilst others use internal controls based on stratification of the recruited group (Brandenburg 2000a, Bont 2000b, Aberle 1999, Renzi 1999, Bont 1999).

(iii) In all studies, peripheral venous blood is collected for analysis. However, subsequent processing and treatment of the blood specimens can differ widely. Stimulation assays are generally performed with either whole blood cultures (Bendelja 2000, Bont 2000b, Bont 1999) or with isolated mononuclear cells (Renzi 1999, Roman 1997, Renzi 1997). Various polyclonal activators are then employed including IL-2 (Renzi 1999, Renzi 1997), IFN-γ (Bont 2000b), PMA /Ionomycin (Bendelja 2000), PHA (Bont 1999, Roman 1997), LPS (Bont 2000b) and Pokeweed Mitogen (Roman 1997). The culture time also varies considerably with a wide range from 6 hours (Bendelja 2000) to 3 days (Renzi 1999, Renzi 1997).

The reported studies also have other significant design weaknesses. Cytokine responses are known to vary considerably in the first year of life with a rapid suppression of the type 2 response characteristic of the neonate (Prescott 1999). However, only one study to date has actively controlled for age differences between groups (Bont 2000b). Furthermore, no previous study has attempted to study infants at a standardised point during the RSV disease process despite evidence that the immune response to RSV changes appreciably with time (Sheeran 1999). Finally, the antigenic load has a significant influence on the nature of the host immune response (Secrist 1995). Virus culture techniques have demonstrated that the viral load during RSV infection can vary by a factor of up to $5x10^4$ (Hall 1976a). Despite this there has been no previous attempt to quantify this important variable.

The prospective birth cohort study described in this thesis was therefore carefully designed to investigate the systemic type 1/ type 2 cytokine responses to RSV infection and to control for all the above confounding factors. The study examined the immune responses of infants to their first natural proven RSV infection through analysis of cytokine production by stimulated blood mononuclear cells taken at identical times from onset of symptoms of infection. Subjects were prospectively monitored to determine if infection resulted in bronchiolitis or upper respiratory signs and symptoms alone. Cytokine responses were then analysed in relation to age and disease outcome and virus load was assessed.

Aims

The aims of this part of the study were:

(1) to analyse the systemic immune response to RSV infection

(2) to examine the relationship between systemic type 1 and type 2 immune responses during early RSV infection

(3) to examine the relationship between systemic cytokine responses and RSV disease severity

Results

Cytokine mRNA production by stimulated PBMCs

To investigate whether the nature of the host immune response differed between those who developed AB and those with URTI alone, venous blood was collected from infants with a proven RSV positive respiratory tract infection. Blood was collected between days 5 and 7 from the start of the illness. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation and suspended at 1 x 10^6 cells/ml in culture medium supplemented with 10% filtered autologous plasma and stimulated with phytohaemaglutinin (PHA, 1μg/ml) or lipopolysaccharide (LPS, 5μg/ml) for 24 hrs at 37°C with 5%CO_2. Cultures were centrifuged and total cellular RNA extracted and analysed by RT-PCR using specific primers for IFN-γ, IL-4, IL-12 and IL-18 mRNA and internal control glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. PCR products were electrophoresed on 2% agarose gels containing VISTRAGreen and analysed by direct fluorescence on a Storm 850 imager (Molecular Dynamics, USA) using ImageQuant software.

As cytokine values and the derived cytokine ratios were skewed to the right, the data was logarithmically transformed to obtain approximate normality before analysis. To account for age differences between groups, the transformed cytokine levels and cytokine ratios were compared between AB and URTI groups using analysis of covariance (ANCOVA), in which age (in days) was incorporated as a covariate. The magnitude of difference between groups for this data is summarised using the geometric mean ratio (gmr) together with a 95% confidence interval. In the figures the centre of location for each variable is represented by the geometric mean.

PHA-stimulated PBMCs from infants with signs of an URTI alone expressed significantly more IFN-γ mRNA [p=0.002; gmr 24.04 (3.81,151.71)] than PBMCs from infants with acute bronchiolitis (Figure 5.1). There was no significant differences in IL-4 mRNA levels observed between the two groups.

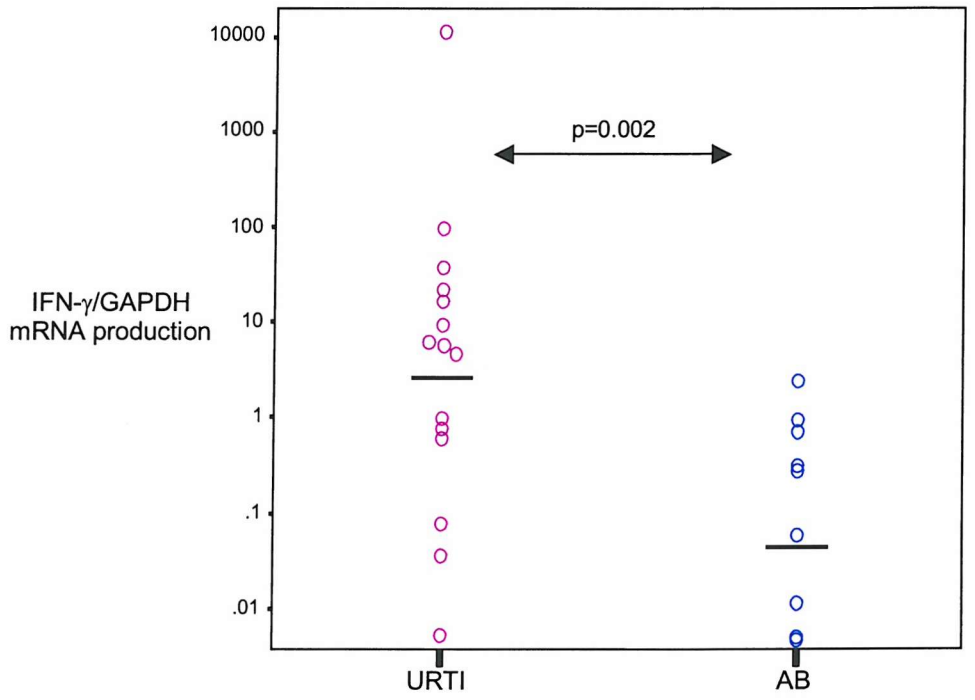

Figure 5.1 IFN-γ mRNA production by PHA stimulated PBMCs

Peripheral blood was collected on days 5-7 of each illness. PBMCs were isolated by density gradient centrifugation, resuspended in culture medium and stimulated with PHA (1mg/ml) for 24 hours at 37oC with 5%CO_2. Purified mRNA was analysed by RT-PCR using specific primer pairs for IFN-γ. Results are expressed as the IFN-γ/GAPDH ratio. Figure shows individual values and the geometric mean. An increased IFN-γ/GAPDH ratio was detected in the URTI group.
(AB = Acute Bronchiolitis; URTI = Upper Respiratory Tract Infection)

In order to examine the balance between type 1 and type 2 cytokine responses, the results were expressed as a ratio (IL-4/IFN-γ). The ratio of IL-4 / IFN-γ mRNA production was significantly higher [p=0.010; gmr 67.92 (3.13,1472.31)] in the AB group when compared to the URTI group (Figure 5.2).

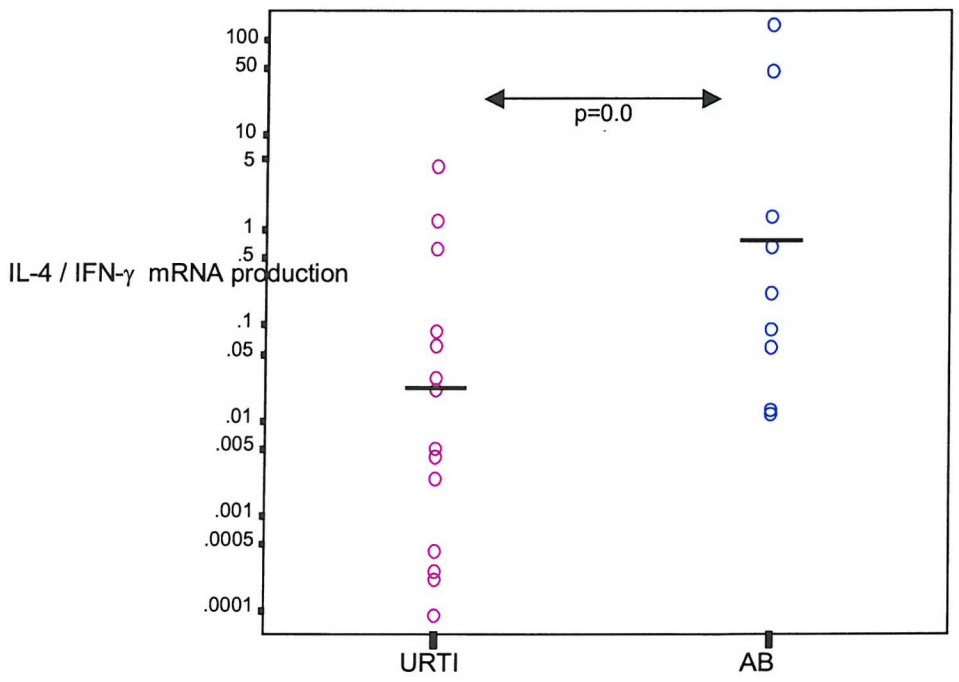

Figure 5.2 IL-4/IFN-γ mRNA production by PHA stimulated PBMCs

Peripheral blood was collected on days 5-7 of each illness. PBMCs were isolated by density gradient centrifugation, resuspended in culture medium and stimulated with PHA (1mg/ml) for 24 hours at 37oC with 5%CO_2. Purified mRNA was analysed by RT-PCR using specific primer pairs for IFN-g and IL-4. Results are expressed as the IL-4/IFN-γ ratio. Figure shows individual values and the geometric mean. An increased IL-4/IFN-γ ratio was detected in the AB group.
(AB = Acute Bronchiolitis; URTI = Upper Respiratory Tract Infection)

LPS-stimulated PBMCs from infants with acute bronchiolitis also expressed significantly less IL-18 mRNA [p=0.019; gmr 14.45 (1.61,129.71), Figure 5.3] than PBMCs from infants with signs of an URTI.

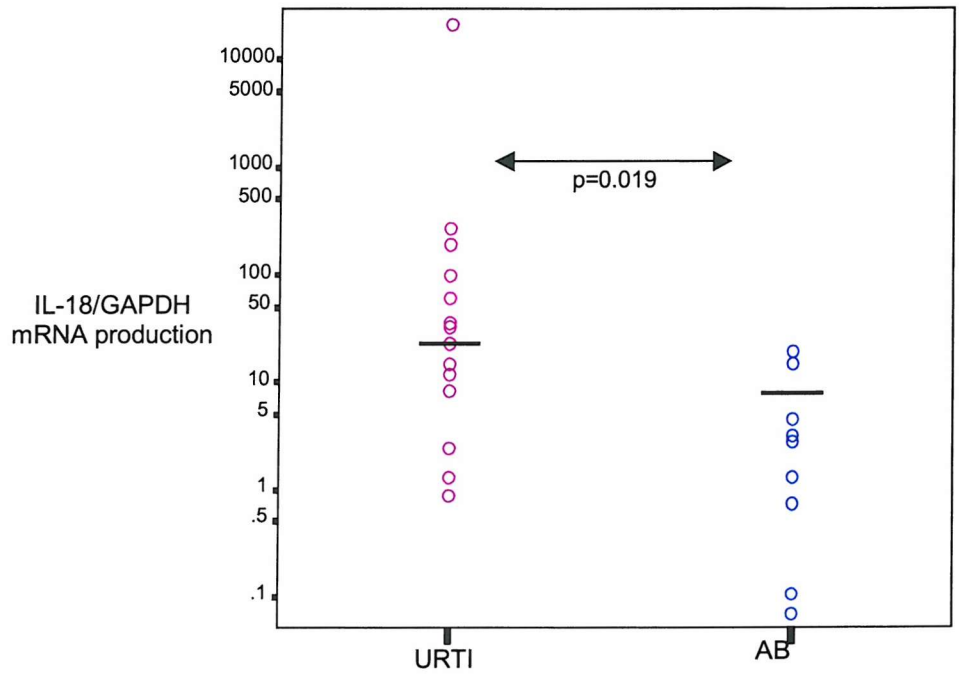

Figure 5.3 IL-18 mRNA production by LPS stimulated PBMCs.

PBMCs were isolated from peripheral blood by density gradient centrifugation, resuspended in culture medium and stimulated with LPS (5mg/ml) for 24 hours at 37oC with 5%CO2. Purified mRNA was analysed by RT-PCR using specific primer pairs for IL-18. Results are expressed as IL-18 mRNA relative to GAPDH mRNA. Figure shows individual values and the geometric mean. IL-18 mRNA production was reduced in the AB group.
(AB = Acute Bronchiolitis; URTI = Upper Respiratory Tract Infection)

IL-12 mRNA expression in LPS-stimulated PBMCs was not significantly different [p=0.215; gmr 2.7 (0.54,13.58)] between the two groups.

Discussion

The main finding of this investigation is the association of bronchiolitis with a relative type 2 systemic cytokine response. PHA-stimulated PBMCs from infants with bronchiolitis were found to produce a significantly higher IL-4/IFN-γ mRNA ratio compared to PBMCs from infants with URTI alone (Fig 5.2). This observation substantiates the nasal lavage cytokine profiles presented in Chapter 4. Together, these data demonstrate reduced IFN-γ and increased IL-4 production and a highly significantly increased IL-4/IFN-γ ratio in RSV bronchiolitis in both the respiratory tract and systemically. This provides strong evidence for an excess type 2 and a deficient type 1 immune response in infants with bronchiolitis.

The current study was designed to enable a sensitive analysis of the systemic immune response during RSV infection. Polyclonal stimulation was used in preference to specific T cell cloning in order to avoid the acute selective pressures observed during the cloning process and to enable a more 'global' overview of the systemic immune response rather than having to extrapolate findings from a small component of the T cell repertoire. Moreover, live virus stimulation was not employed so as to avoid the effects of cell death on cytokine production. A relatively short period of stimulation (24 hours) minimised the potential for deviation of the intrinsic cytokine response by polyclonal activation. Cellular mRNA production was analysed rather than the corresponding translated protein - whilst recognising the difficulties of relating mRNA expression to protein production, this enabled the analyses to have maximal sensitivity. This was deemed particularly important in the light of previous attempts to quantify extremely low levels of protein production (Roman 1997).

RSV infection in infancy has a wide spectrum of clinical outcomes ranging from asymptomatic infection to severe lower respiratory tract disease requiring mechanical ventilation. The current study was specifically designed to analyse the immunological differences between infants with and without lower respiratory tract involvement by comparing infants with bronchiolitis to infants with signs and symptoms of an URTI alone. This division was made using *a priori* definitions of both URTI and bronchiolitis. The definitions used were based on published criteria (Everard 1996). However, the recognised criteria for the diagnosis of bronchiolitis are not consistent internationally. In the UK, for example, audible crackles are an important part of the diagnosis whereas in North America wheezes but not crackles are regarded as a diagnostic sign (Everard 1996). Further analysis of the current data has shown that no infant with wheeze and no crackles on auscultation fulfilled the other diagnostic criteria for bronchiolitis (i.e. subcostal retractions and a

respiratory rate greater than 60 per minute). Consequently, the findings are consistent whether crackles or wheeze are used as the primary auscultatory finding in bronchiolitis.

This study's principal finding of a relative type 2 immune systemic response in severe RSV disease, corresponds with the results from several previous studies (Bendelja 2000, Bont 2000b, Aberle 1999, Roman 1997). However, the current data contradict the findings of a study published in the Journal of Medical Virology in October 2000 (Brandenburg 2000a). Brandenburg *et al* studied 111 infants admitted to Sophia Children's hospital (Rotterdam, Holland) with a suspected RSV respiratory infection. A subgroup of 13 patients with RSV infection (7 severe infection and 6 mild infection) were selected for the characterisation of RSV-specific T cell responses. B lymphoblastic cell lines were generated from PBMCs, infected with RSV and then used for the stimulation of T cells in culture (in association with IL-2 and PHA). Specific ELISAs were used to quantify cytokine production in culture supernatants. IFN-γ was detectable in 4 infants with severe RSV infection and 2 infants with mild infection. IL-4 was detected in only 1 patient (mild infection). These results led the authors to conclude that, irrespective of clinical severity, immune responses to RSV infection were dominated by a type 1 response. However, there are considerable differences between this study and the current study. Firstly, the techniques employed in the current study to assess the relative contributions of type 1 and type 2 immunity (i.e. RT-PCR) are more sensitive than those used by Brandenburg *et al.* This enables a quantitative analysis of the immune response as opposed to the qualitative responses observed by Brandenburg *et al.* Secondly, the groups of infants studied were significantly different. Brandenburg *et al* compared infants with severe RSV infection (raised carbon dioxide levels, poor oxygen saturations or artificial ventilation) against infants with mild infection (none of the aforementioned criteria). Both groups had evidence of lower respiratory tract infection. By contrast, the current study compared infants with bronchiolitis to infants with signs / symptoms of upper respiratory tract involvement alone. Thus, the current study has analysed the immune factors important for progression of RSV infection to bronchiolitis whereas Brandenburg *et al* studied those factors associated with severe lower respiratory tract disease. In addition, the current study controlled for several factors that are known to influence the immune response including time of specimen collection relative to symptom onset and age differences between groups. There were no such controls incorporated into the design of the Brandenburg *et al* study. Furthermore, the findings of the two studies are not necessarily conflicting. Brandenburg *et al* have concluded that they could find little evidence for a type 2 immune response in RSV infection. However, this statement is based on their inability to detect IL-4 in a large proportion of patients (12 of 13) using a relatively insensitive assay (lower detection limit 7 pg/ml). These qualitative data made it impossible

to assess the type 1 / type 2 cytokine balance in the recruited infants. It is therefore difficult to compare these data with the quantitative cytokine ratios obtained in the current study and raises the possibility that the findings of Brandenburg *et al*'s study may have been different if quantitative cytokine ratios had been available (possibly through the use of assays with a higher sensitivity).

These data for the first time demonstrate deficient IL-18 expression by stimulated PBMCs in infants with bronchiolitis (Figure 5.3). IL-18 (previously known as IFN-γ inducing factor) is a recently described cytokine that is synthesised by activated macrophages and plays an important role in type 1 immune responses. IL-18 acts synergistically with IL-12 to induce IFN-γ production in T cells and natural killer cells and to promote the differentiation of naïve T cells into type 1 T cells (Okamura 1998). IL-18 has a central role in the development of protective immunity against bacteria. IL-18 deficient mice have been found to suffer from uncontrolled bacterial infections (Sugawara 1999) whereas the administration of IL-18 enhances bacterial clearance (Kawakami 1997). Likewise, recent murine studies have highlighted the protective properties of IL-18 in several viral infections. Local IFN-γ production is a crucial component of the early defence against pulmonary adenovirus infection in mice and has been shown to significantly depend on an appropriate IL-18 response (Xing 2000). The production of endogenous IL-18 is also essential for a protective IFN-γ response against murine cytomegalovirus (Pien 2000). In the current study, IL-18 was found to be deficient in those infants with a severe clinical response to RSV infection in accordance with this animal data. These findings raise the possibility that deficiency of this cytokine may be important in contributing to the deficiency of IFN-γ observed in infants with RSV bronchiolitis. Further studies are required to confirm this finding and to investigate the interrelation between IL-18, IL-12 and IFN-γ in these infants.

Conclusions

- Stimulated PBMCs from infants that develop signs and symptoms of bronchiolitis following RSV infection demonstrate an excessive type 2 or deficient type 1 cytokine response compared to infants with an upper respiratory tract infection alone.
- Stimulated PBMCs from RSV infected infants with acute bronchiolitis express significantly less IFN-γ and IL-18 mRNA than PBMCs from infants with signs of an URTI.

Chapter 6:

Viral load changes during early RSV infection.

Introduction

Methods of Viral Quantitation

Virus numbers within biological fluids can be quantified by culture-based and non-culture-based techniques. Culture-based techniques involve the serial dilution of collected specimens and their subsequent inoculation into a suitable cell line for culture. The most commonly used technique is the plaque assay, which relies on the spread of virus from cell to cell, and the resultant formation of a localised plaque. After an incubation period, the infectious plaques are enumerated under microscopic examination. This enables the initial viral concentration to be estimated. Other more rapid techniques have been described which quantify viral spread (and hence viral concentration) by immunofluorescence or the formation of syncytia (Domachowske 1998a). The principle advantages of culture are it's ability to quantify viable virus (albeit viable within the chosen cell line) and the subsequent production of an isolate for further study. However, culture is relatively slow, labour intensive and somewhat insensitive. Moreover, routine cell culture is unable to detect a number of clinically important viruses such as Epstein-Barr virus and Human herpes virus and specimen transport conditions can be critical to the success of this technique.

Culture-independent molecular-biology-based methods have been developed which enable precise, accurate and reproducible quantitation of viral nucleic acids. These techniques rely on the amplification of selected regions of the viral nucleic acid and subsequent quantitation of the amplified product. They include quantitative PCR, branched chain signal amplification and nucleic-acid sequence- based amplification (Hodinka 1998). Quantitative molecular assays are extremely sensitive, quick to perform and reproducible. Molecular assays are also not significantly affected by specimen transport and storage conditions. The quantitation of nucleic acid does not, however, enable an assessment of viable virus.

The Clinical Application of Viral Quantitation

Over the past decade there has been huge interest in the clinical application of viral quantitation techniques. The ability to assess viral load or burden has had dramatic consequences both for our understanding and the management of numerous viral diseases.

Human Immunodeficiency Virus

Many clinical and laboratory markers have been used to estimate prognosis in HIV infection. The absolute $CD4^+$ T cell count is an accurate prognostic indicator but is useful only in the later stages of disease after substantial immune destruction has occurred. In the early 1990s, quantitative molecular assays were developed that could accurately measure HIV RNA in cells or plasma. It was soon realised that HIV load is the single best predictor of disease outcome (Mellors 1998) and it is this parameter that is now used predominantly in clinical practice for prognostic calculations.

Prior to the development of viral load assays, HIV disease was thought to have a prolonged phase of relative virologic latency in the period before symptoms became evident. However, quantitative molecular assays have shown that HIV is characterised by high rates of virus production and clearance of both infected cells and virions (Ho 1995). This discovery has led to a new emphasis in management with the development of highly active retroviral treatments which aim to cause prolonged maximal suppression of virus replication (Anon. 1997).

HIV quantitation has also proven extremely useful in the clinical management of patients. Viral load testing is now an essential investigation during antiretroviral therapy, guiding decisions to initiate or change therapy as well as enabling the physician to evaluate compliance and dosing requirements.

Hepatitis C Virus

Hepatitis C virus (HCV) can only be detected by molecular based assays because no cell culture system, which permits a reliable isolation of clinical specimens, is currently available. HCV RNA levels in the blood do not appear to correlate well with long-term prognosis. However, viral load prior to treatment has been shown to be predictive of response to interferon-α treatment, with increased viral load associated with decreased rates of response (Weiland 1999). Commercially available molecular quantitation assays are now

available and are used widely to initiate and monitor appropriate therapy in chronic HCV infection (Davis 1994).

Hepatitis B virus

Hepatitis B virus (HBV) cannot be cultured *in vitro* and serological assays are, therefore, used for the diagnosis and follow-up of acute or chronic infection. However, serum levels of HBV DNA provide a more sensitive means of establishing infectivity and enable carrier status to be accurately assessed in patients with ambiguous serology (Berger 2001). In addition, quantitation of HBV DNA allows early recognition of patients who do not respond to therapy and so prevents the use of ineffective and expensive therapy.

Cytomegalovirus

Cytomegalovirus (CMV) infection remains an important cause of morbidity and mortality in immunosuppressed patients, particularly those post transplant. Newer antiviral therapies have dramatically improved the prognosis of CMV infection. However, the toxicity associated with the currently available antiviral agents remains a significant problem. Quantitative molecular assays are now available that enable the CMV load to be accurately determined. Studies have shown that a high CMV load is associated with a higher risk of progression to CMV disease (Gor 1998). This observation has led to a strategy aimed at identifying individuals at risk for disease prior to the onset of disease, thereby focusing antiviral treatment to at risk patients only. In addition, viral quantitation is used to direct antiviral therapy in established disease, enabling the physician to alter drug dosing and treatment period appropriately (Evans 1998).

Respiratory Syncytial Viral Load

With one exception, previous studies of viral load in human RSV disease have used culture-based techniques to quantify virus. The study outcome measures investigated have essentially fallen into two main categories:

(1) changes in viral load following treatment (Malley 2000, Malley 1998, Englund 1990, Hall 1985, Hall 1983)

(2) relationship between viral load and disease severity (Buckingham 2000, Sheeran 1999, Hall 1976a, Hall 1975a).

Changes in RSV Load following treatment

Ribavirin is one of the most investigated and controversial treatments for RSV. Early laboratory studies demonstrated its strong antiviral properties *in vitro* against a variety of both RNA and DNA viruses (Sidwell 1972). These observations led to a large number of studies into the potential clinical use of ribavirin in viral bronchiolitis. Whilst it's clinical efficacy remains in doubt (Randolph 2000), its ability to expedite viral clearance *in vivo* has been demonstrated consistently. Using standard plaque assay techniques, it has been demonstrated that both conventional (Hall 1985, Hall 1983) and high-dose short-course (Englund 1990) ribavirin therapy result in increased viral clearance from infants infected with RSV.

A humanised monoclonal antibody to the F protein of RSV, Palivizumab, has now been developed (Johnson 1997) and its effects on viral load *in vivo* have recently been investigated (Malley 2000, Malley 1998). Recognising the limitations of standard culture-based techniques, the investigators developed a quantitative enzyme-linked immunosorbent assay (ELISA) and evaluated it against standard culture techniques. Quantitative ELISA and plaque assay correlated well for both baseline tracheal aspirate and nasal wash specimens. However, whereas palivizumab treatment resulted in significantly reduced RSV levels on plaque assay, there was no reduction following palivizumab when assessed by ELISA. The authors suggest that this apparent discrepancy can be explained by the ability of ELISA to detect antibody-bound, and therefore neutralised, virus. The ELISA system described is not significantly affected by time from collection to assay or specimen storage and may prove useful in community-based studies of RSV disease. It's clinical value remains to be seen.

RSV load and disease severity

Various clinical parameters have been used to study the relationship between the quantity of RSV in respiratory tract specimens and disease severity.

Hall *et al* studied viral titres in daily nasal wash specimens from 19 infants with RSV infection (Hall 1976a). Severity of illness was determined using both clinical and radiological parameters. Serial dilutions of nasal specimens were inoculated onto cell cultures to determine tissue culture infectious dose 50 (TCID50). Quantities of RSV were significantly greater in infants with evidence of pulmonary consolidation on chest X-ray and viral shedding was found to be significantly prolonged in those with lower respiratory

tract disease compared to those with upper respiratory illness.

A recent study by Buckingham and colleagues classified RSV disease according to need for mechanical ventilation (Buckingham 2000). Nasal aspirates were cultured using a standard plaque assay technique to establish RSV quantity expressed as plaque forming units per ml. RSV concentrations were found to be significantly higher in infants with "severe" disease (ventilated) compared to infants with "non-severe" disease.

Whereas the above studies have found evidence of a positive correlation between nasal RSV load and disease severity, Sheeran *et al* have recently published data that questions this relationship (Sheeran 1999). Nasal wash (NW) specimens were obtained serially from non-intubated patients with RSV disease and paired NW and tracheal aspirate specimens collected from intubated patients. RSV concentrations were determined using standard plaque assays. No significant correlation was found between viral load and markers of disease severity including need for mechanical ventilation, days on supplemental oxygenation and days of hospitalisation.

All of the reported studies to date have been limited to infants requiring hospitalisation as a result of RSV infection. There have been no community-based studies and, therefore, no assessment of viral load in early RSV infection. No previous study has attempted to analyse samples collected at consistent times during the infective process so it has been difficult to construct an exact timeline of viral load changes during RSV infection. Furthermore, virus quantitation has previously been carried out predominantly using culture-based techniques with their inherent transport / storage difficulties as discussed above. The current study was designed to counter these issues by studying nasal lavage specimens collected in early RSV infection at set time points following initial symptoms and using a molecular-based assay to quantitate viral load.

Real-Time Quantitative Polymerase Chain Reaction

PCR is extensively used for the detection of specific nucleic acid sequences. Target sequences are amplified exponentially, using two oligonucleotide primers (complimentary to each end of the target sequence) and a heat-stable DNA polymerase (which catalyses extension of the primers using the DNA strand as template). The detection of the resultant PCR product is usually detected after the amplification process. An aliquot of PCR product is loaded, along with appropriate molecular-weight markers, onto an agarose gel that contains 0.8-4.0% ethidium bromide. Product is then size separated using electrophoresis

and DNA bands on the gel can then be visualized under ultraviolet trans-illumination. The relative intensity of each band enables a qualitative assessment of the relative starting concentrations of the amplified templates.

Real-time PCR uses continuous monitoring of PCR products to follow the time course of the PCR reaction and eliminates the need for post-PCR processing. In 1992, Higuchi *et al* first described a system to continuously monitor the PCR reaction (Higuchi 1993, Higuchi 1992). This system includes the intercalator ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with a computer controlled camera. Amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. Other similar intercalator systems have since been described. However, the principal drawback of any intercalator-based system is that non-specific PCR products also generate signal and can thus interfere with accurate quantitation of the specific product. The development of probe-based real-time PCR systems has addressed this potential flaw.

5' exonuclease PCR

The DNA polymerase most frequently used for PCR is Taq polymerase which is derived from *Thermus aquaticus*, a yeast that is native to hot springs, and is able to withstand the high temperatures generated during the PCR reaction without denaturing. Taq polymerase also has significant 5' exonuclease activity.

During 5' exonuclease PCR, a specific oligonucleotide probe is annealed to a target sequence located between the two primer binding sites. The probe is labelled with a reporter fluorescent dye at the 5' end and a quencher fluorescent dye at the 3' end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. The probe has a 3'-blocking phosphate that prevents extension of the annealed probe during amplification. During the extension phase of PCR the probe is cleaved by the 5' exonuclease activity of Taq polymerase as the primer is extended. This cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye signal. Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. With each PCR cycle, additional reporter dye molecules are cleaved from their respective probes resulting in an increase in fluorescence intensity proportional to the amount of PCR product generated.

Holland *et al* were the first to demonstrate that cleavage of a target probe during PCR by the 5'nuclease activity of Taq polymerase could be used to detect amplification of the target-specific product (Holland 1991). Subsequent advances have resulted in the development of the TAQMAN system (Applied Biosystems (ABI), USA) (Livak 1995).

TAQMAN™ Quantitative Polymerase Chain Reaction

The TAQMAN™ system uses double labelled probes. The reporter dye 6-carboxy-fluoroscein (FAM) is attached to the 5' end of the probe with the quencher dye 6-carboxy-tetramethyl-rodamine (TAMRA) attached to the 3' end (Figure 6.1).

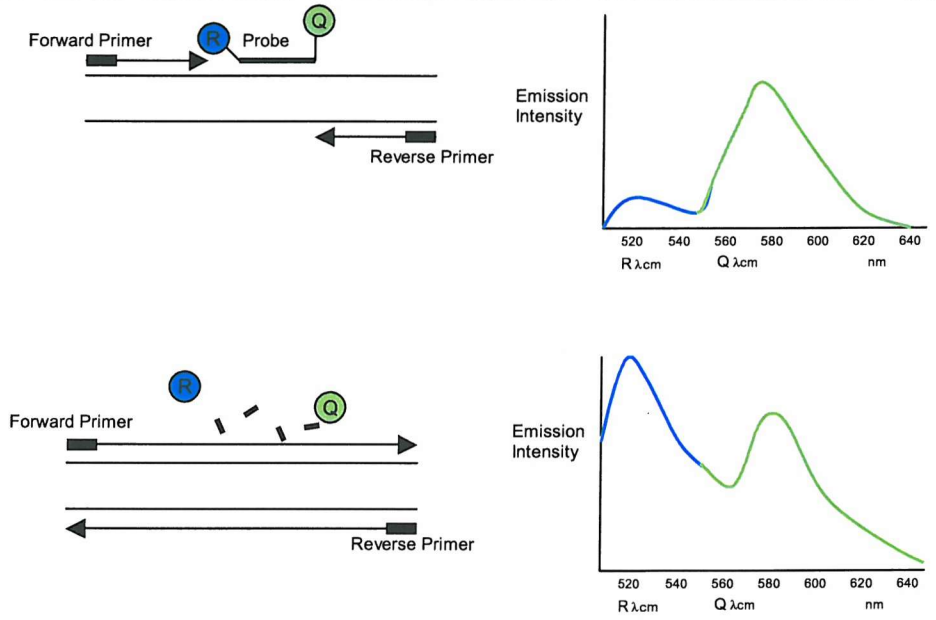

Figure 6.1 Fluorescence emission by Taqman probe reaction

The fluorogenic probe aligns itself along the genomic sequence which is to be amplified. When both dyes are attached to the probe the reporter dye emission is prevented by the quencher dye. The sense and anti-sense primers attach to the relevant sequences of the genome and the extension phase of PCR starts under the influence of DNA polymerase. The extension process causes cleavage of the probe and separates the reporter dye from the quencher dye thus increasing the reporter dye specific fluorescent emission at 518nm.Cleavage removes the probe from the target strand allowing primer extension to continue unimpeded to the end of the template strand.

The cleavage of the probe by the Taq polymerase liberates the FAM dye from the quenching effects of the TAMRA dye. The resultant increasing fluorescence is detected using the ABI PRISM 7700 system. The ABI PRISM 7700 sequence detector is comprised of a 96-well thermocycler, argon laser and charged- coupled device (CCD) camera. The argon laser is used to excite electrons from the fluoroscein reporter molecules. The fluorescence emission is detected by the CCD camera and the signal is analysed by

computer software. The heat cycling process can lead to changes in overall fluorescence of the reaction and to account for this there is a passive internal reference dye (ROX) also present in the reaction buffer. The change in the fluorescence in each single reaction vessel is monitored and the changes expressed relative to the passive reference dye. This is expressed as the ΔR_n, and for each reaction this can be plotted against the number of PCR cycles completed (Figure 6.2).

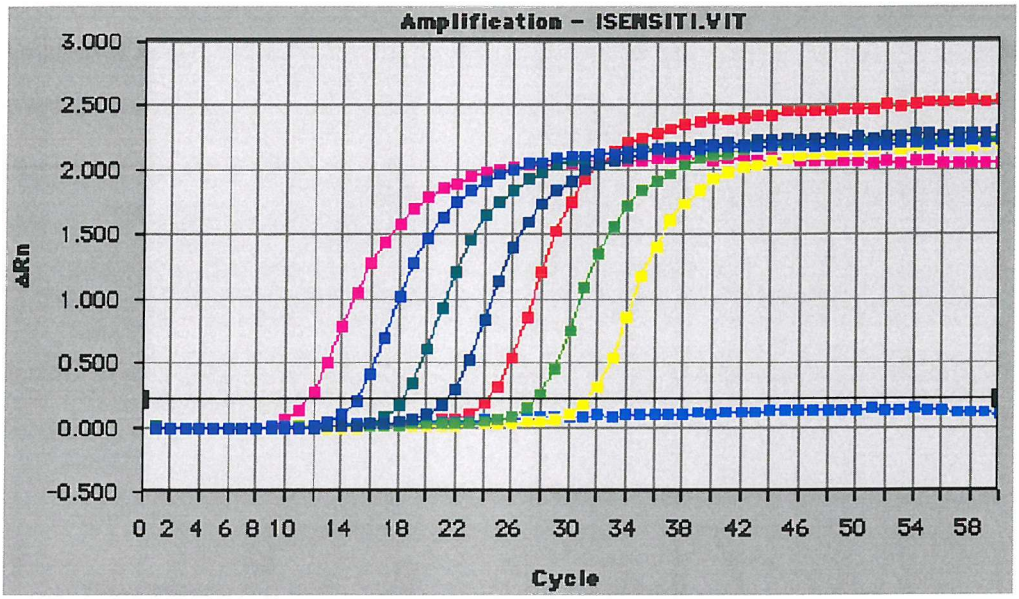

Figure 6.2 Typical plot of ΔR_n vs Cycle number produced by the TAQMAN system.

In this example the cycle threshold is depicted by the dark horizontal line at a ΔR_n of 0.023. This results in a Ct value determined by it's intersection with the amplification curve of each specimen (e.g. 11.7 for the specimen depicted by the pink line).

A reaction is considered to be positive when it crosses a predetermined threshold referred to as the threshold value. The threshold value is defined as 10 times the standard deviation from the average baseline between cycle number 3 and 15. The fractional cycle number at which the fluorescent intensity rises above the threshold value is referred to as the threshold cycle (Ct) and is inversely proportional to the target sequence concentration. If known quantified standards are also run alongside the unknown samples, a plot of the log of starting DNA quantity can be plotted against threshold cycle – this has been shown to produce a straight line (Higuchi 1993). Using this graph, the starting DNA quantities for the unknown samples can be extrapolated (Figure 6.3).

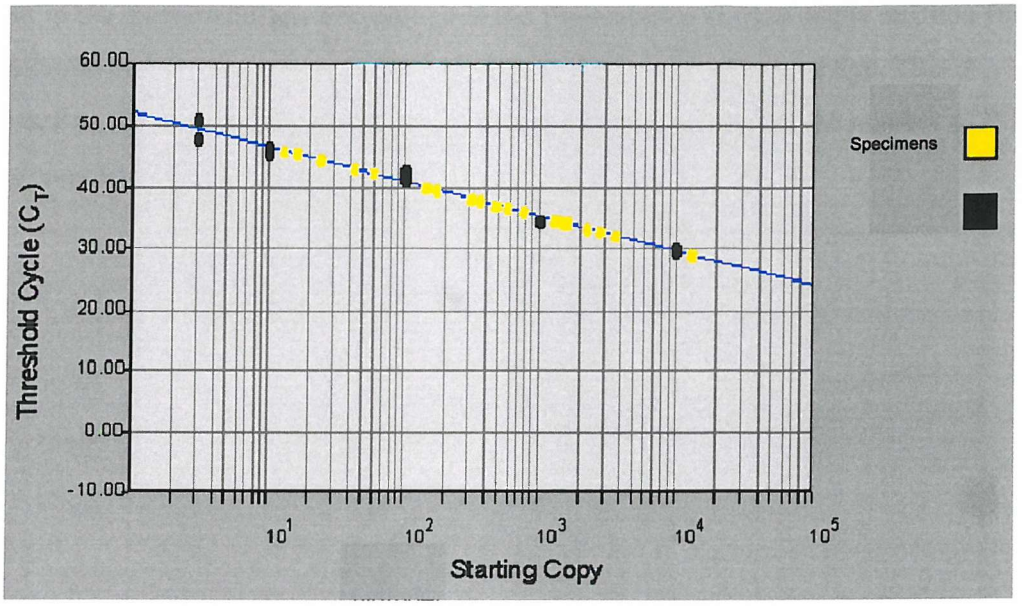

Figure 6.3 Typical plot of starting copy vs threshold cycle.

Black markers depict quantified standard specimens that are used to produce the blue line from which starting copies of the specimens analysed can be extrapolated.

Aims

The aims of this part of the study were to:

(1) determine viral load during RSV infection using quantitative PCR on nasal lavage specimens

(2) assess changes in viral load at different time points during the infection

(3) assess the relationship between viral load and clinical response to RSV infection.

Results

Patient characteristics and outcome

Of the 28 children with RSV detected by EIA, nine developed signs and symptoms consistent with acute bronchiolitis. The remaining 19 infants developed signs of an URTI alone. Table 6.1 represents demographic data for the 2 groups of patients (AB and URTI). No statistically significant differences were observed

Characteristic	WholeGroup (n=28)	Acute Bronchiolitis (n=9)	Upper Respiratory Tract Infection (n=19)	p (AB vs URTI)
Sex				
Male	15 (54%)	3 (33%)	12 (63%)	0.228
Female	13 (46%)	6 (66%)	7 (37%)	
Mean (SD) age at RSV infection (days)	195 (116)	147 (84)	219 (125)	0.132
Breast Fed >3 months	15 (54%)	5 (56%)	10 (53%)	0.604
Nasal lavage collection - mean (SD) time from start of illness (hours)				
Days 1-2	50 (17)	47 (16)	51 (18)	0.616
Days 5-7	138 (29)	129 (32)	143 (26)	0.293
Mean (SD) Gestational age (weeks)	39.54 (1.0)	39.15 (1.2)	39.37 (0.9)	0.324
Mean (SD) Birth Weight (kg)	3.52 (0.60)	3.55 (0.77)	3.5 (0.52)	0.872

Table 6.1 Patient Characteristics

RSV F-protein in nasal lavage

To assess the possible role of differences in initial viral load and to determine relative rates of viral clearance between the two infant groups, RSV F protein mRNA expression was quantified using quantitative PCR. Initial viral load was similar - F protein gene copies in the nasal lavage fluid obtained on days 1-2 of the illness did not differ significantly between the 2 groups (Mann-U Whitney non-parametric test, p=0.308, Figure 6.4).

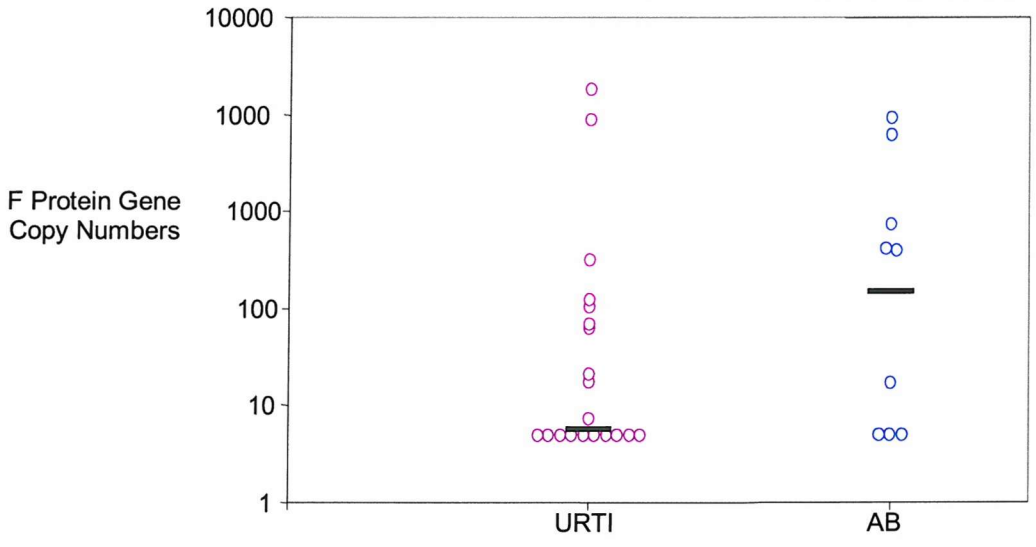

Figure 6.4 F protein gene copy numbers on Days 1-2 of illness.

No significant difference between the two groups was noted. Figure shows individual values and median for the two groups.
(AB = Acute Bronchiolitis; URTI = Upper Respiratory Tract Infection)

However, there was a trend towards impaired viral clearance in the AB group, in that 4 of the 9 infants with AB had a rise in F protein gene copies between days 1-2 and days 5-7 compared to only 2 of the 19 infants with URTI (Fisher's exact test, p=0.064, Figure 6.5).

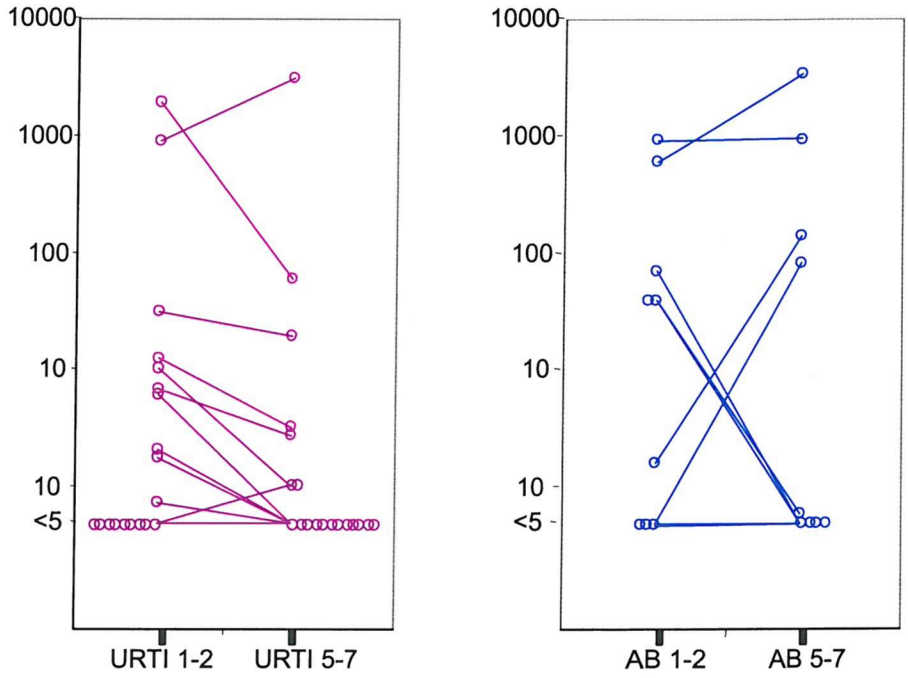

Figure 6.5 Change in F protein gene copy numbers.

Figure shows individual values at both time points. Values are connected to demonstrate change within each subject. Initial viral load was similar between groups with a trend towards impaired viral clearance in the AB group (p=0.064).
(AB = Acute Bronchiolitis; URTI = Upper Respiratory Tract Infection)

Discussion

These data demonstrate that, in the early stages of infection, respiratory tract viral load is not significantly different between infants with an RSV-URTI and those with signs of acute RSV bronchiolitis. Hall *et al* have previously studied viral shedding during RSV infection and its relation to disease severity (Hall 1976a). A total of 23 infants were assessed during a single RSV outbreak, each child having required hospitalisation for RSV infection. Nasal washes were performed on a daily basis and analysed for the presence of RSV using viral culture. Daily collections were continued until there was no evidence of further RSV shedding. Those infants with evidence of lower respiratory tract involvement were found to have significantly greater quantities of virus shed than those with upper respiratory tract

signs alone. Viral shedding continued for between 1 and 21 days following hospitalisation with a mean of 6.7 days. Those infants with lower respiratory tract disease shed virus for a significantly longer period (mean 8.4 days) than those with clinical manifestations limited to the upper respiratory tract (mean 1.4 days). Using quantitative PCR, the current study has corroborated the observed prolongation of viral shedding in infants with lower respiratory tract disease but contradicts Hall *et al*'s finding of a significantly higher viral load in this group. There are however considerable differences between the 2 studies. The current study has analysed an earlier phase in the course of RSV infection, i.e. pre-hospitalisation, and has utilised predetermined diagnostic criteria to establish the severity of RSV disease. Moreover, the current study has quantified viral load and not simply viral positivity, to study the dynamics of viral clearance. Certainly the current study is limited by the use of only 2 time points and daily (or even twice daily) specimen collections would give further weight to the study findings. In addition, extending the period of follow up beyond days 5-7 of illness would provide further valuable information about RSV clearance. Personnel limitations unfortunately prevented more regular or prolonged specimen collection, and these factors should undoubtedly be considered in the design of any future studies.

The mechanism of impaired clearance is unclear. The immune processes contributing to virus clearance are complex. Humoral responses do not appear to have a major role in clearance with no apparent temporal relation between RSV antibody generation and subsequent viral clearance in mice (Graham 1991a). By contrast, cell mediated immunity appears to be crucial in the elimination of virus. Cytotoxic T lymphocytes (CTL) are very effective at destroying virus-infected cells infected. Experimentally infected mice develop a significant lung CTL response which is temporally related to a reduction of viral shedding (Anderson 1990). Furthermore, athymic mice that become chronically infected with RSV can successfully clear the virus following injection with primed RSV-specific CTLs (Cannon 1987). $CD4^+$ T cells also have a role in viral clearance as evidenced by studies in T cell depleted mice. RSV infection of mice depleted of their $CD4^+$ T cells results in significant prolongation of viral shedding (Graham 1991b). Recent interest has focussed on the contribution of type 1 and type 2 immune responses to viral clearance. Immunisation of mice with formalin-inactivated (FI) RSV primes for an increase in IL-4 mRNA expression relative to IFN-γ mRNA expression upon RSV challenge (Graham 1993). The administration of recombinant IL-12 at the time of FI-RSV vaccination shifts the cytokine response pattern from type 2 to type 1 with subsequent live virus challenge, and results in enhanced viral clearance (Tang 1995). Similarly, anti-IL-4 treatment at immunisation

modulates the cytokine response on live virus challenge, with an increased type 1:type 2 cytokine ratio and a reduction of virus replication (Tang 1994). Further murine studies have highlighted the pivotal role of IL-4 levels in viral clearance. Transgenic mice that over express IL-4 demonstrate markedly delayed RSV clearance following live virus challenge compared to their parent controls (Fischer 1997). The mechanisms by which IL-4 impairs the clearance of RSV is unclear although recent data suggests that high levels of IL-4 may inhibit viral clearance through a reduction in $CD8^+$ cytotoxic T-lymphocyte activity (Aung 1999). Whilst the presence of such a process in humans is merely speculative, it is intriguing to note the findings of the current study with both increased nasal IL-4 levels and evidence of impaired viral clearance identified in infants with RSV bronchiolitis.

One possible limitation of the PCR reaction used to determine F-protein gene copy numbers is its inability to detect RNA from all possible RSV genotypes. Various RSV strains are known to co-circulate during the course of an RSV epidemic. Genetic studies have demonstrated distinctly different patterns of RSV genotype between communities. However, there is far less genotypic diversity within an individual community particularly when the isolates from only 1 epidemic season are considered (Peret 2000). One genotype typically predominates during the course of each season, with local factors, such as immunity induced in the previous season, possibly determining the prevailing strain. In the current study, the fact that all infants were recruited prospectively and exposed to RSV infection during the same short season in the same localised geographical area minimised the possibility of substantial genotypic diversity in the RSV strains collected. Furthermore, the primers were designed to enable PCR amplification of an area in the conserved region of the F gene. The PCR was thus able to detect both A and B strains of RSV and the common A strain sub-types. These factors together would suggest that the presence of an RSV genotype that was undetectable by the PCR system was extremely unlikely.

The nature of an immune response can be categorised according to the pattern of cytokine production and which arm of the immune system is predominantly activated (Mosmann 1996). Type 1 cytokines promote cell-mediated immunity and are required for effective responses to intracellular pathogens (such as viruses). Type 1 cytokines include interferon-γ (IFN-γ) (produced primarily by Type 1 T cells), IL-12 (from antigen presenting cells) and IL-18 (from activated macrophages). Type 2 cytokine production favours humoral immunity against extracellular pathogens and allergic responses. Examples include IL-4, -5 and -10, all produced by Type 2 T cells. Cross regulation occurs between the two types of response, some type 1 cytokines decreasing type 2 cytokine levels and vice versa. Many factors have been shown to influence the class of the immune response that develops on

exposure to a foreign antigen. Genetic factors appear to play an important role with many studies identifying gene loci associated with certain patterns of immune response. Similarly, environmental factors can affect cytokine production including the physical form of immunogen, the type of adjuvant and the route of antigen entry. The antigen dose at the time of immune priming is another variable that has significant consequences for the immune response. Very low and very high antigen doses have been suggested to promote a type 2 response, while moderate antigen levels predispose naïve cells to become type 1 cells (Murray 1998). The cytokine differences that were observed in the current study (as detailed in chapters 4 and 5) could therefore be explained by differences in antigen dose between the 2 groups (AB and URTI). To address this possibility, quantitative PCR was used to determine viral load (and hence antigen dose) in the collected specimens. No difference in initial viral load was observed between those with and without bronchiolitis thus making differences in antigen dose an unlikely explanation for our findings.

Conclusions

- During the first 24-48 hours of an RSV infection, there was no difference in viral load between infants who subsequently develop bronchiolitis and those with upper respiratory tract signs only.
- Infants who develop symptoms and signs of bronchiolitis, have impaired clearance of RSV during early RSV infection, compared to infants with upper respiratory tract infection alone.
- Infants with symptoms and signs of acute bronchiolitis demonstrate a relative type 2 immune response compared to infants with an upper respiratory tract infection alone. The disparate cytokine profiles observed are unlikely to be explained by differences in antigen dose.

Chapter 7:

Pathogens Associated with Respiratory Infection in Infancy

Introduction

Acute respiratory infections are the most common disease of the human host. The majority of morbidity and mortality accompanying these infections takes place in early childhood. All classes of microorganism can infect the respiratory tract, but viruses are by far the most frequent cause of infection. The current study examines infection with a panel of common respiratory pathogens in infants with an acute respiratory illness. The pathogens chosen for this investigation represent those most commonly associated with respiratory disease in infancy as detailed in Chapter 1. The following section outlines those pathogens studied and our current understanding of their contribution to respiratory disease during infancy.

Pathogens Causing Acute Respiratory Infections in Infancy

Respiratory Syncytial Virus (RSV)

RSV has been discussed in detail in Chapter 1.

Parainfluenza Viruses

The parainfluenza viruses are medium sized, enveloped viruses having a single stranded, non-segmented, negative-sense RNA genome. They are important respiratory pathogens in infants and young children and are reported to be the most common identifiable agent in the croup syndrome and second only to RSV as a cause of lower respiratory tract disease in infants (Glezen 1997a). The four human parainfluenza viruses were first recovered in the late 1950s from infants and young children with lower respiratory tract disease (Johnson 1960, Chanock 1958, Chanock 1956).

Human parainfluenza (PIV) type 1 and PIV-3 belong to the genus *Paramyxovirus* in the *Paramyxoviridae* family. PIV-2 and PIV-4 are part of the *Rubulavirus* genus, which also contains the mumps virus.

Epidemiology and Pathogenesis

Parainfluenza viruses are most commonly recovered in young children with upper respiratory tract illness. The epidemic behaviour and age and sex distribution of severe illnesses caused by PIV-1 and PIV-2 differ from those caused by PIV-3. PIV-4 infection accounts for a small proportion of PIV-related illness and generally causes a mild URTI only (Downham 1974). PIV-1 and PIV-2 tend to occur as biennial epidemics in the autumn, while PIV-3 occurs throughout the year with peaks typically in the spring and summer (Laurichesse 1999). PIV-1 and PIV-2 tend to cause severe disease (particularly croup) more frequently in boys and children between 6 months and 6 years of age. By contrast, PIV-3 affects both sexes equally and causes severe disease primarily in young infants. Serological studies have shown that a large majority of children are infected by PIV-1 and PIV-2 by 5 years of age and by PIV-3 by 2 years of age (Glezen 1997a, Chanock 1963).

The parainfluenza viruses are distributed worldwide and are transmitted from person to person by aerosol or by contact with infected secretions. Following an incubation period of between 2 and 6 days, parainfluenza viruses replicate within the nasopharyngeal epithelium with spread to the lower respiratory tract 2 to 3 days later in a proportion of cases.

Clinical Features

The clinical effects of parainfluenza viruses have been best described in hospitalised children (Korppi 1988, Downham 1974), although informative studies of lower respiratory tract illness exist in outpatient cohorts of children (Knott 1994, Welliver 1982, Monto 1973). The spectrum of clinical manifestations due to parainfluenza virus infection in children varies from no illness to life threatening croup or bronchiolitis. Factors that influence the clinical severity of the illness include the viral serotype and whether it is the primary or re- infection with a particular serotype.

The majority of children with a primary parainfluenza infection have only mild disease. At 2 years of age over 60% of children have serological evidence of previous infection with a history of a significant respiratory illness in less than half of this group (Glezen 1997a). Commonly recognised symptoms of primary infection in children include coryza, cough, hoarseness and anorexia. A significant percentage of symptomatic children will have an associated fever of over 38°C with signs of lower respiratory tract involvement (crackles and wheezes on auscultation) in a small number (Reed 1997). During primary infection of infants, PIV-3 has been shown to produce a febrile illness in 80% of individuals with involvement of the lower respiratory tract in 13 to 30% of cases (Glezen 1984, Chanock 1963). Primary infection with PIV-1 and PIV-2 produces fever in a similar proportion of

infants although acute laryngotracheobronchitis is observed in only 2-3% of those infected (Glezen 1997a). Following a primary infection, further infections with the same virus strain produce markedly less severe disease. However, there is no evidence of cross-strain protection against lower respiratory tract involvement (Welliver 1982). Reinfections can be frequent with reported intervals of less than 3 months in certain cases.

Adenovirus

The adenoviruses are medium-sized, non-enveloped, double-stranded DNA viruses of the family *Adenoviridae,* genus *Mastadenovirus*. They were first identified in 1953 in surgically removed adenoidal tissue which was found to degenerate when maintained in culture for several weeks (Rowe 1953). Adenoviruses were subsequently established as a cause of acute respiratory disease in military personnel and later in adults and children (Hilleman 1957).

Human adenoviruses are classified into 6 subgroups (A-F) by the haemagglutination pattern of red blood cells, the ability to cause tumours in rodents or the percentage of guanine plus cytosine content of their DNA (Benko 2000). Within each subgroup several serotypes have been identified on the basis of antigenicity. At the present time, 49 distinct adenovirus serotypes have been recovered from humans. Approximately one-third of these serotypes are associated with most of the infections caused by adenovirus.

Epidemiology and Pathogenesis of Respiratory Disease

Human adenoviruses are ubiquitous agents with a worldwide distribution. Circulating serotypes have been found to vary in different locations around the world (Marshall 1996). Serological evidence suggests that the majority of individuals have had at least one adenovirus infection by 10 years of age (Cherry 1998a). Longitudinal epidemiological studies have demonstrated that many infections are asymptomatic and that approximately 10% of febrile illnesses in childhood are due to adenoviruses (Vihma 1969).

The age of the patient has a significant bearing on the clinical consequences of infection and the probable infecting serotype. Respiratory disease is far more common in young children than in older children and adults. Infection with serotypes 1, 2 and 5 primarily occurs in children under 5 years of age and causes either no symptoms or upper respiratory tract disease. Serotypes 3 and 7 have been associated with lower respiratory tract infection (LRTI) in young children. Whilst, adenoviruses are a less frequent cause of LRTI in children than are respiratory syncytial virus and parainfluenza virus, they can cause a severe

LRTI that can result in fatal outcome or residual sequelae. The severity of adenovirus LRTI varies according to serotype, age and socio-economic status.

The typical manifestations of adenovirus disease are the result of the cytolytic properties of the virus as well as the immune and cytokine responses of the host. Adenoviruses have been shown to cause lysis of cells by inhibition of both mRNA transport and host macromolecular synthesis (Marshall 1996). There is some evidence to suggest a contribution of the immune response to disease pathogenesis with elevated levels of IL-6, IL-8 and TNF-α associated with more severe disease in adenovirus infection (Mistchenko 1994).

Clinical Features

Adenoviruses most commonly infect the respiratory tract causing mild and self-limited nasal congestion, coryza and cough. Approximately 5% of all URTIs in children younger than 5 years are caused by adenoviruses (Brandt 1969). However, adenoviruses are also a significant cause of lower respiratory tract disease. Serotypes 3, 4, 7 and 21 are most commonly implicated as causes of laryngotracheobronchitis or pneumonia. In the majority of cases the lower respiratory tract involvement is mild with a complete subsequent recovery but a small minority suffer severe disease with significant mortality or long-term sequelae (Marshall 1996). In infants particularly, adenovirus type 7 may cause fulminant pneumonia with subsequent chronic airways obstruction, bronchiectasis and, in the most extreme cases, bronchiolitis obliterans (Sly 1984).

Picornaviruses

The *Picornaviridae* family is composed of five genera including three that are pathogenic for humans: *Hepatovirus, Rhinovirus and Enterovirus.* The hepatoviruses, which include hepatitis A, do not affect the respiratory system and will not be considered further.

Rhinoviruses

The rhinoviruses are small, non-enveloped viruses having a single-stranded, positive sense genome surrounded by a symmetric protein shell. The group derives its name from the predominant site of replication and symptomatology, the nose. Rhinoviruses represent the predominant cause of respiratory disease in humans accounting for over a half of all common colds (Couch 1996). Despite early observations of a "filterable agent" causing common cold symptoms in volunteers, it was not until the mid 1950s that rhinovirus was first isolated using rhesus monkey kidney tissue cultures (Price 1956).

Since 1967, a collaborative program has established a uniform classification for the known rhinovirus immunotypes (Hamparian 1987). Rhinoviruses have been numbered 1-100 and subtype 1A on the basis of their surface antigens. Identification is based on neutralisation of viral growth with hyper immune animal antiserum containing 20 units of antibody. Field studies have found that most isolates have already been identified but have discovered antigenic variants of current serotypes and some intertypes raising the possibility of a degree of antigenic drift (Halfpap 1983, Schieble 1970).

Epidemiology and Pathogenesis

Rhinoviruses are the most common cause of acute respiratory illness in humans and have a global distribution. These infections begin in early childhood and continue throughout the adult years. Rates of infection vary from 1.2 infections per person per year in infancy (Cooney 1972) to 0.7 in young adults (Hamre 1966). Infection rates have a well-established seasonal pattern with peaks in early autumn and spring in temperate climates (Monto 1971). Rhinovirus transmission can occur during person-to-person contact, by exposure to fomites and by large or small particle aerosols.

Human rhinovirus infection can be established following exposure to extremely low quantities of virus. Under experimental conditions, nasal drops containing less than one 50% tissue culture infective dose ($TCID_{50}$) of rhinovirus cause infection and illness (Couch 1996). Rapid replication occurs within the nasal epithelium and rhinovirus is usually detectable in nasal secretions within 24 hours, with peak concentrations between 2 and 3 days following inoculation (Couch 1996). Both the rate of replication and the final peak concentration of virus in secretions, correlate with severity of illness (Douglas, Jr. 1966). The mechanisms by which replicating rhinoviruses produce symptoms and signs of the common cold are currently under investigation. Studies thus far have revealed a complex relationship between virus replication, neurogenic reflexes and host inflammatory response (Papadopoulos 1999).

Clinical Features

Rhinoviruses produce the well-recognised signs and symptoms of the common cold. Sneezing, nasal obstruction, nasal discharge and sore throat are the predominant symptoms observed usually reaching peak severity within the first 48 hours (Couch 1996). Other less frequent observations include fever, headache, cough, hoarseness and minor gastrointestinal symptoms. Examination typically reveals clear or mucoid nasal discharge with a hyperaemic or pale oedematous nasal mucosa. Symptoms and signs typically persist for 5 to 7 days although symptoms may persist for up to 2 weeks in a quarter of cases.

Rhinovirus infection in infancy is most commonly associated with URTI. However, there is increasing evidence that rhinoviruses can cause significant lower respiratory tract disease in early childhood. McMillan *et al* isolated rhinovirus from respiratory tract specimens collected from 48 infants requiring hospitalisation with respiratory symptoms (McMillan 1993). Bronchiolitis was the single most frequent diagnosis with 9 of the infants sufficiently unwell to be diagnosed with sepsis. The use of RT-PCR has been shown to increase the frequency with which infections are identified compared to conventional culture techniques (Johnston 1995). The studies performed to date have used culture to identify rhinovirus infection and, therefore, represent under estimates of infection frequency. Consequently, the role of rhinoviruses in lower respiratory tract disease is yet to be accurately quantified.

Enteroviruses

The enteroviruses are small, non-enveloped viruses having a single-stranded, positive sense genome surrounded by a symmetric protein shell. The enterovirus genus contains almost 70 antigenically distinct species that infect humans. There are three traditional enterovirus groups based on their effects in tissue cultures and in animals – the polioviruses, the coxsackieviruses and the echoviruses. The clinically useful association of specific diseases with individual groups has ensured the continued use of this classification scheme despite recent molecular analyses that have shown this division to be somewhat artefactual (Hyypia 1997). Over 20 clinical disease syndromes have been associated with enterovirus infection including various respiratory illnesses.

Epidemiology and Pathogenesis of Respiratory Disease

Enteroviruses have a worldwide distribution with most strains recovered during isolation studies in many different geographical regions (Cherry 1998b). Transmission appears to occur by two main routes – faecal-oral and via contact with respiratory secretions (person to person and fomites). The relative importance of each transmission route depends on the virus and the environmental setting – for example, coxsackievirus 21 is a well-recognised cause of URTI and is spread by respiratory secretions. Enteroviral infections occur throughout the year with peaks during summer and autumn in temperate climates. Certain enteroviruses have been associated with local and occasionally national epidemics (Cherry 1998b). The prevalence of individual enteroviral strains varies both temporally and geographically. At a given location, the predominant circulating enteroviruses can vary dramatically year to year. Following complete absence for many years, a particular strain

may reappear to infect susceptible persons of different ages who have been born since the previous outbreak. Enteroviruses are predominantly pathogenic in childhood with the highest incidence of both infection and illness during infancy.

The specific pathogenic mechanisms for many of the enteroviruses are poorly defined. During respiratory tract infection there is cytopathic destruction of the respiratory epithelium producing epithelial shedding and a local inflammatory response. Typically, this is followed by a minor viraemia resulting in involvement of secondary infection sites such as the tonsils and cervical lymph nodes. In severe cases, a major viraemia may occur some days later resulting in secondary organ involvement.

Clinical Features of Respiratory Disease

Over 60% of enterovirus infections are asymptomatic. Of those infections producing clinical disease, the majority are associated with signs and symptoms of a mild URTI. Of the numerous cocksackieviruses and echoviruses that have been implicated in upper respiratory infection, only coxsackievirus A21 has been comprehensively studied. Coxsackievirus A21 produces the common cold syndrome in artificially infected volunteers and has caused epidemics in military personnel although this pattern has not been observed in children (Cherry 1998b). Pharyngitis is often associated with enterovirus infection and is characterised by a rapid onset and a high accompanying fever. During large surveillance studies of respiratory infection in young children, enteroviruses have sporadically been associated with croup, bronchiolitis and pneumonia (Cherry 1998b). Several outbreaks of both croup and pneumonia have been reported in the literature.

Enteroviral culture is relatively insensitive and recent studies have highlighted the superiority of RT-PCR for the detection of enteroviral infection (Gorgievski-Hrisoho 1998). Serological testing is impractical because of the large number of enteroviral serotypes.

Coronaviruses

The genus *Coronavirus*, of the family *coronaviridae*, consists of medium sized, pleiomorphic, enveloped viruses having a single-stranded positive polarity RNA genome. They were first identified in 1965 by culturing nasal wash specimens from adults with the common cold in human embryonic tracheal organ cultures (Tyrrell 1965). The *Coronaviridae* family comprises a large number of viruses largely associated with respiratory and enteric infections in mammals and birds.

Coronaviruses have been divided into four major antigenic groups based on serological analyses (Holmes 1996). The best-studied human coronavirus strains are 229E (antigenic group 1) and OC43 (antigenic group 2), both of which can be cultured under stringent conditions in tissue culture or animals. The other human coronaviruses that have been isolated from the respiratory tract are related antigenically to one or other of these strains, but have proved extremely difficult (or impossible) to culture.

Epidemiology and Pathogenesis of Respiratory Disease

Serological studies form the bulk of available epidemiological data as a result of the difficulties associated with coronavirus culture. Serology has identified evidence of coronavirus infection worldwide (Holmes 1996). Antibody to the two main serotypes, OC43 and 229E, appears in early childhood and increases in prevalence rapidly with age (McIntosh 1970). Over 85% of adults have been found to have antibodies to OC43 and 229E (Holmes 1996).

Coronavirus respiratory infections display significant seasonality with peaks in the winter and spring in temperate climates. The proportion of respiratory infections due to coronavirus varies both seasonally and on a yearly basis with often dramatically different rates year to year (Hamre 1972). Little is known of coronavirus infection in infancy and early childhood. One recent paper from Scandinavia used a reverse transcription-PCR-hybridisation assay to detect OC43 and 229E serotypes in children under 24 months of age with respiratory infection (Nokso-Koivisto 2000). Coronaviruses were detectable in 2.4% of nasopharyngeal and 3% of middle ear fluid samples. This represents a lower rate than that observed in older children – possible explanations include age differences or specific epidemiological factors of the study population.

Transmission of coronaviruses probably occurs in a similar manner to rhinoviruses with good airborne stability identified for the 229E strain (Ijaz 1985). The precise cells initially infected are not known, but a wide variety of cells in the respiratory tract bear the hAPN receptor for 229E on their apical surfaces (Yeager 1992). Coronaviruses have a cytolytic effect during cell culture and it is likely that destruction of the epithelium occurs *in vivo* resulting in an inflammatory host response.

Clinical Features

The clinical pattern of coronavirus infection has been most extensively studied following experimental infection of adult volunteers. Following an incubation period of 2 to 5 days the principal symptoms are those of an upper respiratory tract infection with nasal

discharge, malaise, headache, sore throat and cough (Bradburne 1967). Approximately 20% of infected adults develop low-grade pyrexia. Lower respiratory tract infection is uncommon although coronavirus infection has been associated with the exacerbation of pre-existing cardiopulmonary conditions in hospitalised patients (El Sahly 2000).

Coronavirus infection of infants and children generally produces an URTI although signs and symptoms in this age group are less well defined. Otitis media appears to be a reasonably frequent consequence of infection with 3% of middle ear fluid specimens proving positive for coronavirus by PCR in 2 separate studies (Nokso-Koivisto 2000, Pitkaranta 1998). The incidence of lower respiratory tract involvement is not clear. Two large studies of hospitalised children with respiratory disease, failed to identify evidence of coronavirus infection either by serology or immunofluorescence (McIntosh 1978c, McIntosh 1970). However, another study recovered several strains resembling 229E from infants with pneumonia and found antibody titre rises to coronavirus in 8% of this group (McIntosh 1974). More recently, coronaviruses were identified by PCR in 5 children less than 2 years of age with LRTI (Nokso-Koivisto 2000).

Mycoplasma Pneumoniae

Mycoplasmas form a genus of the *Mycoplasmataceae* family, which is included within the class *Mollicutes*. Mollicutes are the smallest and simplest free-living organisms. They are distinct from bacteria as they lack a cell wall and are bound only by a plasma membrane. Unlike viruses, they contain both RNA and DNA and can grow on cell-free media. Mycoplasma was first was isolated from a human in 1937 (Dienes 1937). Four mycoplasmal species are now known to be pathogenic for humans but only *Mycoplasma pneumoniae* is an important cause of respiratory disease in humans accounting for approximately 15 to 20% of all community acquired pneumonias (Foy 1970). *Mycoplasma pneumoniae* was first isolated in the early 1960s following extensive study of its properties and disease features during the second world. It has a filamentous morphology and is somewhat motile. *Mycoplasma pneumoniae* has a circular genome of double stranded DNA of only 800 kilobases (less than half the size of most bacteria). This extremely small genome has been the subject of an intense research effort to establish the prerequisites of the theoretical "minimal cell" capable of self-replication (Mushegian 1996).

Epidemiology and Pathogenesis of Respiratory Disease

Mycoplasma pneumoniae has a worldwide distribution. The incidence of infection peaks in

children 5 and 15 years of age accounting for between 30 and 60% of community acquired pneumonias in this age group (Foy 1979). Rates of infection reduce with increasing age accounting for less than 10% of pneumonias in adults over 20 years of age. Infections occur throughout the year with no evidence of seasonality. Epidemics have been described with infection rates increasing up to 5 fold in older children and young adults during these periods (Foy 1979). Transmission of the organism between individuals is slow and requires close contact for an extended period. The incubation period is 2 to 3 weeks. Pre-existing immunity determines predominantly an individual's susceptibility to infection.

Infection occurs via the respiratory route following close contact with an infected individual. Large droplets of secretion appear to be a more prevalent form of transmission then small-particle aerosols. *Mycoplasma pneumoniae* appears to produce most of its physiologic and cytolytic effects while remaining extracellular. The organism can be first isolated from respiratory secretions 2 to 8 days before clinical illness and persists for many weeks following disease resolution. The mechanism of disease progression and spread through the respiratory tract is not clear and may depend largely on the initial site of infection.

Clinical Features of Respiratory Infection

The majority of symptomatic respiratory infections caused by *Mycoplasma pneumoniae* involve only the upper respiratory tract. There is typically an insidious onset of symptoms with fever, malaise, headache and cough followed 2-4 days later by rhinitis and/or pharyngitis. Recent evidence has also implicated *Mycoplasma pneumoniae* as a cause of otitis media in infants (Raty 2000).

In a small minority of cases, the infection progresses to tracheobronchitis or pneumonia. The initial manifestations of fever and malaise persist and coughing becomes increasingly prominent. In children, the cough tends to be non-productive and is sometimes associated with muscular chest pain. Fever over 38°C is a frequent sign and this is often accompanied by chilly sensations. Physical signs are variable with chest auscultation frequently revealing crackles and occasionally wheeze over the involved areas (Stevens 1978). Signs of severe lower respiratory tract infection such as tachypnoea and cyanosis are unusual. Pneumonia due to *mycoplasma pneumoniae* rarely requires hospitalisation.

Mycoplasma pneumoniae infection in infancy appears to cause minimal disease. Monitoring of infants in a day-care setting revealed that the majority of infections were asymptomatic or associated with mild non-specific coryza (Fernald 1975). Lower respiratory tract involvement was more likely in those children over 3 years of age. The

high incidence of asymptomatic infection in young children has been reaffirmed by recent studies using PCR detection of *mycoplasma* (Dorigo-Zetsma 2001).

Influenza Viruses

Influenza viruses are enveloped viruses of the *Orthomyxoviridae* family containing a single stranded negative polarity RNA genome. There are three major antigenic types of influenza - A, B and C. Influenza A and B cause the well-recognised, acute febrile illness of influenza whereas influenza C causes symptoms of the common cold and rarely, if ever, produces the influenza syndrome. Pandemics of influenza appear to have occurred since ancient times with historians identifying at least 12 pandemics over the last 400 years. Under the electron microscope, the influenza viruses appear as irregular spherical particles 80 to 120 nm in diameter with occasional filamentous and icosahedral forms also observed. Anchored in the virus membrane, 400-500 spikes project out of the influenza virus particle. Each of these projections consists of a glycoprotein, either haemagglutinin (HA) or neuraminidase (NA).

Epidemiology and Pathogenesis of Respiratory Disease

Influenza infection occurs in seasonal (winter months) yearly epidemics, with more severe epidemics every 2 to 3 years and major worldwide pandemics every 20 to 40 years. Influenza typically has its highest attack rate in the young while causing the highest mortality in the elderly (Glezen 1991). Influenza infection during infancy appears to be a significant problem. A recent large retrospective study found influenza related hospitalisation rates were similar in both healthy infants and adults at high risk for influenza (Neuzil 2000).

The long-term epidemiological success of influenza viruses is primarily due to antigenic variation that takes place in HA and NA surface glycoproteins of the virus. Antigenic variation renders an individual susceptible to new strains despite previous infection by influenza viruses or previous vaccination. This variation is the consequence of two processes – antigenic drift and antigenic shift (Murphy 1996).

Influenza virus is transmitted in airborne respiratory secretions. Infection occurs in the tracheobronchial epithelium, where the first cycle of replication takes approximately 4-6 hours. During this initial stage of infection high titres of virus are shed that, combined with the short incubation period (18-72 hours), produces the typically explosive nature of influenza outbreaks. Bronchial biopsy specimens from infected individuals demonstrate

necrosis of ciliated epithelial cells with local oedema and cellular infiltration by lymphocytes, plasma cells and polymorphonuclear leucocytes (Murphy 1996). These findings extend to the level of the alveolar cells in cases of influenza pneumonia.

Clinical Features

The clinical manifestations of influenza are similar in both children and adults. There is an acute onset of systemic symptoms with fever, chills, headaches, myalgia and malaise predominating. Respiratory symptoms such as a dry cough and coryza are often observed but are usually minor compared to the systemic symptoms. The symptom complex usually persists for 3 days at which point the fever subsides followed closely by the other symptoms.

Infants infected with influenza typically present with a non-specific febrile upper respiratory tract illness that is difficult to differentiate from other respiratory tract viruses. Gastrointestinal symptoms such as nausea, anorexia and diarrhoea are frequent symptoms in infancy (Paisley 1978) and very young infants (<2 months of age) often present with features indistinguishable from bacterial sepsis. Other common presentations in infancy include bronchiolitis, croup and febrile convulsions (Glezen 1998).

Detection of viral RNA using RT-PCR has recently been shown to provide a rapid, sensitive alternative to virus culture for the detection of influenza viruses in clinical specimens (Magnard 1999).

Chlamydia Pneumoniae

Chlamydiae are obligate intracellular bacteria that have a unique biphasic developmental cycle involving an extracellular form (elementary body) and an intracellular replicating form (reticular body). *Chlamydia pneumoniae* was first isolated in 1965 but was not recognised as a respiratory pathogen until 1983 (Grayston 1986). It has since been recognised as an important cause of community acquired pneumonia in both children and adults.

Epidemiology and Pathogenesis of Respiratory Disease

Serologic studies have demonstrated that *Chlamydia pneumoniae* has a worldwide distribution, has at some stage infected over 50% of 30-year olds and has caused respiratory infections since at least 1958. Seropositivity is low under 5 years of age but then increases rapidly through the school-age years (Grayston 1992). However, there is a poor humoral response to infection in young children so that serology may underestimate the incidence of

infection in this age group (Normann 1998a). *Chlamydia pneumoniae* causes between 7 and 10% of community acquired pneumonias in adults (Grayston 1989) with a similar incidence reported in childhood (Principi 2001, Forgie 1991). Sensitive molecular analyses have recently highlighted a significant incidence of infection in both adults and children with URTI (Normann 1998b). In infancy, *Chlamydia pneumoniae* appears to be an infrequent cause of LRTI (Jantos 1995, Forgie 1991) although it's contribution to upper respiratory tract symptoms in this age group remains to be defined.

Clinical Features

The most frequent clinical presentations of *Chlamydia Pneumoniae* infection are pneumonia and URTI including sinusitis and pharyngitis (Principi 2001, Normann 1998b, Grayston 1989). Symptoms typically have an insidious onset with initial rhinitis followed by an escalating cough over the ensuing weeks. Symptoms due to *Chlamydia Pneumoniae* infection are often chronic in nature, persisting for many weeks despite appropriate antibiotic therapy. Interestingly, asymptomatic infection appears to be widespread in both children and adults (Normann 1998b, Hyman 1995) and these individuals may act as reservoirs for dissemination of the organism. The clinical features of infection in infancy are not as well described but appear to follow a similar pattern with upper and lower respiratory tract symptoms described, as well as asymptomatic infection (Normann 1998a, Normann 1998b, Jantos 1995, Forgie 1991). .

Chlamydia Pneumoniae can be isolated in cell culture but is fastidious and slow growing and therefore unlikely to provide clinically useful results. By contrast, PCR provides rapid results and has been shown to have a similar if not superior sensitivity to culture (Dalhoff 1996).

Epidemiology of Infection with Multiple Respiratory Pathogens in Infancy

Many studies have examined the utility of nucleic acid amplification for the identification of respiratory pathogens from clinical samples. However, the majority of studies have focussed on a single pathogen only and have, therefore, been unable to identify a specific aetiology when the result for that pathogen has been negative. Furthermore, such studies are unable to examine simultaneous infections involving more than one pathogen.

Four published studies have used molecular techniques to study multiple respiratory pathogens during infancy (Table 7.1). Gilbert *et al* used a panel of RT-PCR protocols to

detect RSV, parainfluenza viruses and picornaviruses in respiratory specimens from 80 infants (Gilbert 1996). All infants were hospitalised with a variety of clinical diagnoses (URTI, croup, bronchiolitis and pneumonia). Nucleic acid for all three virus groups was identified in a substantial number of specimens (35% specimens positive for RSV, 35% parainfluenza, 33% picornavirus). RT-PCR was found to be significantly more sensitive than culture for the detection of picornaviruses and mixed infections. No association was identified between the infecting virus and clinical diagnosis. In a similar study, Freymuth *et al* used RT-PCR followed by enzyme immunoassay detection of PCR product to detect RSV, parainfluenza 3 virus, adenoviruses and rhinoviruses in nasal aspirates from 277 infants (Freymuth 1997). All infants were hospitalised with an acute respiratory infection (both upper and lower respiratory tract illnesses were included). RSV was detected in 64% samples, PIV-3 in 8%, rhinoviruses in 14% and adenoviruses in 10%. Simultaneous viral infections were identified in 18% of cases. RT-PCR was found to be significantly more sensitive than both immunofluorescence assay and culture. Using a similar RT-PCR-EIA technique, Eugene-Ruellan *et al* simultaneously examined the presence of RSV and PIV-3 in nasal aspirates of 261 infants hospitalised with bronchiolitis (Eugene-Ruellan 1998). They detected RSV and PIV-3 sequences in 39.4% and 5.7% of specimens respectively. No mixed infections were identified.

The most comprehensive study of respiratory pathogens in infancy to date utilised a previously optimised multiplex RT-PCR technique to study nine microorganisms simultaneously (Weigl 2000). Nasopharyngeal aspirates from 1281 children (527 infants) admitted with an acute respiratory infection were analysed. A pathogen was identified in almost 40% of specimens including RSV (23% of samples), adenoviruses (7.2%), influenza-A (5.3%), PIV-3 (2.5%) and *Mycoplasma pneumoniae* (1.3%). Dual infection was identified in 3.4% of cases.

Reference	Study Period	Type of Study	Study Site	Method Used	Specimen Type	Symptoms	Total Number of Infants Studied (% in- or out-patients)	Percentage of Specimens Positive for Organism (NE= Not Evaluated)											
								Rhino	RSV	Para-1	Para-2	Para-3	Adeno	Corona	Flu-A	Flu-B	Chlamydia	Myco-plasma	Mixed Infection
Weigl et al 2000 (Weigl 2000)	Dec 1995 to March 1999	Retrospective	Germany	Nine-Valent Multiplex RT-PCR	Nasopharyngeal Aspirates	ARTI	527 (100% in-)	NE	23	0.8	NE	2.5	7.2	NE	5.3	0.9	0	1.33	3.4
Eugene-Ruellan et al 1998 (Eugene-Ruellan 1998)	October 1995 to March 1996	Retrospective	France	RT-PCR-EIA	Nasal Aspirate	Bronchiolitis	261 (100% in-)	NE	39.4	5.7	NE	NE	NE	NE	NE	NE	NE	NE	0
Freymuth et al 1997 (Freymuth 1997)	October 1995 to March 1996	Retrospective	France	RT-PCR-EIA	Nasal Aspirate	ARTI	277 (100% in-)	12.6	62	NE	NE	8	10.8	NE	NE	NE	NE	NE	18
Gilbert et al 1996 (Gilbert 1996)	October 1993 to April 1994	Retrospective	Canada	RT-PCR-EIA	70 nasopharyngeal washes; 6 nasopharyngeal aspirates; 4 tracheal aspirates	ARTI (20 URTI, 20 croup, 20 bronchiolitis, 20 pneumonia)	80 (100% in-)	34	35	NE	NE	35	NE	NE	NE	NE	NE	NE	20

Table 7.1 **Previous studies examining multiple respiratory pathogens in infancy using PCR.**
Values represent percentage of specimens positive for pathogen.

Key: Rhino, rhinovirus; Para-1, parainfluenza virus 1;Para-2, parainfluenza virus 2; Para-3, parainfluenza virus 3; Adeno, adenovirus; Corona, coronavirus; Flu-A, influenza A virus; Flu-B, influenza B virus; Chlamydia, chlamydia pneumoniae; Mycoplasma, mycoplasma pneumonia ARTI, acute respiratory tract infection

Aims

The aims of this part of the study were:

(1) to establish the incidence of infection with a number of respiratory pathogens during infancy

(2) to establish the incidence of multiple infection with two or more respiratory pathogens during infancy

(3) to analyse the association between infecting pathogen and symptoms/signs as reported by parents

(4) to analyse the association between infecting pathogen and clinical diagnosis

Results

Patient Characteristics

Ninety-one babies were recruited antenatally. Three of the recruited infants were withdrawn from the study before the start of the study period. Two infants developed symptoms and signs consistent with asthma and one infant was withdrawn for personal reasons. Eighty-eight infants were subsequently monitored through their first winter - 1st November 1997 to 31st March 1998. This study period was used to coincide with the peak rate of infantile RSV infections as determined from Southampton General Hospital Public Health Laboratory virological data. Table 7.2 represents demographic data for the study group.

Characteristic	**Whole Group** (n=88)	**Male** (n=44)	**Female** (n=44)
Number of URTIs during study period			
0	19	12	7
1	34	15	19
2	22	10	12
3	7	3	4
>3	6	4	2
Breast Fed >3months	39 (44%)	17 (39%)	22 (50%)
Mean (SD) Gestational Age (weeks)	39.50 (1.23)	39.16 (1.40)	39.84 (0.94)
Mean (SD) Birth Weight (kg)	3.43 (0.53)	3.42 (0.57)	3.43 (0.50)

Table 3.2 Patient Characteristics

Incidence of Respiratory Symptoms and Pathogen Detections

88 infants were followed through their first winter. Whenever a baby developed respiratory symptoms, parents were asked to telephone the study team within 24 hours. One of the research team (JPL) then visited the family at home. During this visit a respiratory questionnaire was completed to establish the exact nature of the respiratory symptoms and their impact on feeding and sleep (Appendix 3). A clinical examination was then performed to elicit any signs of respiratory disease. Following this a nasal lavage specimen was collected. Parents were given a daily diary sheet (Appendix 4) complete for 7 days from the visit date and asked to send the sheets in a prepaid envelope to the research team on completion.

As part of the study protocol, a further home visit was made 5 to 7 days after the initial onset of respiratory symptoms if the initial nasal lavage specimen tested positive for RSV by enzyme immunoassay (EIA). At this visit, a further respiratory questionnaire and clinical examination were performed. In addition, another nasal lavage was carried out. If the initial nasal lavage specimen tested negative for RSV, the family was contacted by telephone 3 to 4 days later to ascertain the infant's progress. If the child had developed symptoms and signs of lower respiratory tract involvement, a further home visit was made 5-7 days after the initial visit as detailed above. To ensure compliance with the study protocol, study families were contacted by telephone or mail every 3 weeks to enquire as to the health of their infant and as a reminder of the indications for contacting the research team.

A clinical diagnosis was made according to the predetermined diagnostic criteria discussed in Chapter 2. In brief, (a) Upper Respiratory Tract Infection (URTI) was defined as new-onset rhinorrhea with or without fever or cough but without signs of bronchiolitis, (b) Acute bronchiolitis was diagnosed if the infant had all 3 of tachypnoea (>60 breaths per minute), subcostal recession at rest and inspiratory crackles on chest auscultation, (c) Croup was defined as a clinical syndrome consisting of inspiratory stridor and barking

A total of 123 episodes of acute respiratory infection were reported from which 151 nasal lavage specimens were collected (28 episodes had 2 lavage specimens collected for the reasons stated above). As detailed in Chapter 2, respiratory pathogens were identified using specific RT-PCR for picornaviruses, RSV, parainfluenza viruses, coronaviruses, influenza viruses, adenoviruses, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*. Respiratory pathogens were identified in 125 (83%) of the 151 specimens analysed and in 102 (83%) of the 123 episodes studied.

Picornaviruses were the most frequently detected pathogen being identified in 46% (56/123) of episodes (Table 7.3). RSV (27%, 34/125) was the next most frequent pathogen followed by the parainfluenza viruses (13%, 16/125) and the coronaviruses (9%, 11/125). *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, adenoviruses and the influenza viruses were found infrequently.

Dual pathogen infections were identified in 25 (17%) of the 151 specimens analysed representing 22 (18%) of 125 episodes (Figure 7.1). No specimen contained more than two pathogens. The most frequent combination was picornavirus and RSV (8% of episodes, 10/123) followed by picornavirus and parainfluenza virus (6%, 7/123), picornavirus and coronavirus (1.6%, 2/123), RSV and parainfluenza viruses (1.6%, 2/123), picornavirus and adenovirus (0.8%, 1/123), picornavirus and *Chlamydia pneumoniae* (0.8%, 1/123) and RSV and coronavirus (0.8%, 1/123).

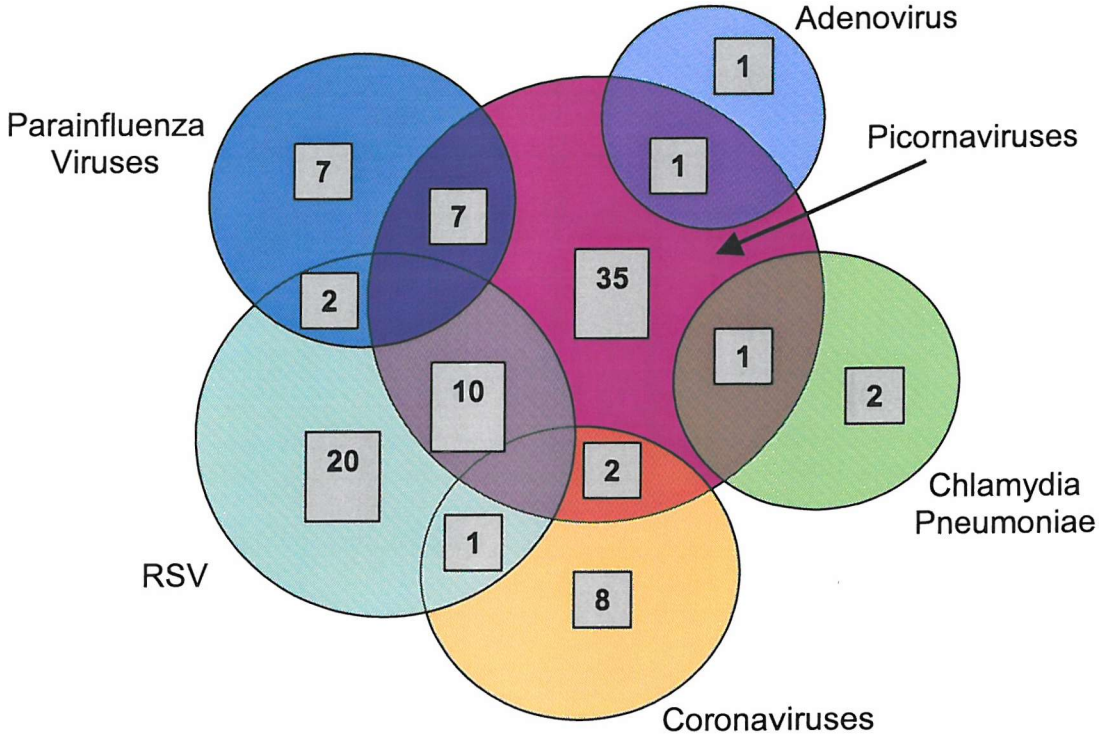

Figure 7.1 Graphical Depiction of Multiple Respiratory Pathogen Infections.

Each circle represents an individual respiratory pathogen. Dual infection is represented by circle intersections. Figures represent total number of episodes where pathogen(s) was identified.

Age and Sex Distribution of Pathogen-Specific Episodes

The age and sex distribution of infants at the time of infection are presented in Figure 7.2 and Table 7.3. RSV, influenza A and *Chlamydia pneumoniae* infections were more frequent in males. All other pathogens were detected predominantly in females.

In the first 9 months of life picornaviruses were the most common pathogen detected, identified in 51% (50/98) of all episodes of acute respiratory infection in this age group (Figure 7.2). After 9 months of age picornaviruses (24% of episodes), RSV (36%) and parainfluenza viruses (20%) were the most frequent organisms. Picornavirus and influenza virus infections reduced in frequency with increasing age, whilst coronavirus infections appeared to increase in frequency with age. RSV infections were most common after 3 months of age. Influenza A, adenovirus, chlamydia pneumoniae and mycoplasma pneumonia were detected throughout the first year of life although distinct patterns were difficult to identify due to low numbers.

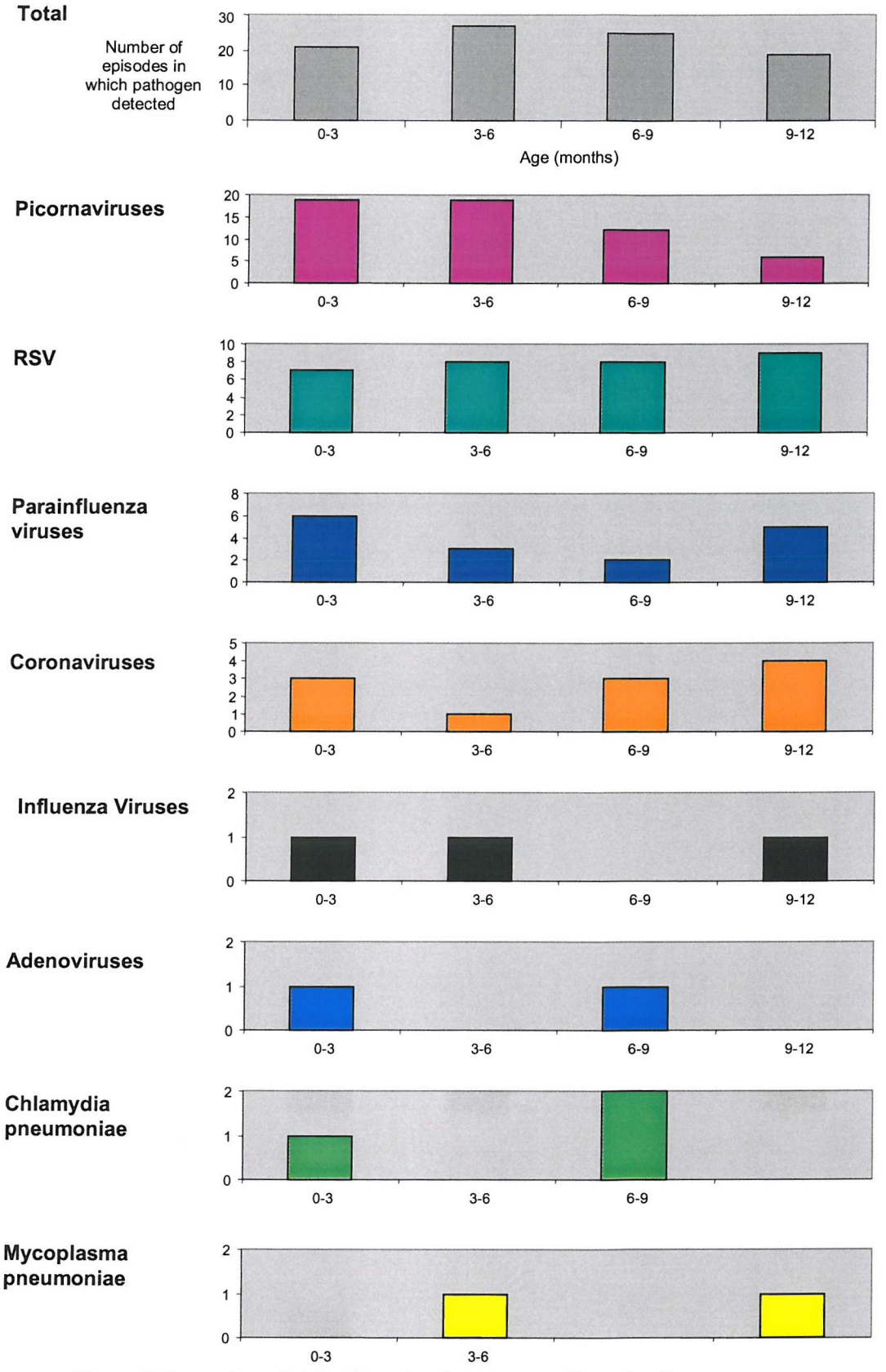

Figure 7.2 Age distribution of pathogen-specific episodes.

Age is divided into 4 three-monthly periods on the Y-axis. The X-axis represents the total number of episodes in which the pathogen was detected (i.e. out of a total of 125 episodes).

Pathogen	Number of Cases (%age episodes)	Mean Age ± SD (months)	Min Age	Max Age	Male: Female (Ratio)
Picornaviruses	56 (46%)	4.9 ± 3.1	8 days	392 days	0.81: 1
RSV	33 (27%)	6.7 ± 4.0	34 days	408days	1.75: 1
Parainfluenza viruses	16 (13%)	6.2 ± 4.6	26 days	408 days	1: 1
Coronavirus 229E	8 (6.5%)	7.9 ± 4.6	25 days	398 days	1: 1
Coronavirus OC43	3 (2.4%)	4.0 ± 4.5	18 days	271 days	All Female
Influenza A	3 (2.4%)	5.6 ± 4.7	26 days	305 days	2: 1
Influenza B	0	ND	ND	ND	ND
Adenoviruses	2 (1.6%)	5.6 ± 4.8	64 days	269 days	All Female
Chlamydia pneumoniae	3 (2.4%)	5.7 ± 3.8	47 days	271 days	2: 1
Mycoplasma pneumoniae	3 (2.4%)	8.0 ± 5.5	94 days	418 days	0.5: 1
Total	127*				

*including 24 episodes where 2 pathogens were detected.

ND- Not Detected

Table 7.3 **Incidence of pathogen detection and age and sex distribution of infants**

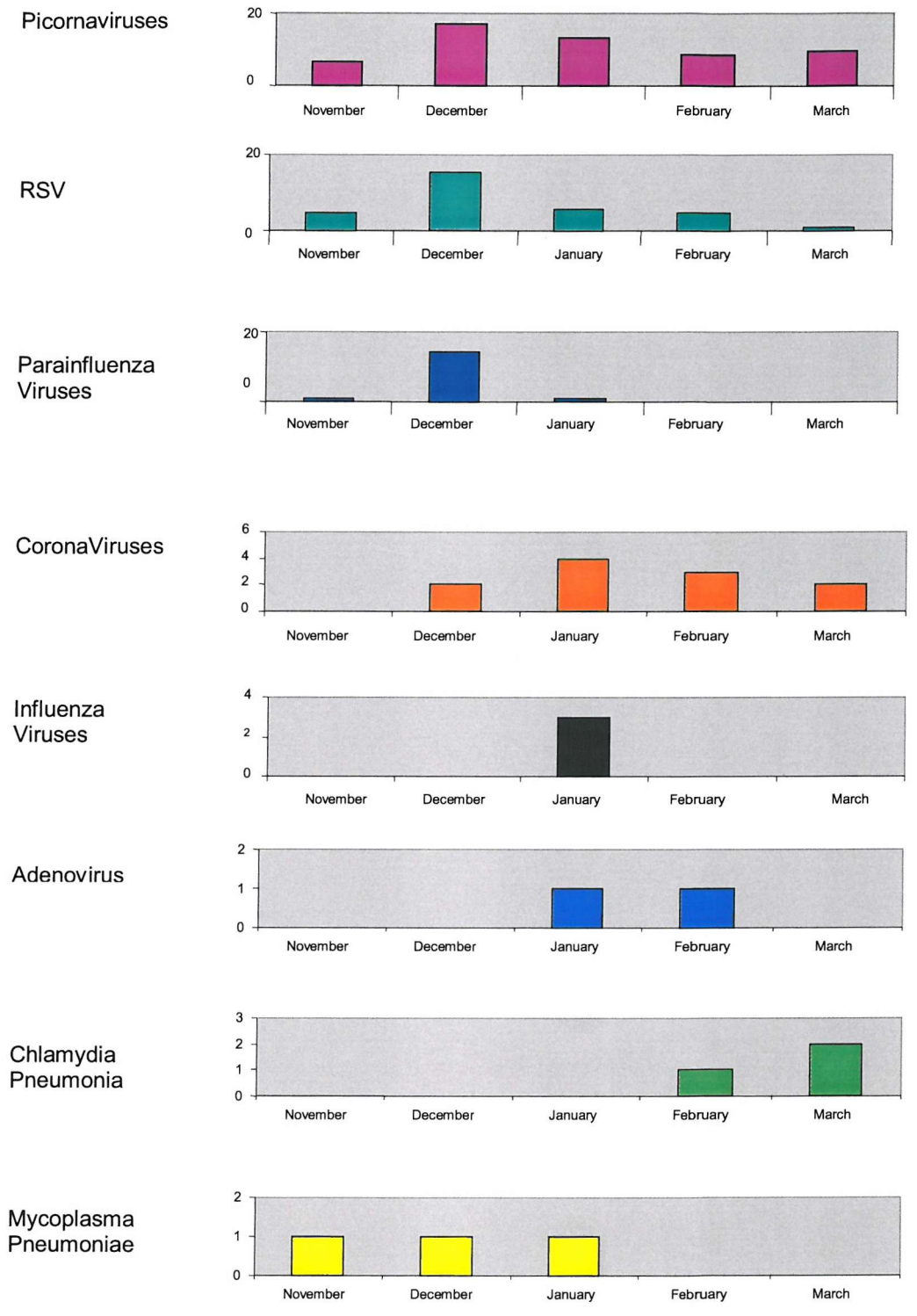

Figure 7.3 Monthly Distribution of Respiratory Pathogen

Monthly Distribution of Pathogen-Specific Episodes

The study period covered late autumn (1st November 1997 to 21st December 1997), winter (22nd December 1997 to March 20th 1998) and early spring (March 20th 1998 to 31st March 1998). For clarity, Figure 3.3 represents the distribution of infections on a monthly basis. Overall, the peak months for respiratory infection were December (32% of all episodes) and January (24%). RSV, picornavirus and parainfluenza virus infections were at their peak in December, whilst coronavirus peaked in January. *Mycoplasma pneumoniae* infections occurred during the early part of the study period, whilst *Chlamydia pneumoniae* and adenovirus infections were more frequent during the later months. In the current study, Influenza A infection was confined to January with 3 separate episodes detected. This is in keeping with PHLS data showing influenza A detections starting in the first week of January 1998 and peaking in mid February 1998 (Dedman 1998).

Relationship between Pathogen and Reported Symptoms and Signs

Parents were asked to complete a daily diary sheet for 7 days following the initial visit (Appendix 4). Diary sheets were returned for 95 (77%) of the 123 acute respiratory episodes studied. The results from the diary sheets were tabulated using computer software (Excel 97, Microsoft, USA). The relationship between the infecting pathogen(s) and the presence or frequency (number of days out of 7) of the specific signs and symptoms was analysed. No significant associations were identified.

Relationship between Pathogen and Clinical Diagnosis

Table 3.4 presents the distribution of pathogens and clinical diagnoses for the respiratory episodes studied. There was a significant relationship between clinical diagnosis and infection with RSV. Children whose first acute respiratory episode was bronchiolitis were more likely to have a RSV infection than children whose first diagnosis was URTI or croup ($p=0.013$, Fisher's exact test). No other significant associations were identified.

There was no significant association between dual infection and a diagnosis other than URTI.

	URTI	Bronchiolitis	Croup	Total
Picorna	35 (100%)	0	0	35
RSV	14 (74%)	5 (26%)	0	19
PIV	6 (86%)	0	1 (14%)	7
Corona	8 (100%)	0	0	8
Flu	3 (100%)	0	0	3
Adeno	1 (100%)	0	0	1
Chlam	2 (100%)	0	0	2
Myco	3 (100%)	0	0	3
Picorna & RSV	6 (60%)	4 (40%)	0	10
Picorna & PIV	3 (43%)	3 (43%)	1 (14%)	7
Picorna & Corona	2 (100%)	0	0	2
RSV & PIV	2 (100%)	0	0	2
Picorna & Chlam	1 (100%)	0	0	1
Picorna & Adeno	1 (100%)	0	0	1
RSV & Corona	1 (100%)	0	0	1
No Respiratory Pathogen Detected	20	1	0	21
Total	108	13	2	123

Table 7.4 Distribution of Clinical Diagnoses and Detected Pathogens

Discussion

Picornavirus infections, and particularly rhinovirus infection, represent the most common cause of acute respiratory illness in humans. RT-PCR has been demonstrated to have a considerably higher diagnostic yield than culture for picornavirus infection (Johnston 1993). This study is the first to analyse picornavirus respiratory infections using RT-PCR in non-hospitalised infants. Previous studies have identified the picornaviruses as the most common cause of acute respiratory infection in children and adults (Makela 1998, Arruda 1997, Monto 1994). The current data extends this observation to infants. The infection rate identified (Table 7.3, 46% of all respiratory infections) is in keeping with adult studies of acute respiratory infection (Makela 1998) and represents a significantly increased incidence of infection compared to that reported in previous culture-based studies in non-hospitalised infants (Kellner 1988).

In adults, picornavirus infections typically account for between 50% and 92% of colds depending on the time of year. In temperate areas of the northern hemisphere, picornavirus respiratory infections peak in September / October with a second peak in April and May (Makela 1998, Arruda 1997). The study period chosen for the current investigation falls between these peaks and it is therefore likely that the picornavirus infection rate detected in the studied infants represents an underestimate of the annual infection rate. Furthermore, the study period was chosen to coincide with the annual RSV epidemic, with RSV infections accounting for 27% of all respiratory infections (Table 7.3). Undoubtedly, this high rate of RSV infection has also reduced the proportion of infections due to the picornaviruses. A more prolonged period of study will be necessary to uncover the true impact of picornavirus infection in infancy although the current data implies the relative importance of these viruses in the first year of life.

Dual pathogen infections were identified in 24 (20%) of the 125 episodes of acute respiratory infection studied. The majority of dual infections were due to mixed picornavirus / RSV or picornavirus / parainfluenza infections (17 (14%) of 123 episodes, Figure 3.1) with picornaviruses identified in the greatest proportion of mixed infections (21 (17%) of 123 episodes). Two previous studies have utilised RT-PCR to detect simultaneous infection with picornavirus, RSV and parainfluenza virus infection in infancy. Gilbert *et al* reported a mixed infection rate of 20% in specimens obtained from a group of 80 children hospitalised with various acute respiratory diagnoses (Gilbert 1996). Similarly, researchers at Caen University Hospital in France, found mixed infection in 18% of 277 nasal aspirates from hospitalised infants with upper or lower respiratory tract infection (Freymuth 1997).

Both studies found RSV to be the most frequent virus recovered in conjunction with another virus. The current investigation has several fundamental differences from these studies that may explain the somewhat lower incidence of mixed infection and the discrepant pathogen findings. Firstly, the current investigation was community-based and, therefore, included infants with less severe respiratory disease. Whereas significant numbers of children had lower respiratory tract infection in both of the previously reported studies, only a very small percentage (12%) of the current study had respiratory diagnoses other than URTI. Secondly, there are considerable age differences at the time of infection and sampling between the studies. The average age in Gilbert *et al*'s study was 9.6 months (Gilbert 1996) and whilst no detailed age data is provided for Freymuth *et al*'s study, the recruited children were noted to be "mostly less than 2 years of age" (Freymuth 1997). The infants in the current study were considerably younger than either of these studies with a mean age of only 5.1 months. A third discrepancy between studies is the seasonal timing of investigation - both previous studies having a longer study period than the current investigation. Overall, it is likely that one or more of the above factors have resulted in the differing findings of the current study.

RSV was the only pathogen associated with a distinct clinical diagnosis (i.e. acute bronchiolitis). Study numbers were not large enough to identify other associations although some interesting observations are possible. Croup, for example, was only diagnosed twice during the study and a parainfluenza virus was detected in both cases. The study does, however, have significant limitations with regard to the diagnostic categories attributed to each respiratory episode. A clinical diagnosis was made during a short (usually under 40 minutes) visit to the infant's home and therefore represents only a "snapshot" of the child's clinical course. The diary sheets completed on a daily basis by the parents (Appendix 4) were not designed to emulate a clinical examination but to study common observations made by parents when describing their child's illness (e.g. cough frequency and timing). During each visit, parents were taught how to identify subcostal retractions by the researcher and asked to note any such observations on the diary sheet. Parents were also asked to contact the researcher again should the infant's clinical condition significantly deteriorate. There were no instances where a child diagnosed with URTI was subsequently reported in the diary sheets as having retractions or where the researcher was contacted because of clinical deterioration. Whilst these are obviously crude measures of subsequent respiratory disease, it nonetheless suggests that those diagnosed with an URTI did not subsequently develop bronchiolitis. Further studies of this nature would benefit from regular visits to each patient during the course of their illness, so that any developing

clinical signs could be observed and a more definitive clinical diagnosis made.

Picornaviruses, parainfluenza viruses and RSV were the only respiratory pathogens associated with diagnoses other than URTI. This finding is possibly due to the relatively large numbers of infections due to these organisms compared to the other pathogens investigated. However, it is interesting to note that respiratory viruses such as adenoviruses and influenza viruses, which have previously been associated with more severe disease in hospitalised infants, produced symptoms and signs indistinguishable from the other pathogens. Serological evidence suggests that these viruses cause mild, self-limited infections of the upper respiratory tract in the majority of cases during infancy with a large proportion of both adenovirus and influenza virus infections not requiring hospital care in early childhood (Cherry 1998a, Glezen 1997b). The findings of the current study are in keeping these previous observations albeit in a small number of infants.

Picornaviruses were detected in 54% (7/13) of episodes with a clinical diagnosis of bronchiolitis. This is a somewhat surprising finding given that bronchiolitis has previously been widely regarded as synonymous with RSV infection (Everard 1996). However, using a similar RT-PCR protocol, a recent study detected picornavirus in 29% of infants hospitalised with bronchiolitis (Papadopoulos 2002). Interestingly, the authors found that dual infection with both picornaviruses and RSV was associated with an increased bronchiolitis severity compared to RSV infection alone. Whilst the current study did not identify such an association, the findings further substantiate the relative importance of the picornaviruses in bronchiolitis.

Respiratory infections generally have a higher prevalence in male infants with male to female ratios of almost 2:1 repo

Chapter 8:

Discussion and Conclusions

The primary aim of this thesis was to determine the importance of type 1 and type 2 immunity in determining the outcome of RSV infection during infancy. In addition, this thesis aimed to study the potential occurrence of prenatal sensitisation to RSV and it's immunological consequences. A further secondary objective was to establish the relative incidence of respiratory pathogen infection during infancy through a community-based study.

Initial cord blood studies found evidence for prenatal sensitisation in one third of infants exposed *in utero* to a RSV epidemic after 22 weeks gestation (Chapter 3). Cord blood mononuclear cells were cultured with RSV that had been inactivated through ultraviolet light exposure. The use of inactivated RSV instead of live virus enabled an evaluation of the secondary (memory) immune response to RSV antigen whilst avoiding the effects of cell death on cytokine production. Further characterisation of the sensitisation process revealed a primarily type 1 immune response with IFN-γ production from RSV induced proliferating cells. A major concern was the possibility that the observed responses were merely due to contamination of the cord blood specimens with maternal blood during collection. Since IgA is not produced by the foetus and cannot cross the placenta, the effects of contamination were negated by the absence of significant IgA levels in the specimens.

A further prospective, community-based study was then performed (Chapters 4-7). Eighty-eight babies were recruited antenatally and monitored closely through their first winter for the development of respiratory infection. Nasal lavage specimens were collected on day 1-2 of each respiratory illness and also on day 5-7 if the initial specimen proved positive for RSV by enzyme immunoassay. The type 1 and type 2 immune response to RSV infection was determined both locally (nasal lavage) and systemically (peripheral blood collected on day 5-7).

All nasal lavage specimens (151 in total) underwent RT-PCR analysis for 10 pathogens known to account for the majority of respiratory infection in childhood (Chapter 7). Picornaviruses were found to be the most common respiratory infection during infancy in keeping with previous studies of older children and adults. RSV was the second most

common pathogen, followed by the parainfluenza viruses. Only small numbers (<6%) of infections due to the other pathogens were identified. In over 18% of infections more than one pathogen was identified although this was not found to be associated with an increased incidence of lower respiratory tract disease. A vast majority of the respiratory illnesses studied were confined to the upper respiratory tract, as would be expected from a community-based study of this type. The extremely low rate of clinical diagnoses other than upper respiratory tract infection precluded a meaningful evaluation of the association between individual pathogens and particular clinical syndromes. Future studies would require larger study numbers to adequately analyse this interesting area.

This study represents the first prospective evaluation of type 1 and type 2 cytokine responses in infants proven to have RSV bronchiolitis and in infants also with RSV infection but with mild clinical illness without bronchiolitis. Both cytokine protein and mRNA production were examined in infants from the same birth cohort, all with at least 1 atopic asthmatic parent, who lived in the same geographical area, and who were infected at the same age with the same virus, but had markedly different clinical outcomes. The study design permitted controlling for age and dilutional factors in nasal lavage, sampling of both airway and peripheral blood, and sampling of the two groups concurrently in relation to onset of signs of infection. The main immunological finding of the study was that RSV bronchiolitis is associated with a profound imbalance in type 1/type 2 cytokines with deficient type 1 and excess type 2 responses. This imbalance was manifest in both inducing (IL-12 and IL-18) and effector cytokines (IFN-γ and IL-4) and was present in both airway secretions and in peripheral blood (Chapters 4 & 5). The consistency of the trends and the magnitude of the differences observed between the groups (5-6 fold differences in protein ratios and >60 fold difference in mRNA ratios) provide strong direct evidence that bronchiolitis is associated with deficient type 1 cytokine production. It has been further demonstrated that this imbalance cannot be attributed to differences in age, dilution or in initial viral load and that it is associated with impaired virus clearance in the bronchiolitic group (Chapter 6).

Antigen dose has been shown to significantly influence the type of immune response induced by exposure to a foreign antigen (Murray 1998). To address the possible contribution of virus load to the observed cytokine responses, quantitative real-time RT-PCR was used to determine the quantity of RSV F protein mRNA present in the day 1-2 nasal specimens. No difference in initial viral load was observed between those who subsequently did or did not develop bronchiolitis thus making differences in antigen dose an unlikely explanation for our findings. The fact that all infants were recruited

prospectively and exposed to RSV infection during the same short season in the same localised geographical area also makes differences in RSV subtypes between groups an unlikely explanation for our findings.

Cytokine responses to acute respiratory viral infections are known to rise and fall during the first days of infection, with varying time courses depending on the cytokines measured (Hayden 1998). Infants admitted to hospital with acute bronchiolitis can present several days after the onset of infection, while uncomplicated URTI is a short-lived condition. For this reason, cytokine responses measured in cross sectional studies of infants admitted to hospital with bronchiolitis cannot be reliably compared with control infants with mild URTI, as the timing of sampling relative to the onset of infection is certain to be different between groups and can never be reliably established. This confounding influence can only be removed by employing a prospective study design in which sampling of the index and control groups is simultaneous relative to the onset of signs/symptoms of infection. This study is the first to employ such a design and therefore remove confounding by timing of sampling.

A further possible confounding influence in this study is the age of the children. Prescott et al demonstrated a rapid suppression of type 2 responses in non-atopic children but a persistence of these responses in atopic children over the first 6 to 12 months of life (Prescott 1999). To take account of the small difference in age between the groups, all comparisons of cytokine values were adjusted for age using ANCOVA. The imbalanced cytokine responses we have observed therefore cannot be accounted for by differences in age between the two groups.

The immunological findings of this study therefore support the results of previous studies (Aberle 1999, Roman 1997, Rabatic 1997) and are the first to identify both respiratory tract and systemic cytokine responses, to control for age, to ensure simultaneous timing of sampling relative to onset of infection, to investigate initial virus load and to monitor virus clearance. It is also unique in using prospectively identified controls from the same birth cohort and geographical area who are infected in the same season with the same virus but who do not develop bronchiolitis. It is likely that the absence of one or more of these design features explains the conflicting results reported in 2 other previous studies (Brandenburg 2000a, van Schaik 1999).

RSV bronchiolitis is an important risk factor for the development of asthma (Stein 1999) and atopic sensitisation (Sigurs 2000), both of which are characterised by an excess of type 2 cytokines (Robinson 1992). However it is debated whether RSV bronchiolitis causes asthma by deviating subsequent immune responses towards a type 2 or away from a type 1

phenotype, or whether asthma and bronchiolitis simply have common risk factors, with bronchiolitis being the first presenting illness in a child already at risk of developing asthma as a result of risk factors that predated RSV bronchiolitis. The present data clearly demonstrate an excess type 2 and deficient type 1 response in RSV bronchiolitis and are very consistent with the increased risk of subsequent asthma in these patients. The fact that we observed these skewed patterns of cytokine production as early as 1-2 days into the clinical illness strongly suggests that the skewed production was present before the onset of the illness, rather than as a result of the RSV infection itself, as it is highly unlikely that a viral infection could itself skew immune responses so early when virus-specific responses are just beginning to develop.

Each winter outbreak of RSV infection results in 1-3% of infants under 1 year of age requiring hospitalisation (Phelan 1994). The healthcare costs of these annual epidemics are substantial, amounting to an estimated $300 million per annum in the United States alone (Heilman 1990). Moreover, recent evidence suggests that there has been a significant increase since 1990 in the healthcare impact of these epidemics (van Woensel 2002). Despite the magnitude of the problem, advances in both prevention and therapy have been slow and vaccination has been held back over 30 years by the increased mortality observed with a formalin-inactivated vaccine (Wright 2000, Hall 1999). The current study has demonstrated the association of a deficient type 1 cytokine response to RSV infection in the respiratory tract with severe illness and with impaired virus clearance. This raises the possibility that treatments which augment type 1 cytokine responses will ameliorate RSV disease in those at risk. However the opportunity to achieve this post-natally in a disease with a peak age incidence of 2-4 months is very limited.

Animal data indicate that prior sensitisation to F or G RSV surface proteins followed by infection with live RSV induces polarised type 1 responses associated with efficient virus clearance, or type 2 responses associated with augmented illness and eosinophilia, respectively (Alwan 1994). There is therefore the theoretical possibility that prior sensitisation to RSV in infants could result in either protective (augmented type 1) or deleterious (augmented type 2) immune responses upon subsequent encounter with natural RSV. The initial part of this thesis investigated RSV-induced type 1 and type 2 cytokine production in cord blood mononuclear cells from potentially exposed infants who were sensitised and not sensitised to RSV antenatally. In all six infants sensitised to RSV antenatally, stimulation of cord blood mononuclear cells with RSV induced augmented IFN-γ production and in no case was induction of IL-4 observed, confirming a likely protective potential of such antenatal sensitisation.

As mentioned previously, eighty-eight babies were recruited antenatally and monitored through their first winter as part of the community-based study of RSV infection. Cord blood was obtained at delivery wherever practicable and cultured with UV-inactivated RSV as for the initial studies. Unfortunately, inadequate numbers of cord blood analyses were successfully performed to enable worthwhile statistical analysis. Several factors contributed to this including poor quality of collected cord blood, delayed laboratory processing of the collected specimen, failure to collect cord blood at delivery, poor communication to laboratory at time of specimen collection and insufficient specimen for the desired analyses. Consequently, it is not possible to directly identify the influence of prenatal sensitisation to RSV on the subsequent response of these infants to natural infection. However, it is possible to make certain recommendations for any further studies of this kind. Firstly, it is imperative that families that have been recruited to a study of this nature are clearly identified so that appropriate specimens can be collected at the time of birth. Secondly, all personnel involved with the collection of cord blood should undergo adequate training in the procedure and the appropriate storage / handling of the collected specimens. Finally, sufficient laboratory personnel are required and an efficient communication route to the laboratory established to prevent any unnecessary delays in processing.

In conclusion, the data presented identify a profound deficiency in type 1 cytokine responses in infants with RSV bronchiolitis and suggest that this deficiency was present prior to the RSV infection occurring. It has also been demonstrated that antenatal sensitisation to RSV can occur in infants of mothers potentially exposed to RSV from 22 weeks gestation and that sensitisation is associated with induction of protective type 1 cytokine responses on stimulation with RSV.

These latter data suggest that antenatal RSV infection/immunisation of mothers after 22 weeks gestation might represent a new strategy for prevention of RSV bronchiolitis and possibly subsequent wheezing illness. Antenatal immunisation with RSV is currently being explored in a clinical trial with the aim of augmenting maternal antibody responses and protecting infants as a result of passively transferred IgG antibody (Dr Janet Englund, Baylor College of Medicine, Houston, Texas; personal communication). However such protection is known to wane around 6 weeks postnatally. The data presented here indicate that such a strategy might have additional benefits, by also augmenting the infant's own type 1 specific cellular immune response to RSV. Further large prospective birth-cohort studies are now required to confirm the proposed immunological benefits of antenatal exposure to RSV.

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Appendices

Appendix 1: Time and Dose Response Curves for Cord Mononuclear Cell Proliferations in Response to UV-inactivated RSV

Fig 1. Stimulation Indices Following 3 Days Culture
(Cord Blood 1,2,3 & 4)

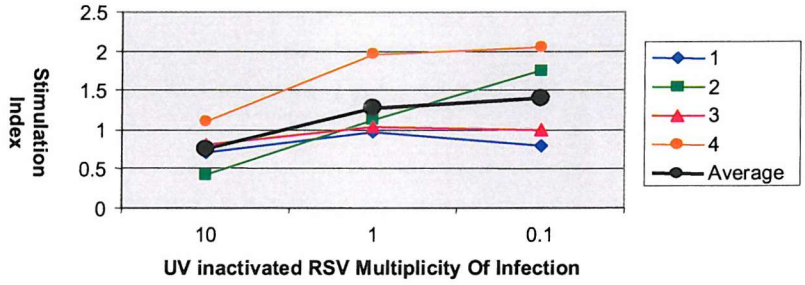


Fig 2. Stimulation Indices Following 5 Days Culture

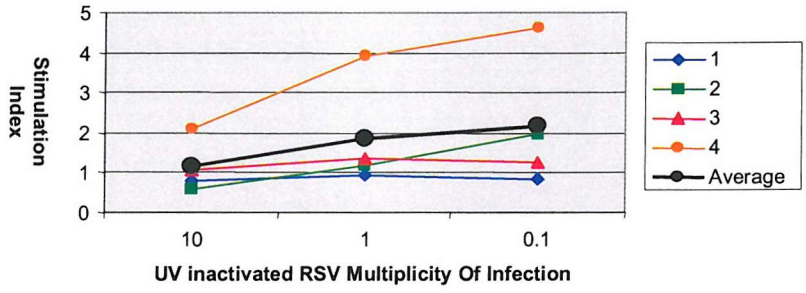


Fig 3. Stimulation Indices Following 6 Days Culture

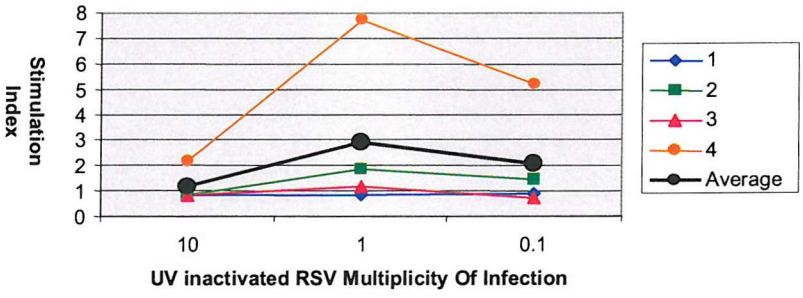
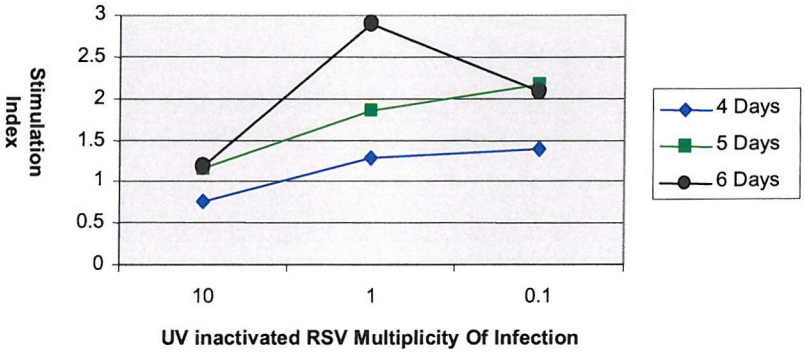


Fig 4. Average Stimulation Indices For All Time Points



Appendix 2: Recruitment Questionnaire Completed by Both Parents

1. Have you ever had wheezing or whistling in the chest at any time in the past? — Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 6

2. Have you had wheezing or whistling in the chest in the last 12 months? — Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 6

3. How many attacks of wheezing have you had in the last 12 months? — None ☐ 1 to 3 ☐ 4 to 12 ☐ More than 12 ☐

4. In the last 12 months, how often, on average, has your sleep been disturbed due to wheezing?
   Never woken with wheezing ☐
   Less than one night per week ☐
   One or more nights per week ☐
   Never woken with wheezing ☐

5. In the last 12 months, has wheezing ever been severe enough to limit your speech to only one or two words at a time between breaths? — Yes ☐ No ☐

6. Have you ever had asthma? — Yes ☐ No ☐

7. In the last 12 months, has your chest sounded wheezy during or after exercise? — Yes ☐ No ☐

8. In the last 12 months, have you had a dry cough at night, apart from a cough associated with a cold or chest infection? — Yes ☐ No ☐

9. Do you usually cough first thing in the morning in the winter? — Yes ☐ No ☐

   9.1 If yes, do you cough like this on most days for as much as 3 months each year? — Yes ☐ No ☐

10 Do you usually bring up any phlegm from your chest *during* the day, or at night, in the winter? — Yes ☐ No ☐

   10.1 If yes, do you bring up phlegm like this on most days for as much as 3 months each year? — Yes ☐ No ☐

Appendix 2: Recruitment Questionnaire (Continued)

11. Have you ever had a problem with sneezing, or a runny, or blocked nose when you DID NOT have a cold or the flu? Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 16

12. In the past 12 months, have you had a problem with sneezing, or a runny, or blocked nose when you DID NOT have a cold or the flu? Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 16

13. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes? Yes ☐ No ☐

14. In which of the past 12 months did this nose problem occur? (Please tick any which apply)

January	☐	May	☐	September	☐
February	☐	June	☐	October	☐
March	☐	July	☐	November	☐
April	☐	August	☐	December	☐

15. In the past 12 months, how much did this nose problem interfere with your daily activities?

Not at all ☐
A little ☐
A moderate amount ☐
A lot ☐

16. Have you ever had hayfever? Yes ☐ No ☐

17. Have you ever had an itchy rash which was coming and going for at least six months? Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 20

18. Have you had this itchy rash at any time in the last 12 months? Yes ☐ No ☐

IF YOU HAVE ANSWERED "NO" PLEASE SKIP TO QUESTION 20

19. Has this itchy rash at any time affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes? Yes ☐ No ☐

Appendix 2: Recruitment Questionnaire (Continued)

20. Has this rash cleared completely at any time during the last 12 months?
- Yes ☐
- No ☐

21. In the last 12 months, how often, on average, have you been kept awake by this itchy rash?
- Never in the last 12 months ☐
- Less than one night per week ☐
- One or more nights per week ☐

22. Have you ever had eczema?
- Yes ☐
- No ☐

Appendix 3: Questionnaire completed at initial home visit

Name..... D.O.B.....

Age.....

Postcode.....

Cold first Noticed (Date/Time).....

Date/Time of visit.....

Breast Fed for How Long?.....

No. Of other children (Ages/At School?).....

.....

Smokers in home?.....

.....

Pets in home?.....

Symptoms

Feeding Well?.....

Blocked Nose/Breathing Through Mouth?.....

Breathing Fast/Short of Breath?.....

Cough? Worse at night?.....

Wheeze heard?.....

Recession?.....

Sleeping O.K?.....

Blue episodes?.....

Medications?.....

Appendix 3: Questionnaire completed at initial home visit (Continued)

Signs

Mouth/Nasal Breathing.....

Nature of nasal secretions.....

Respiration Rate.....

Cyanosis.....

Nasal Flaring.....

Heart Rate.....

	0	1	2	3	4	Total
Wheezing						
Expiration	None	End	½	3/4	All	
Inspiration	None	Part	All			
Location	None	Segmental :<= 2 of 4 lung fields	Diffuse: >=3 of 4 lung fields			
Retractions						
Supraclavic	None	Mild	Moderate	Marked		
Intercostal	None	Mild	Moderate	Marked		
Subcost	None	Mild	Moderate	Marked		
						/17

Additional Findings.....

Appendix 4: Daily Diary Sheet for Completion by Parent

Diary Sheet (Please could you return all 7 sheets at the end in the prepaid envelope)

Date..... Name.....

(1) Has your baby had a runny nose today? YesY NoY

If so what colour have the nasal secretions been?
(E.g.Thick,green,clear).....

(2) Has your baby been feeding well today? YesY NoY

If not, how much was your baby taking compared to usual?.....

(3) Has your baby had a fever today? YesY NoY

If so how did you measure it (i.e.thermometer or felt hot) and if you used a thermometer what was the temperature?.....

(4) Has your baby had a cough today? YesY NoY

If so when was it at it's worst (ie.what time day or night).....

(5) Have you heard your baby wheezing today?..... YesY NoY

If so when was it at it's worst.....

(6) Has your baby been short of breath today more than you would expect? YesY NoY

If so when did this shortness of breath occur?.....

(7) Did your baby sleep well last night? YesY NoY

If not what disturbed his/her sleep?.....

(8) Has your baby had any "recession" today (as shown to you by Dr Legg)? YesY NoY

If so when did it occur?.....