THE DELAYED EFFECTS OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

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by

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Abstract

Respiratory Syncytial (RS) virus is the most important respiratory pathogen of infants. The role of RS virus, in the pathogenesis of wheezing and asthma has been a topic of medical interest for many years. Many questions about the pathogenesis of RS virus disease remain unanswered. With new techniques, answers to some of these questions can be attempted. In this thesis, novel techniques were used in studies of RS virus infected infants, children and adults, and mice.

Using a nested reverse transcriptase polymerase chain reaction (RT-PCR), it was shown that cell associated viraemia occurs in some infants hospitalised with bronchiolitis but that RS virus cannot be detected in serum or cerebrospinal fluid. RT-PCR was also used to determine, first, the frequency of RS virus in nasopharyngeal aspirates from children admitted with respiratory illnesses, and, in bronchoalveolar lavage fluid from adults infected with human immunodeficiency virus being investigated for unexplained respiratory symptoms.

In mice, RT-PCR showed that RS virus persists for at least 100 days after acute infection. Almost all the isolates sequenced from these mice contained an unchanged dominant epitope for cytotoxic lymphocyte (CTL) recognition and all the mice tested had vigorous CTL recognising this epitope. A mutant epitope was also detected, but extensive studies using synthetic peptides did not show interference or inhibition of CTL responses by this mutant.

The effect of RS and influenza A virus infections on the immune response to an inhaled protein antigen (ovalbumin) was studied. In the presence of viral infection, acute anaphylaxis and exaggerated T_H2 responses to ovalbumin were shown, without the use of adjuvant. This comprises a novel finding of possible clinical significance. By showing viral persistence and altered responses to other antigens, these studies may in part explain the delayed effects of RS virus infection in man.

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Abbreviations:

Ab	Antibody
AEC	3-amino 9-ethylcarbazole
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BALT	Bronchial associated lymphoid tissue
βGal	Beta galactosidase enzyme (of Escherischia Coli)
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary DNA
CDV	Canine distemper virus
cm	Centimetre
CMV	Human cytomegalovirus
CO_2	Carbon dioxide
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
cpm	Counts per minute
^{51}Cr	Chromium-51
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxy (nucleotide) triphosphate
dGTP	Deoxyguanine triphosphate
dCTP	Deoxycytosine triphosphate
dTTP	Deoxythymidine triphosphate
dATP	Deoxyadenosine triphosphate
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECACC	European collection of animal cell cultures
EDTA	Ethylenediamine-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
F	Fusion protein of RS virus
Fabs	Antigen binding fragments
FCM	Flow cytometric
FCS	Fetal calf serum
G	Attachment protein of RS virus
g	Gravity or grams
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony stimulating factor
HCl	Hydrochloric acid
hCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSA	Heat shock antigen
HSV	Herpes simplex virus
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
KCl	Potassium chloride
kd	Kilodaltons
L	litre or polymerase of RS virus

LCMV	Lymphocytic choriomeningitis virus
LFA	Leucocyte function antigen
LPS	Lipopolysaccharide
M	Matrix protein of RS virus or Molar.
m	Meter
M2	Second matrix protein of RS virus
MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
mCi	Millicurie
$MnCl_2$	Manganese chloride
mg	Milligrams
$MgCl_2$	Magnesium chloride
ml	Millilitres
mM	Millimolar
mm	Millimetre
MMLV-RT	Murine Moloney leukaemia virus reverse transcriptase
moi	Multiplicity of infection
μCi	Microcurie
μg	Microgram
μl	Microlitre
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NK	Natural killer
nm	Nanometre
ns	Not significant
OD	Optical density
OPD	O-Phenylenediamine
ORF	Open reading frame
p	Probability
P	Phosphoprotein of RS virus
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
pfu	Plaque forming unit
PHA	Phytoerythrin
PMA	Phorbol 12-myristate 13-acetate
PVM	Pneumonia virus of mice
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Royal Park Memorial Institute (medium)
R10F	RPMI with FCS, glutamine, antibiotics (as in methods)
RS	Respiratory Syncytial
RV	Rhinovirus
rVV	Recombinant vaccinia virus
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
sem	Standard error of the mean
TCR	T cell receptor
T_H	T helper cell
TNF	Tumour necrosis factor
TRTV	Turkey rhinotracheitis virus
u	Units
VCAM-1	Vascular cell adhesion molecule
W	Watts
2ME	2-mercaptoethanol

Chapter 1 Introduction

Viruses are obligate intracellular organisms, in most cases the acute disease and its sequelae can be directly linked to the destruction of cells by the infecting virus (311). Respiratory syncytial virus is associated with little direct cell pathology and yet it can cause severe illness and long lasting effects in susceptible individuals, especially the very young. Consequences of severe RS virus bronchiolitis in infancy include recurrent wheezing episodes with a higher likelihood of being diagnosed as having asthma. Complex and subtle interactions exist between many viruses and the host immune response. Many strands of evidence suggest that the illnesses associated with RS virus are a result of the immune response to it. Some viruses, including some paramyxoviruses, can persist in immunocompetent hosts. Persistence implies avoiding recognition or clearance by the immune system. New methods have become available, such as flow cytometry and sensitive techniques for detection of viral genome, that allow studies both of viruses and the host immune response.

This thesis describes a series of studies of RS virus, both in man and an established mouse model. The mouse model has previously been used to provide evidence of the role immunopathology in acute primary infection and secondary infection after vaccination. To obviate the limited sensitivity of conventional virological methods of RS virus detection, a nested RT-PCR was developed and refined for use in different tissues. The first chapter is about the detection of RS virus in human tissues using this method and the discovery of viraemia in primary infection. Prospective study of nasopharyngeal aspirates from a series of children admitted to hospital with wheezing was performed. Data on persistence of RS virus were investigated and evidence for escape from immunity sought. The possible

interaction between infection with RS virus and sensitisation to other irrelevant antigens was shown. Conclusions are drawn about the links that may exist between acute virus infections of respiratory mucosal surfaces and the development of allergy or atopy.

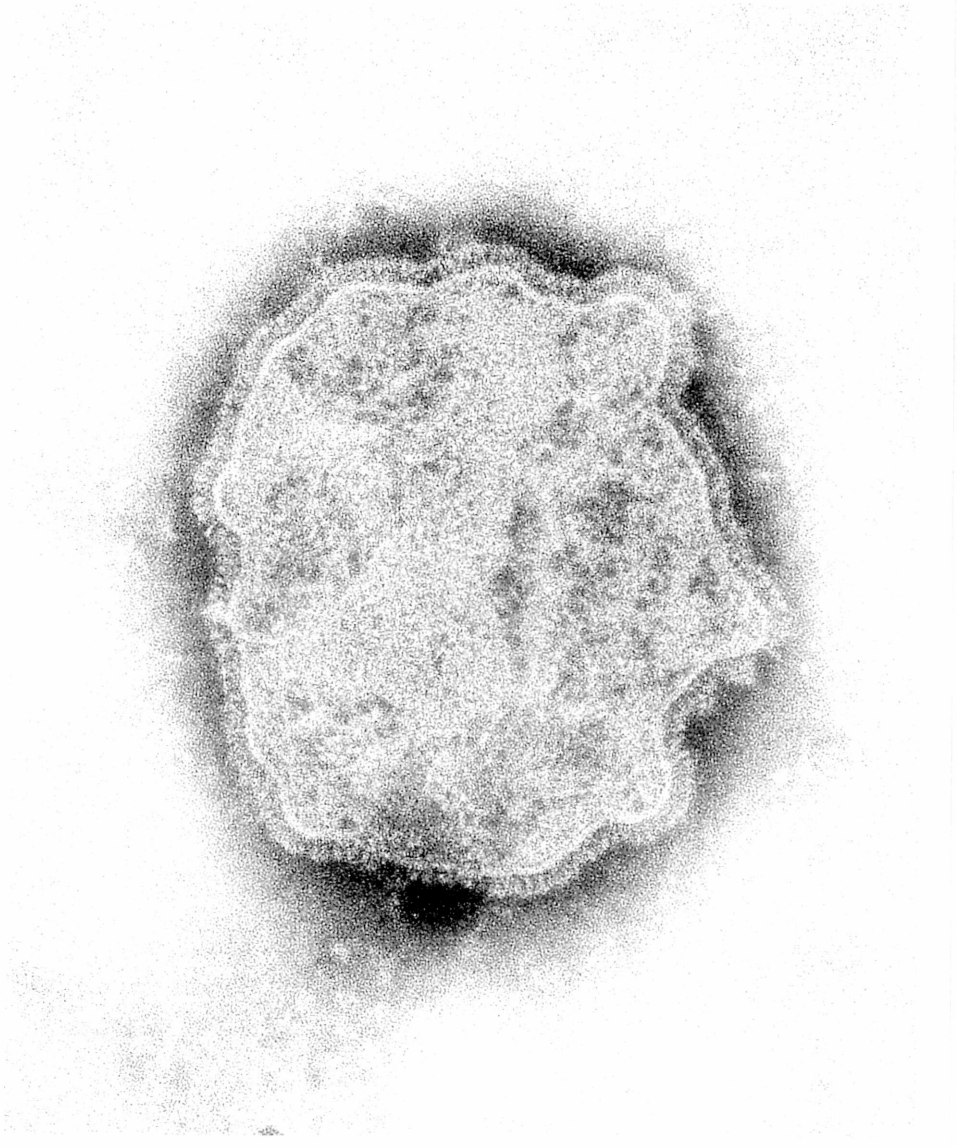


Figure 1.1 Electronmicrograph of RS virus (original magnification × 140 000). RS virus is pleomorphic and has filamentous and spherical forms. In this electronmicrograph a spherical RS virus is shown. Club like surface glycoprotein protein spikes approximately 12nm long protrude from the surface at regular intervals. The outer lipid membrane is derived from the host cell during budding. This electronmicrograph was kindly given to me by Professor Peter Watt and Dr Paul Lambden, Department of Molecular Microbiology, University of Southampton.

Features of Respiratory Syncytial Virus:

History

Outbreaks of feverish respiratory illnesses have been described for many centuries and the word "influence" (from which 'influenza' comes) was used from the fourteenth century to imply that the disease was affected by astrological events (326). Goodpasture published the first account of pneumonia in infants caused by epidemic virus infection in 1939 (117) and Adams was probably the first to give a detailed description of an outbreak of RSV in 1941. He described a nosocomial infection in a newborn nursery that affected 32 children causing 9 deaths (3) and found cytoplasmic inclusions in the lung at post mortem. The virus itself was first isolated from a chimpanzee with a coryzal illness in 1956. The virus was cultured and shown to be able infect other chimpanzees and was named "Chimpanzee Coryza Agent" (247). The following year, Chanock and others isolated the same virus from a child with croup in Baltimore (48). The virus was renamed respiratory syncytial virus to describe the site of infection and characteristic syncytium formation found in cell culture and infected tissues.

Epidemiology

RS virus is distributed worldwide with similar patterns of disease in all climates. Wherever it is found, children are the ones most likely to have the severe illnesses. It is the single commonest reason for a child under one year of age to be admitted to hospital in the western world. Reinfections occur throughout life, in spite of good levels of neutralising serum antibody. As well as the very young the very old are also at risk from RS virus infection. Outbreaks of RS virus pneumonia in the elderly are probably as important as influenza in

causing excess deaths (95). Annual outbreaks occur which are usually of sharp onset and follow regular predictable patterns. In temperate climates most cases of RS virus infection are reported during the winter months, sometimes extending into spring. Most hospital admissions occur during a narrow peak period lasting only a few weeks. In the UK the peak incidence is usually seen between the beginning of January and the end of March (131). In tropical climates such as in Hong Kong and Trinidad, epidemics are seen during the rainy season. Influenza epidemics also occur in the winter but do not usually coincide with the peak of the annual epidemic of RS virus infection (131,134). Clinical isolates of RS virus are rare in the UK during the summer months (373) and it is not clear where the virus goes or how it re-emerges so rapidly during the next winter season. No animal reservoir for human RS virus is known to exist. Some domestic animals such as dogs, cats and sheep have been shown to have serum antibody to human RS virus but the significance of this is not clear (25,131,222).

Until the last few years the only studies of strain variation in RS virus came from monoclonal antibody typing into A and B strains. Both strains commonly cocirculate in one outbreak (154,339,343,353). There is probably little difference in the type or severity in clinical disease produced by A or B strains (236). The possibility still exists however, that some strains, not defined by monoclonal studies, may cause more severe disease. The recent development of molecular biological techniques has allowed a more detailed study of subtype variation within RS virus, by classification according to genome sequences (35,37) especially of the highly variable G protein gene. Interestingly, viruses sequenced from children all over the world fall into the same lineage classifications although the relative frequency of each lineage differs. Unlike influenza virus several local strains of RS virus

commonly circulate alongside one another with similar frequencies. However some evidence of evolution of RS virus strains has emerged, some strains becoming less common over the years and others increasing in frequency (36). This suggests that sequential accumulation of genomic sequence changes probably occurs as seen with other viruses such as the antigenic drift of influenza virus haemagglutinin (294).

RS virus causes between 50 and 90 percent of the cases of bronchiolitis admitted to hospital each year. Between one and two percent of the infants born in the UK each year will require admission because of bronchiolitis or viral pneumonia. It is also associated with 10 percent of the cases of croup and up to 25 percent of the cases of pneumonia under one year of age (50,110,131,153).

Taxonomy: the Mononegavirales

The order mononegavirales consists of viruses with nonsegmented negative stranded RNA genomes. It includes three families of virus: the Filoviridae, Paramyxoviridae and the Rhabdoviridae. All three have similar genomic organisation and patterns of morphogenesis and are differentiated by their biological differences (292-294). Paramyxoviridae, including RS virus are transmitted by contact or aerosols and have mainly been isolated from warm-blooded vertebrates suggesting the family evolved relatively recently. They are generally associated with respiratory illnesses. Filoviruses have only been isolated in sporadic cases or outbreaks, and cause with severe haemorrhagic illnesses in primates and man. Rhabdoviruses are widespread and infect both mammals and other organisms even some plants (178,370). They are mostly spread by arthropod vectors and, with the exception of Rabies, cause only mild illness in humans.

Paramyxoviridae (from this point referred to as paramyxoviruses) are divided into two subfamilies: *Paramyxovirinae,* and the *Pneumovirinae*, to which RS virus (genus *Pneumovirus*) belongs. The *Paramyxovirinae* can be further subdivided into three genera: *Rubulavirus* (e.g. mumps), *Morbillivirus* (e.g. measles) and *Paramyxovirus* also known as *Parainfluenzavirus*, (e.g. Sendai). Mononegavirales share a common pattern of 5-10 genes arranged linearly with the viral polymerase (L) at the 5' terminus. Except for the *pneumoviruses*, the nucleoprotein is located at the 3' end. Some 95 percent of the gene sequence codes for polypeptides. Features common only to the *Pneumoviruses* are: 1) two non structural genes at the 3' end of the genome before the nucleoprotein (NS1 or 1C and NS2 or 1B) and 2) two genes SH and M2 (22K), in the middle of the genome. Comparison of gene sequences from several paramyxoviruses, rhabdoviruses and the filoviruses Ebola and Marburg suggested that *Pneumoviruses* were indeed a separate lineage. However the L protein of RS virus has closer homology to Ebola and Marburg than to the other *Pneumoviruses* and this was also found for sequence homologies between the N proteins (19,90,322).

Molecular Biology

RS virus virions are very heterogeneous in size and shape with pleomorphic spherical or filamentous forms. Spherical particles are approximately 60-100nm in diameter. The nucleocapsid contains the single strand of nonsegmented negative sense RNA which has been shown to be 15222 nucleotides in length in A2 RS virus (58). Virions assemble at the plasma membrane of an infected cell and are released by budding taking a lipid bilayer membrane derived from the host cell. The envelope of the virus is covered by projecting spikes of 11-20nm separated by 6-10nm (56). These are made up of the three surface

glycoproteins G, F and SH. Ten RS virus proteins have been identified so far and a description of these is given in figure 1.2.

Sequence diversity between A2 and B18537 strains of RS virus has been studied for different genes. The surface protein G is the most divergent with only 53 percent homology of amino acid sequences, whereas the internal nucleoprotein N is most conserved between A and B strains with 91 percent homology of the amino acid sequences. The sequence of the M2 matrix protein overlaps with the polymerase (L) and both the length of the overlap and the nucleotide sequences are highly conserved in this region (92 percent between A and B strains) (56,185,186,188).

Proteins of RS virus

10 proteins of RS virus have so far been identified. Three are surface transmembrane proteins: G, F and SH, 2 are nonglycosylated matrix proteins: M and M2, 3 are associated with the viral genome and make up the nucleocapsid: N, P and L, and 2 are nonstructural proteins NS1 and NS2.

F protein is closely related in structure and function to surface proteins found in other paramyxoviruses. It is involved in virus penetration into cells and syncytium formation. Efficient syncytium formation also requires the presence of the other surface proteins, G and SH (58). F protein is approximately 70kd and is *N* glycosylated (63.5kd unprocessed). The G protein mediates attachment and plays a role in syncytium formation. It is highly serine threonine rich (31%) and becomes extensively *O* glycosylated which is an unusual feature in viral proteins. There is a high degree of variability in the extracellular region and has a

molecular weight of 90kd when glycosylated (32.5kd nonglycosylated). The *O* linked glycosylation is not found in any other paramyxovirus, orthomyxovirus or rhabdovirus. However *O* linked glycosylation has recently been described for the G protein of filoviruses (58). The extracellular region of G although highly variable contains a 13 amino acid region which is conserved between all strains studied so far making it a candidate for receptor binding (187). This region includes four closely placed cysteines (at positions 173, 176, 182 and 186). In bovine RSV these cysteines are also found and six of the other amino acids, but in turkey rhinotracheitis virus (TRTV), a closely linked avian *Pneumovirus*, there is no evidence of this conserved motif.

SH is a small hydrophobic protein of 64 amino acids. It has its C terminus extracellularly, a hydrophobic transmembrane region and there are several forms of its intracellular portion (14,59). Its function is not known but its role in syncytium formation mentioned above might suggest a role in initiating virus infection. The N, P and L proteins are the nucleocapsid protein, the phosphoprotein and the polymerase respectively. The N protein is smaller than and has no close sequence homology to that of other paramyxoviruses. The P protein is also smaller than in other paramyxoviruses (241 aminoacids). In Sendai virus the P protein has been shown to have 2 roles, first as a transcription and replication factor and second as a chaperone in binding soluble L and NP proteins before association with envelope (165); it is possible that the same is true for RS virus. The L protein is the viral polymerase and is highly conserved between strains. There are two non structural proteins NS1 and NS2 that are expressed only in small amounts in virions but accumulate within infected cells (170). NS2 has a short half life and is possibly secreted suggesting a potential role in interacting with the host (58).

Pneumoviruses are unusual in that most of the mononegavirales have a single matrix protein. Whereas M protein is thought to correspond with the matrix protein seen in other negative sense nonsegmented viruses, the M2 protein does not appear to have a counterpart in other mononegavirales. Each matrix protein in RS virus contains a further potential protein encoded as internal open reading frames one within M and the other within M2, neither of these gene products has been identified during RS virus replication to date. The ORF within M2 would code for a 90 amino acid protein and this ORF has been found to be present in all *Pneumoviruses* including pneumonia virus of mice (PVM) and turkey rhinotracheitis virus (TRTV) (58,220,379) suggesting it may be functionally significant. The second matrix protein of RS virus is also unusual in that its genes overlap with the L by 68 nucleotides although overlap has also been described in filoviruses (58). The matrix proteins M and M2 are nonglycosylated.

Infectious cycle

In Vivo

Transmission of RS virus is by introduction of infected secretions onto the mucosa of the eyes or nose. Usually this is by self inoculation with the hands after touching infected secretions or contact with virus on objects such as doors, surfaces or fomites. Aerosolisation seems to be a less frequent means of spread (132). The virus appears to be capable of surviving several hours on inanimate objects given optimal conditions (133,176). RS virus infects the upper respiratory mucosa of the nose and the conjunctivae but does not appear to be able to infect buccal and oral mucosa (135). The mechanism of spread to the lower respiratory tract is not known, but it is assumed this is via the respiratory epithelium or aspiration of infected secretions. The incubation period between inoculation and disease is

4 to 5 days from studies in adults. Lower respiratory tract signs appear 1 to 3 days after the onset of rhinorrhoea. Post mortem studies of lung have indicated a difference in the titre of virus in the lower respiratory tract; in bronchiolitis virus levels are low but in pneumonia high levels of virus have been found (111). In those studies, using immunofluorescence, RS virus was only found in the superficial epithelial layer. Viraemia has not been shown although viral antigens have been reported to be present on circulating mononuclear cells from some individuals (73). Virus shedding is often for more than 2 weeks, after the peak of the illness during resolution. RS virus can be detected by conventional methods even 4 weeks after the onset of bronchiolitis in immunocompetent children.

The principal hosts for RS virus are humans, chimpanzees, and cattle (131). Other animals can be infected by RS virus including baboons, guinea pigs, mice, hamsters and ferrets. Models of infection have been developed in cotton rats, lambs, chimpanzees and mice.

In vitro

RS virus grows best in cultures of human cells such as HEp-2 and HeLa cells. In culture it is associated with a characteristic effect of syncytium formation with eosinophilic cytoplasmic inclusions (131). The growth cycle consists of an adsorption phase of about two hours followed by an eclipse phase of 12 hours. New virus is produced and released into the medium during a logarithmic growth phase lasting a further 12 to 24 hours. Up to 90 percent of the virus remains cell associated. During replication the virus produces numerous incomplete virions termed defective interfering particles (351,352). RS virus is relatively labile: it is destroyed rapidly at 55°C and does not tolerate freeze-thawing well.

Attachment occurs through the viral surface glycoprotein G (216). It is not known which cellular protein provides the site of attachment for RS virus. In cell culture it has been shown that the cellular receptor or receptors appear to be very abundant on the cell surface (364). In other respiratory virus infections the host receptor has been shown to have immunological significance. CD46, the attachment protein for measles, is a member of the family of proteins involved in complement activation regulation, (74) and ICAM-1, an important adhesion molecule is the receptor for rhinovirus (336).

Cell penetration is by fusion and the viral envelope may become incorporated into the cell surface. Thereafter all the events of replication occur in the cytoplasm and the virus can grow in cells where the nucleus is not present (97). Transcription is by the viral transcriptase L. The RS virus genes are transcribed in their 3' to 5' order into a positive strand RNA from which individual proteins are synthesised. In the case of the sequence for the polymerase L the start overlaps with the second open reading frame of the second matrix protein M2. This does not appear to interfere with protein synthesis (57) but it is not clear how the polymerase deals with this. One possibility is that an end signal from the M2 protein causes the polymerase to relocate back to the start of the L protein or an internal promoter as yet undetermined may exist for L. Unlike viruses such as measles and parainfluenza, in which the intergenic regions contain critical conserved sequences, the RS virus intergenic regions do not appear to contain important gene sequences and wide variations in sequence and length occur (58,70). The frequency of gene transcripts produced intracellularly varies and early transcribed proteins from the viral genomic 3' end are found in higher abundance than later transcripts such as the polymerase L (at the 5' terminus). Viral mRNAs and proteins can be detected 4 to 6 hours after infection.

RS virus mRNA synthesis peaks at about 16 hours while protein synthesis peaks at about 18 hours after infection *in vitro*. Negative RNA strand synthesis for viral replication is via a full length positive strand replicative RNA intermediate and is dependant on continuing protein synthesis in a similar way to that shown for vesicular stomatitis virus and Sendai (58). Virion assembly occurs at the plasma membrane. Nucleocapsids preformed in the cytoplasm aggregate near plasma membrane sections containing concentrations of transmembrane RS virus glycoproteins and virion formation occurs by budding. Budding occurs from apical surfaces in well circumscribed cell surface regions. This might indicate a dependence on local host submembranous structures. Occasionally budding virions are seen to fuse back into the cell when viewed by videomicroscopy (58).

Clinical features:

It is believed that primary infections with RS virus are always symptomatic but may range from a very mild "cold" to severe bronchiolitis with respiratory failure (131). The most serious illnesses associated with RS virus occur in children and the elderly. Only a small proportion of RS virus infected infants require admission to hospital. Despite this it was estimated that RS virus accounted for 91,000 admissions to hospital in the USA in 1987 alone, at a cost of $300 million (150). RS virus is associated with a number of clinical conditions including: upper respiratory tract infections (coryza), acute pharyngitis, acute tonsillitis, acute laryngotracheitis (croup), otitis media, bronchitis, viral pneumonia and bronchiolitis. The relative frequency of RS virus isolation in acute respiratory infections of children is indicated in table 1.1 below.

RS virus spreads rapidly through susceptible populations such as children in crèches and

kindergartens and elderly people in residential institutions. Almost 100% of infants in childcare during their first RS virus season become infected (58). Spread within families is also common and having older siblings increases the risk of infants having bronchiolitis.

	Adenovirus	*Enteroviruses*	*Coronaviruses*	*Influenza virus*	*Parainfluenza viruses*	*Rhinoviruses*	*RS virus*	*Epstein–Barr virus*	*Herpes simplex virus*	*Cytomegalovirus*	*Reoviruses*	*Measles virus*
Common cold	+	++	+++	+	+	++++	+				+	
Sinusitis	+			+	+	+						
Pharyngitis	++++	+++	++	++	++	+	+	++	++	+	+	++
Otitis media	++	+	+	+	+	+++	++		+			++
Croup	++	+	+	+++	++++	+	++		+		+	+
Bronchiolitis		+		+	++	+	++++					
Bronchitis	+	+	++	++	+++	++++	++++					++
Pneumonia	++	+	+	+	++	+++	++++		+			++

Table 1.1: Relative frequency of virus isolation in acute respiratory infections in children. (Reproduced from Clinical Spectrum of Disease in Children by David Isaacs in "Viral and other infections of the human respiratory tract" (176))

Children most at risk for severe illness include those who were born prematurely whether or not they have recognised chronic lung disease of prematurity. These infants are increasingly likely to survive the immediate neonatal period because of rapid advances in neonatal intensive care and bronchiolitis represents a serious threat to them. Children with congenital cyanotic heart disease are at even more risk. In early studies these infants had up to 44% percent mortality with bronchiolitis where pre-existing pulmonary hypertension was present. Advances in paediatric intensive care, especially in ventilation and fluid balance, have improved the outlook considerably (376). Today the mortality rate in this group is nearer 9% (58). Another group of special importance are children with congenital or acquired immunodeficiencies in particular children with human immunodeficiency virus (HIV), undergoing chemotherapy or with congenital severe combined immunodeficiency. Other risk factors for severe bronchiolitis include parental smoking, lower socioeconomic

group and other causes of lung disease such as cystic fibrosis. Breast feeding may have a protective influence against severe RS virus bronchiolitis (76,161) but this may be short lived (21,262,377).

Infants with bronchiolitis present with symptoms of a non-specific viral illness with rhinorrhoea, cough and sometimes a low grade fever. Cough is usually prominent but not always especially in young infants. The child may be irritable, feed less well and may sometimes vomit (376). The respiratory rate usually exceeds 60 breaths per minute with subcostal, intercostal or suprasternal recession. Inspiratory crackles with or without expiratory crackles and wheezes are usually heard. The child may have signs of mild or moderate dehydration. Pulse oximetry is an essential, sensitive non-invasive technique and may show evidence of oxygen desaturation. However increased respiratory rate is a more sensitive indicator of impaired gas exchange and frank cyanosis is relatively uncommon (201). Radiographic manifestations of bronchiolitis are non-specific. There is usually diffuse hyperinflation of the lungs with flattening of the diaphragms. Patchy or peribronchial infiltrates suggesting interstitial pneumonia are usually present, but pleural thickening and fluid are very rarely seen and minimal if present. The chest x ray may be normal. Examples of sequential chest x rays from a child with severe RS virus bronchiolitis are shown in figure 1.3.

Pathophysiology

Airway oedema, occlusion and some muscle spasm all result in abnormal respiratory mechanics (201,376). Infants with bronchiolitis breath at high lung volumes and therefore the lung is stiffer. Compliance is also decreased because of uneven ventilation of different

regions of the lung with areas of atelectasis and hyperinflation (75,201,375). Airways resistance is increased both in inspiration and expiration, however, the obstruction is usually more marked during expiration (201). Ventilation-perfusion mismatch can produce hypoxia. In younger infants hypercapnia may also be present secondary to hypoventilation as a result of the increased work of breathing. Some infants will have evidence of a mild to moderate respiratory acidosis but metabolic acidosis is more commonly seen (376).

Diagnosis of infection with RS virus is usually made by direct immunofluorescence of nasopharyngeal aspirates and simultaneous virus culture. Once the diagnosis is confirmed the mainstays of treatment are still supportive: to ensure adequate oxygenation and hydration. In severe cases positive pressure ventilation is often required. Ribavirin, a synthetic nucleoside resembling guanosine, has been shown to be a potent antiviral agent against RS virus *in vitro*. However, studies of treatment of bronchiolitis with small particle ribavirin nebulisation therapy have shown modest benefits and its use is usually reserved for children at very high risk or with severe disease (209).

The Immune response to RS virus

Infection by RS virus does not provide long lasting protective immunity against reinfection and individuals are regularly reinfected throughout life. However, it is only in the very young and elderly that infections are likely to cause severe illness. An intact immune system is important: children with either congenital or acquired immunodeficiencies are often unable to terminate the acute infection and can shed virus at high levels for many months (47,92,93). Local non specific mechanisms used by respiratory mucosal surfaces appear to

have limited effectiveness in protecting against infection. Clinical studies showed that nasal administration of α_2-interferon could partially protect against infection (156). However protection is only seen when it is administered before exposure. Unlike parainfluenza and influenza, RS virus fails to induce interferon in nasal secretions during infection (136,237). In parainfluenza and influenza infections interferon levels rise during acute infection and peak levels coincide with clearance of the virus (58).

Humoral Immunity

The role of antibody in protection against RS virus is still not clear. Serum antibody levels do not correlate well with protection from infection. Children with specific humoral immunodeficiencies but with intact cell mediated immunity do not appear to have more prolonged or severe illness after primary infection. Most infants are admitted to hospital with bronchiolitis at between two and six months of age. The peak age of admission is at two to three months decreasing steadily after that time. Specific passively received maternal neutralising antibody is present in all newborn infants, but it declines over the first six or seven months. This observation lead to the hypothesis that antibody mediated enhancement of disease could be involved in pathogenesis, in a similar manner to some arbovirus infections such as dengue virus (13,138,139,202). Early data suggested that infection of a human and a mouse monocyte/macrophage cell lines could be enhanced by the presence of antibody (202). Welliver *et al* indicated that IgE against RS virus may have a role in illness. These studies suggested that high levels of anti-RS virus IgE after primary infection correlated with the tendency to wheeze (368,369). However this finding has not been confirmed (275).

More recent studies of anti-RS virus antibody levels in cord blood suggest that high levels may in fact confer some protection (115). After seven months of age anti-RS virus antibody is usually acquired following primary infection. By one year of age between 25 and 50 percent of children will possess antibody to RS virus, by 4 years 80 percent of children have serological evidence of RS virus infection (21) and by adulthood everyone has serum antibody (20,49,131). A recent trial of prophylactic pooled anti-RS virus globulin transfusions in children at high risk for severe illness showed some protection (129). Children had less severe illness, shorter admissions and decreased need for intensive care during primary RS virus infection. However, there is no evidence so far of lower mortality with this expensive form of treatment and the risks of fluid overload and the potential for infectious complications associated with transfusion may limit its use.

Mucosal IgA against RS virus may have a protective effect. Some short term protection against reinfection has been shown in experiments in primates (21). Volunteer infection studies have shown a better correlation between secretory IgA in nasal secretions and protection against infection (242) than serum antibody. However in man, IgA mediated immunity appears to wane approximately 9 months after infection and reinfection is still possible despite high levels of local IgA (58). Local secretory IgA produced in infants is often in lower amounts than in adults in response to RS virus (257) and has been found to neutralise the virus poorly *in vitro* (238).

Cellular Mediated Immunity

The role of cell mediated immunity in the clearance of RS virus is becoming clearer and has demonstrated an interesting balance between the control of acute infection and induction of

pathology. T cells can be classified by cell morphology, cell surface protein phenotype ($\alpha\beta/\gamma\delta$ TCR, CD4/CD8), function and cytokine secretion (T_H0, T_H1, T_H2). T cells are responsible for "help" and cytotoxicity through direct contact with antigen processing cells and by secretion of cytokines. Through their interaction with B cells, T cells also have an important role in humoral immunity. Both $CD4^+$ and $CD8^+$ lymphocytes, are important in the clearance of a wide range of viral infections such as influenza, lymphocytic choriomeningitis virus (LCMV), human cytomegalovirus (hCMV) and human immunodeficiency virus (HIV) (253,360,378). Either subset may be able to clear infection in some models for example, in influenza, specific CTL alone can clear virus from the lung (223).

Antigen recognition by T cells

The central mechanism of all adaptive immune responses is the activation of T cells by antigen. T cells recognise antigens as peptide fragments bound to major histocompatibility (MHC) molecules. A single T cell has been estimated to carry 50,000 T cell receptor (TCR) molecules on its surface (181). Of these molecules probably less than 100 may be required to be engaged for signalling to occur. An antigen presenting cell may similarly only need a small number of its 100,000 MHC molecules to bind peptide to trigger a T cell (69,145,181).

MHC and peptide binding

The MHC complex encodes polymorphic genes for the cell surface glycoproteins, class I and class II MHC molecules. These glycoproteins present antigen derived peptides, generated in the cytosol, to $CD8^+$ and $CD4^+$ T cells respectively. Much is now known about both the

structure and interactions of both class I and class II MHC molecules (33,182,338). Endogenously synthesised proteins are processed by proteasomes into peptide fragments and these are transported into the endoplasmic reticulum (ER). The transport into the ER is dependent on proteins like the T cell antigen processing (TAP) complex also encoded by MHC. TAP complexes in the membrane of the ER pre-select correctly sized peptides. The C-terminal residue of the peptide may have some role in influencing TAP transport into the ER (263). For class I presentation the peptide binds to class I heavy chain which induces a conformational change and allows association with β2 microglobulin. This stabilises the molecule and allows it to be transported to the cell surface. MHC class I tertiary structure features a polymorphic groove between the α1 and α2 domains which acts as a binding site for 8 to 11 amino acid long peptides. Specific hydrophobic or hydrophilic regions, or "pockets", within the MHC groove favour or hinder binding by different residues contained within peptides (89,316). It has also been possible to acid elute peptides from MHC molecules and sequence them. For certain MHC molecules it has been possible to determine consensus sequences for binding which allow putative epitopes to be predicted.

Class II MHC molecules are also highly polymorphic. They consist of heterodimeric assembled α and β subunits that bind at an N-terminal domain (338). Once synthesised, peptide binding during transport is prevented by the invariant chain, a molecule expressed in large excess of the class II molecules. It associates with class II and blocks peptide binding to the groove while the molecule is transported to the endosome. In the endosome, the invariant chain is removed by proteolytic degradation. A fragment of the invariant chain, the class II associated invariant peptide (CLIP), which contains the class II binding site remains transiently bound to the class II (100). It is thought that CLIP is released as part of

peptide loading. CLIP alone is highly inhibitory to antigenic peptide binding in vitro (160). Once peptide loading has taken place the class II molecule is transferred to the cell surface (2). Peptides that bind to the groove in class II molecules are usually consist of at least 13 amino acids, longer than class I peptides. The peptide appears to overhang the ends of the groove and residues outside the groove are important in TCR recognition. Maintenance of MHC expression on the surface of the APC plays a key role in immunity. Increases or decreases in expression of class I and or class II can alter antigen presentation. Down regulation of MHC may be a mechanism by which pathogens or tumours escape from the immune response. The human immunodeficiency virus (HIV) for example, may reduce MHC class I expression on infected cells through its TAT protein (167).

Avidity versus conformational change

Peptides that differ only slightly can stimulate T cells to different degrees (240). Some of these peptides bind to the TCR with lower affinity than others. This has lead to the avidity model of TCR peptide binding. This model suggests that the degree of activation is determined by how well the peptide binds to the MHC molecule or the intensity of the interaction with the TCR after binding to the MHC molecule. Other authors have demonstrated that peptides, closely related to stimulatory epitopes, can fail to activate antigen specific T cells (334) and cause anergy. In many cases affinity for the TCR does not predict the level of stimulation of the T cell (145,181). From these observations it has been postulated that T cell activation requires conformational change of the TCR after binding. High affinity may not be required for conformational change to be induced. De Magristis *et al* (67) found that certain variants could competitively antagonise activation by peptide in a T cell line. Antagonism was shown even when the antigen presenting cells were first

loaded with the stimulatory antigenic peptide, indicating that the antagonism occurs at the level of the TCR recognition rather than MHC binding.

Co-stimulatory molecules

The interaction between TCR and antigenic peptide fragments bound to the MHC molecule initiates the immune response but is not sufficient to activate T cells. Quill and Schwartz observed that this interaction alone leads to anergy (302). APCs also have a role in providing essential co-stimulatory signals to the T cell. As well as CD4 and CD8, several molecules have been described as being important for co-stimulation including: ICAM-1 (291,342), VCAM-1 (346), HSA (195), LFA-3 (194) B1 (194)and B7 (98). The nature of these co-stimulatory signals is still being determined and may prove critical to the nature of the response mounted after T cell activation. Co-stimulatory molecules may play an important role in determining the response generated. B7 molecules may interact with cytokines in stimulating T_H2 or T_H1 responses when associated with the TCR-MHC interaction. Murphy *et al* recently demonstrated that B7-CD28 ligation was associated with IL-2 and IL-4 production only unless IL-12 was added when γ-IFN was produced (260). Selective blockade of B7-1 or B7-2 with antibodies during priming or restimulation of transgenic T cells leads to increased IL-4 production or γ-IFN production respectively (204). Recently the B1 accessory molecule has been described on the surface of LPS stimulated murine B cells. B1 was shown to elicit a dose dependant proliferative response from $CD4^+$ T cells. This stimulation resulted in the production of a dominant secretion of IL-4 and IL-5 consistent with a T_H2 response, a low level of IL-2 and no γ-IFN (358).

Mechanisms of viral clearance

CTLs are associated with a potent and specific lysis of virus infected cells. Lysis can be through two main pathways: secretion and direct contact. In secretion, lysis is achieved by the release of antiviral cytokines such as TNFα and IFN-γ. TNF can induce apoptosis through interaction with cell surface receptors (212,380) and IFN-γ is associated with the release of further mediators and the generation of a general antiviral state (314,320). IFN-γ also increases class I expression on infected cells, enhancing presentation of viral peptide antigens and leading to more efficient recognition and clearance. Direct contact killing of infected and tumour cells can be through one or all of three mechanisms. The first mechanism is through perforin release which is characterised by pore formation and cell death (200). A second pathway involves the intercytoplasmic transfer of granzymes which are believed to be involved in inducing target apoptosis (146,254). Thirdly direct engagement of Fas by the Fas ligand triggers programmed cell death accompanied by DNA fragmentation (23,151,200). CD4 positive T cells may also induce killing through class II in an antigen specific manner (128), also associated with DNA fragmentation. CD4 positive T cells are more usually associated with a helper phenotype and produce cytokines leading to help for antigen specific immune responses and the generation of both effector, memory cells and induction of specific B cell responses.

Mice that are immunodeficient (either mice with genetic mutations causing severe combined immunodeficiency (SCID), or after irradiation) fail to clear RS virus and shed high levels of virus (40). Adoptive transfer of RS virus specific CD8 positive CTL was shown to clear the infection but was also associated with weight loss and enhanced lung pathology. Openshaw *et al.* (276)were the first to show that the 22 kilodalton second matrix protein (M2) was the major target protein for K^d restricted cytotoxic lymphocytes in mice. Further

studies from Dr Openshaw's group have found that the nucleoprotein N, the surface proteins SH and F and both the matrix proteins (M1 and M2) are commonly recognised by CTLs by adult donors (51). In adults there appears to be no correlation between pre-existing CTL and protection from reinfection (177).

In the BALB/c mouse it has been possible to further dissect the cell mediated response. Dr Barney Graham (121) used monoclonal antibodies to deplete T helper subsets and examined the pathological findings in the lung. Normal BALB/c mice cleared RS virus by day 8 after primary infection and the mice became ill with a peak weight loss of 8 grams. They developed lymphoid and monocytic infiltrates around bronchovascular bundles and an increase in alveolar lymphocytes on day 7. During convalescence they had collections of uniform small basophilic lymphocytes around the bronchovascular bundles. When both $CD4^+$ and $CD8^+$ lymphocytes were depleted mice had no signs of illness, weight loss or lung infiltrates. Virus persisted at high levels in the lung beyond day 14. $CD4^+$ lymphocyte depleted mice became mildly ill following RS virus infection and lost 6 grams in weight. Clearance of virus from the nose was delayed until day 11. $CD4^+$ depleted mice had decreased lymphocytes in the bronchovascular spaces but increased alveolar lymphocytes and lacked perivascular lymphocytes during convalescence. $CD8^+$ depleted mice also had less severe illness than normal controls and a mean weight loss of 6 grams. Virus was again present in the nose until day 11. $CD8^+$ depleted mice had few alveolar lymphocytes but perivascular infiltrates and convalescent infiltrates were present. Overall it was clear that although both $CD4^+$ and $CD8^+$ lymphocytes were important in the clearance of RS virus they were also associated with illness and weight loss. Further work from Dr Openshaw's group has determined the cytokine production and time course of lymphocytes during RS virus

infection using bronchoalveolar lavage demonstrating that the dominant cytokine production during primary infection is of IFNγ with few cells producing IL-4 or IL-5 (L.S.Spender and T. Hussell - personal communication).

Formalin inactivated vaccine

In the 1960s there were a series of trials of vaccines containing formalin inactivated preparations of RS virus precipitated with alum (52,104,192,198). Vaccinees developed high levels of serum antibody to RS virus but were not protected when subsequently exposed to natural infection during the RS virus season. Alarmingly, when the vaccinees became infected with RS virus they experienced augmented disease with severe pulmonary disease and a number of children died. Admission rates compared with controls were increased from less than 5% to almost 80%. One of the findings noted at post mortem examination in some of the vaccinees was the presence of pulmonary eosinophilia, suggesting a T_H2 type immune response. Formaldehyde inactivated preparations of measles virus have also been used as vaccines. They too were associated with enhanced disease in some children when they subsequently caught measles (82,103). Cardoso *et al* recently showed that CD46 dependent presentation of measles to CTLs is abolished after formaldehyde inactivation but not presentation to helper T cells (43). Work from Dr Openshaw's group indicates that different T helper subsets may be being primed by different RS virus proteins leading to either a T_H1 or T_H2 phenotype and enhanced disease.

T cell subsets: T helper 1 and T helper 2 after vaccination

Based on the analysis of cytokine production by murine T helper clones Mosmann and Coffman classified T helper lymphocytes into either T_H1 or T_H2 (250). A general outline

of this division is shown in figure 1.4. $CD4^+$ T helper cells were found to produce characteristic clusters of cytokines some directing cell mediated immune responses, others enhancing B cell antibody production. T_H1 cells produced IFN-γ and IL-2 , whereas T_H2 cells produced other cytokines: IL-4, IL-5 and IL-10 (and now IL-13). Subsequently, cells producing cytokines from of T_H1 and T_H2 types were demonstrated and termed T_H0. These may represent precursors of the extreme T_H1 and T_H2 phenotypes or an end stage of commitment for some cells *in vivo*. Since the initial discovery of murine T_H1 and T_H2 cells they have also been described in humans (312). In broad terms T_H1 responses are associated with DTH immunity and in particular tumour killing and virus clearance. T_H2 responses have been described in a number of disease states but are commonly associated with responses to extracellular antigens, particularly helminths, and with allergy and atopy. The relationship between certain disease states and the emergence of T_H1 and T_H2 responses is still unclear. Important evidence is emerging that the antigen dose, the site where antigen is encountered and the type of cell presenting antigen are all important in determining the immune response as well as the nature of the antigen (166). In many chronic conditions polarised T_H1 or T_H2 emerge. This appears to be, to a greater or lesser extent, through counter inhibition by secreted cytokines (figure 1.4). IL-12 seems to be a dominant influence in the development of T_H1 responses (168,227) whereas stimulation of T cells with antigen in the presence of IL-4 induces the T_H2 phenotype (340). Under conditions of repeated stimulation with antigen, highly committed murine clones can be generated that secrete only cytokines associated with either T_H1 or T_H2 (271). Under these conditions, dual secretion is only seen in a small number of cells presumed to be uncommitted precursors.

Vaccinia recombinants constructs encoding single proteins have been used to determine the

contribution of individual RS virus proteins to pathology and protection. BALB/c mice were immunised with different recombinants by scarification, producing immunity to one protein of RS virus only. After allowing recovery for a few weeks, the mice were challenged with live RS virus. The response to challenge was monitored using bronchoalveolar lavage (BAL). Mice scarified with recombinant vaccinia viruses (rVV) containing the G protein, F protein or N protein all developed enhanced pathology reflected by weight loss, pulmonary neutrophil efflux and pulmonary haemorrhage when subsequently challenged with RS virus intranasally. However, the mice sensitised to G also developed pulmonary eosinophilia. Short term lines were generated from splenocytes of mice after prior vaccination. It was found that the phenotype of the lines was altered just by changing the protein to which the mice had been exposed. Lines from mice vaccinated with rVV-G were almost all $CD4^+$ with very few $CD8^+$ lymphocytes. Lines from mice exposed to rVV-F contained both $CD4^+$ and $CD8^+$ cells. Mice exposed to rVV-M2, already determined to be the major target for CTL in $H\text{-}2^d$ mice, produced lines that were exclusively $CD8^+$ and needed exogenous cytokine in the form of rat spleen ConA supernatant to maintain the line. The splenocyte cultures were found to produce different patterns of cytokines. Supernatants from rVV-G lines produced the T_H2 cytokines IL-4 and IL-5 whereas supernatants from rVV-F lines produced IL-2 more akin to T_H1. Other investigators have shown that sensitisation of BALB/c mice with formalin inactivated preparations of RS virus prepared in a similar way to the vaccination trials is associated with similar T_H2 cytokine production (122).

From these studies it can be concluded that subunits of RS virus can prime for different T lymphocyte immune responses. In particular the G protein of RS virus may be capable of stimulating a different subset of T cells to produce a T_H2 type response. T_H2 responses are

not normally associated with viruses but characteristically are found in association with responses to extracellular pathogens such as helminths. T_H2 cytokines have also been shown by *in situ* hybridisation of bronchial epithelium in lung biopsy samples from patients with asthma (71,141). Interestingly, the common nature of mucosa associated lymphoid tissues (MALT) of gut and lung has been recently been suggested by similar studies of duodenal specimens. Benoit Wallaert *et al* (363) examined the duodenal lamina propria of specimens from patients with asthma and atopy and compared them with normal controls and patients with chronic obstructive pulmonary disease (COPD). Patients with atopy or asthma demonstrated IL-3, IL-5 and GM-CSF in their lamina propria with significant accumulation of intraepithelial mast cells and lymphocytes which was not found in patients with COPD or in normal controls. Experiments in animals have previously suggested that precursors from bronchus associated lymphoid tissue may migrate to the intestinal tract and other MALT sites (239).

The delayed effects of respiratory syncytial virus infection

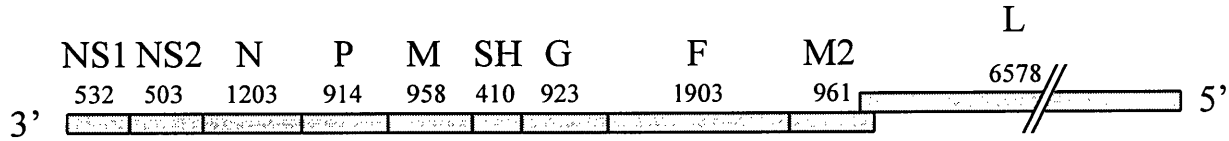
The importance of RS virus in children in causing acute admissions to hospital is clear. RS virus accounts for more admissions to hospital under one year of age than any other pathogen (150). There is also a less well defined area of disease associated with RS virus bronchiolitis highlighted recently by Sigurs *et al* (332). RS virus infection was thought to be limited to superficial epithelial cells and macrophages and yet other similar viruses are known to cause viraemia and infect sites distant from the respiratory tract. In particular measles virus and Sendai virus infect and persist in neurological tissue.

Bronchial hyperresponsiveness and recurrent wheezing after severe bronchiolitis can be

frustrating for parents and paediatricians alike. Some children have many admissions and the condition is difficult to treat especially in children under two (105, 152, 218). The relationship between post bronchiolitis wheezing and asthma remains controversial. More information is needed about the mechanisms that underlie it and in this respect animal models may provide useful information on which to base clinical studies.

After developing a nested RT-PCR to detect RS virus I have used it in a number of ways to add to our information about both the infectious cycle of RS virus its and interactions with the immune system. In the first chapters I will present data from experiments performed on human samples. In primary infection in children, the frequency of respiratory virus infections causing admission is studied and the presence of viraemia is investigated. In the same way the importance of RS virus infection causing respiratory illness in adults with HIV is studied.

By using an established mouse model the duration of infection with RS virus is studied. Evidence for persistence and escape from immunity discussed. Finally a possible role of RS virus in the generation of allergic responses is demonstrated.



Gene	Gene length	Protein length	Comments
Nucleocapsid			
N	1203	391	Major nucleocapsid protein; binds genomic RNA
P	914	241	Viral phosphoprotein; acidic
L	6578	2165	Viral polymerase
Surface			
F	1903	574	Fusion protein; mediates virus penetration and syncytium formation; homotetrameric
G	923	298	Attachment protein; homotri- or tetrameric; extensive O-linked glycosylation; serine and threonine rich.
SH	410	64	Required for syncytium formation; other functions unknown.
Matrix			
M	958	256	Probable counterpart to matrix proteins of other paramyxoviruses
M2	961	194	Role in inhibiting RNA synthesis?
Non Structural			
NS1	532	139	Function unknown (unique to pneumoviruses)
NS2	503	124	Function unknown (unique to pneumoviruses)

Figure 1.2 Proteins of RS virus.
The pneumoviruses (including RS virus) encode 10 mRNAs, more than other paramyxoviruses which encode 6 or 7. The two "non structural" proteins and the second matrix protein M2 are unique to pneumoviruses. The attachment protein G is unusual among viral glycoproteins being highly serine and threonine rich with extensive O-linked glycosylation. Protein length is number of amino acids. Gene length is number of nucleotides. (Adapted from Collins *et al* in Fields Virology 1996)

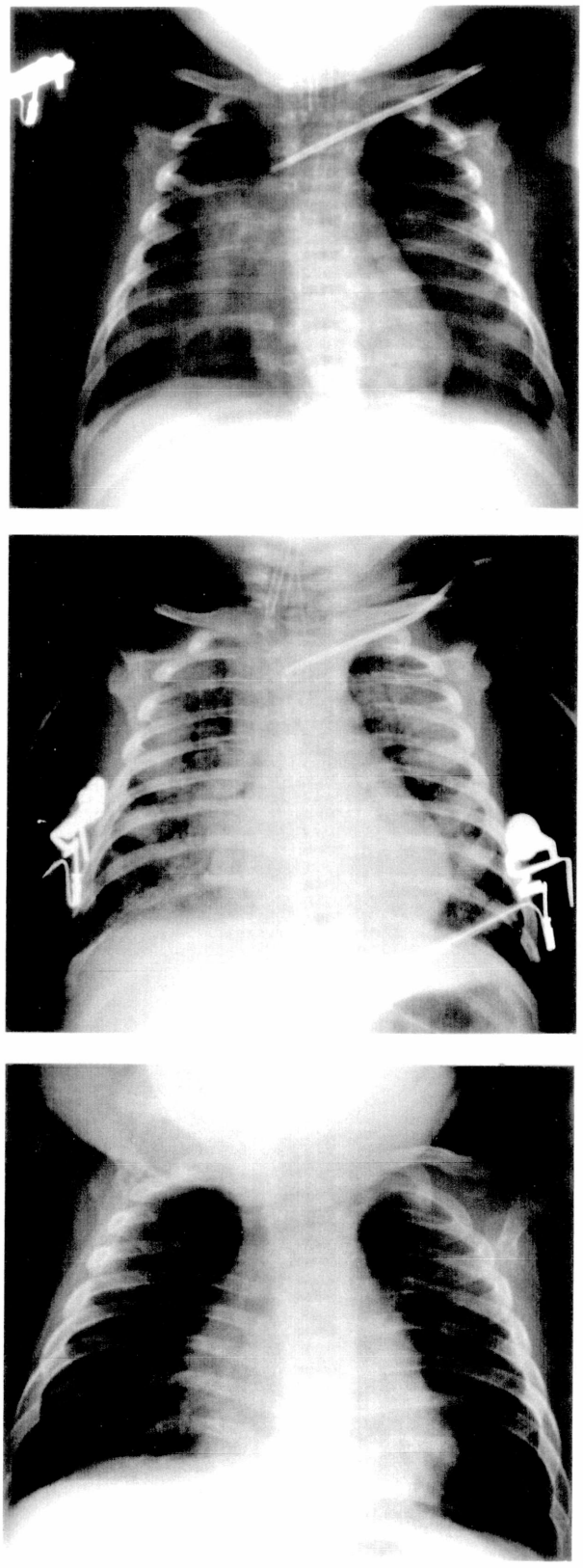


Figure 1.3 Chest X ray appearances in a child with severe RS virus bronchiolitis.
The chest X rays above come from a child admitted to St Mary's Hospital with severe RS virus bronchiolitis. The child required positive pressure ventilation for several days. The top X ray is soon after admission. The middle X ray is from the third day after admission and the bottom X ray is 3 months later.

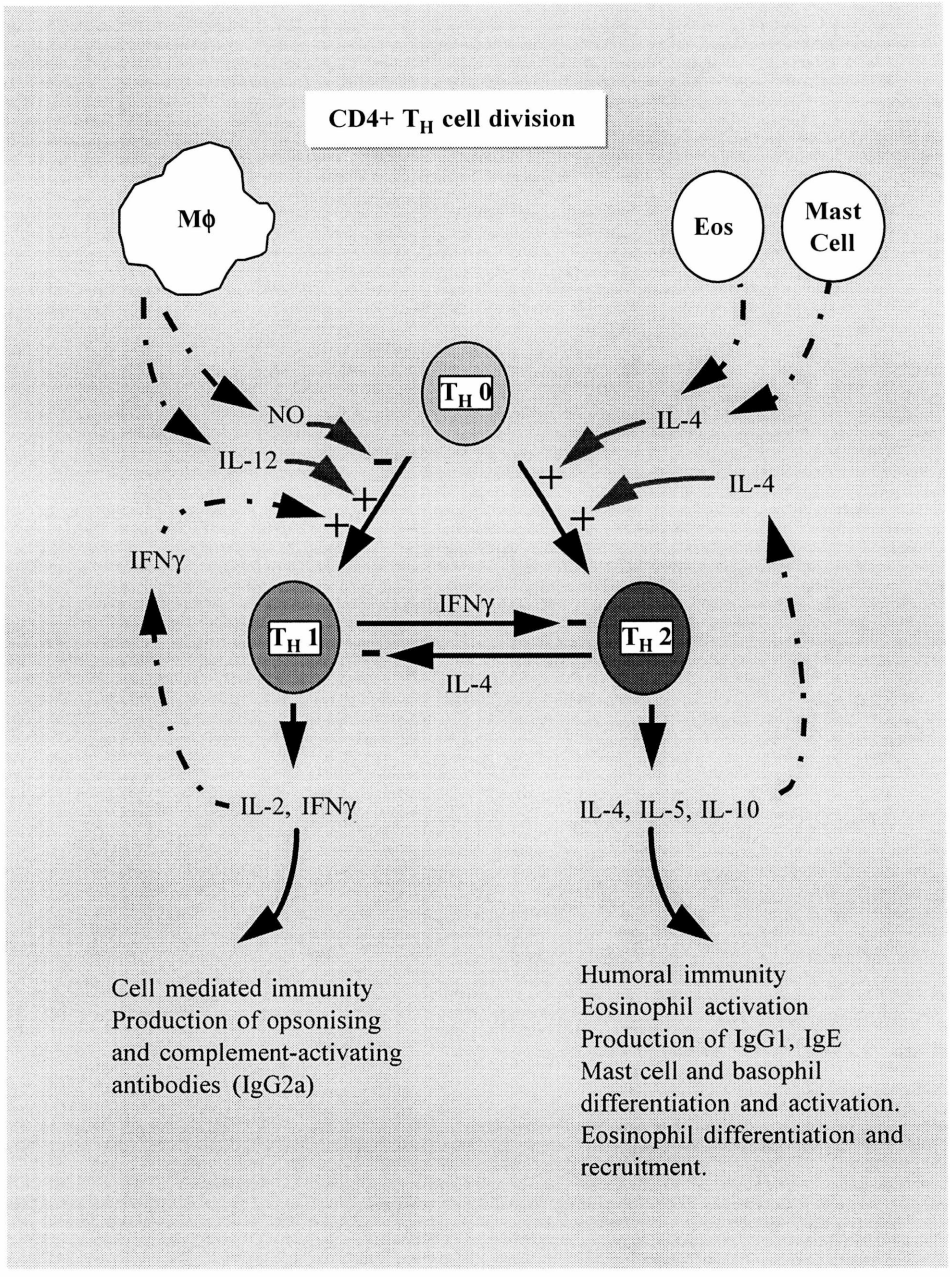


Figure 1.4 CD4+ T_H cell subsets. Division of T helper subsets according to the TH1/TH2 paradigm originally based on the analysis of murine T helper clones by Mosmann and Coffman. Mϕ - macrophage, Eos - eosinophil.

Chapter 2 Materials and methods

Solutions and Chemicals Used

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company, Fancy Road, Poole, Dorset, UK. A further list of specific buffers and reagents is given at the end of this chapter. All virus and cell stocks were checked for mycoplasma using DNA hybridisation (Gen-Probe, San Diego, California).

Respiratory Syncytial Virus

A2 strain was originally obtained from a human isolate and has become the most frequently studied laboratory strain of RS virus. Virus stocks used in these experiments were prepared as described by Bangham *et al* (17). Briefly, human laryngeal carcinoma cells (HEp-2) were obtained from the ECACC (Porton Down, Wiltshire, UK). They were grown in RPMI with 10% fetal calf serum, penicillin 50u/ml, streptomycin 50µg/ml (pen/strep) and glutamine 300µg/ml (R10F) at 36.5-37°C in 5% CO_2 until 70% confluent. The medium was removed and the cells infected with RS virus, in 3ml of serum free RPMI at a final multiplicity of infection (moi) of 0.1pfu / cell. The inoculum was left on the monolayer for 2 hours at 37°C with gentle rocking every 15 minutes. R10F medium was then added to the flasks for further incubation for 24 hours at 37°C. After 24 hours the FCS content was reduced to 2% by removing 80% of the medium, resuspending the cells and replacing the medium with serum free medium. The flasks were then incubated at 37°C for a further 24 hours. When cytopathic effect reached 80-90% the cells were harvested using cell scrapers (Costar). Paired uninfected HEp-2 monolayers were harvested simultaneously as controls. Identical samples were pooled, aliquoted, snap frozen and stored in liquid nitrogen.

Microplaque assay for infective virus (from Cannon *et al* (38))

96 well microtitre plates were seeded with 2×10^4 HEp-2 cells/well. The next day the wells were assessed to confirm almost complete confluence of the monolayer without overgrowth or cell death. Virus samples were then diluted in serum free medium (RPMI with antibiotics, 2ME and glutamine) and ½ log dilutions performed changing the micropipette tips after each step. Medium from the microtitre plates containing the adherent HEp-2 monolayers was removed by inverting the plate and 50µl aliquots of the virus dilutions were added to the plate starting with the most dilute and ending with the most concentrated. The virus was allowed to adhere for 2 hours at 37°C then the wells were made up to 200µl with R10F. The plates were incubated for a total of 24 hours at 37°C. The wells were then inverted to remove the medium and washed with PBS. After removing the PBS by inverting the plates the wells were fixed by adding 100µl per well of methanol containing 0.6% H_2O_2 for 20 minutes. The monolayers were then left overnight if necessary, in PBS with 1% BSA and 0.1% azide (PBS/BSA/azide). The infected cells were then stained by adding peroxidase conjugated anti-RS virus antibody in PBS/BSA/Azide for 30 minutes. After washing 3 times, 50µl of AEC substrate was added. The plate was incubated for between 40 minutes and 3 hours until positive control wells showed characteristic salmon pink staining of infected cells. The substrate was then washed away and the infected cells counted using an inverted microscope.

Vaccinia Recombinants

Vaccinia viruses expressing genes for M2 of RSV or β-gal were used in cytotoxicity assays (CTL assays). The transgenic seed viruses were a kind gift of Prof. G.W. Wertz (University

of Alabama, USA). In brief, they were prepared in HEp-2 cells by infection with 0.3-0.5pfu/cell and cultured for 48-72 hours in serum free medium at 37°C. The cells were then resuspended using a cell scraper and cells pelleted (2000rpm for 10 minutes at 4°C). The supernatant was discarded and the pellet washed in PBS and repelleted. The pellet was sonicated (3 times, 10 minutes, 150W) in an ice bath, large debris removed by centrifugation (1000rpm, 5 minutes, 4°C) and the supernatant tested for infectivity on HEp-2 cells.

Mouse Experiments

BALB/c mice were obtained from Harlan Olac. Mice were kept in pathogen free conditions throughout. All bedding, water and feed was sterilised. Cages were covered with filtered tops and where possible mice were kept in a filter cabinet. Mice used in these experiments were 12-16 weeks old at the start of each experiment unless otherwise stated.

Intranasal infection

Virus stock was diluted in PBS to the required pfu/ml (indicated in each chapter). Mice were lightly anaesthetised with ether. Fifty to 90µl of virus stock was then placed on the nose while the mouse is held upright until aspiration had occurred.

Injection of depleting antibodies i.v.

Depleting antibodies were a kind gift of Dr Steve Cobbold (William Dunn School of Pathology, Oxford). Mice were warmed using a lamp and 200µl containing 1mg anti-CD4 and 1mg anti-CD8 injected into the tail veins. The antibodies were equal mixtures of two synergistic antibodies for CD4 (YTS 191.1 (53), YTA 3.1 (300)) and two for CD8 (YTS 169.4 (53), YTS 156.7 (301)).

Recovery of tissues from mice

Mice were given a lethal dose of pentobarbitone (3mg i.p.). After unconsciousness but before cardiac arrest the femoral vessels on one side were transected. Approximately 300 to 500µl of blood can be obtained per mouse using this method. Splenocytes were obtained by disrupting an intact spleen through a mesh, washing once with fresh medium and resuspending the cells obtained in R10F. To perform bronchoalveolar lavage, complete pneumothorax was induced by nicking the diaphragm, then the trachea was exposed through the anterior neck and transected transversely to allow a 1.22mm cannula (Portex) attached to a 1ml syringe barrel to be inserted. The tip was advanced into the trachea and 1ml of PBS containing 12mM lignocaine used to inflate the lungs 6 times. The cells washed out were then put into prepared tubes on ice containing 10ml of R10F. The lungs were removed by lifting the distal trachea, dissecting posterior to the lungs and removing them *en bloc.* The heart was then dissected away and the lungs either snap frozen, placed in R10F or fixed in formalin. In some studies the olfactory bulbs were obtained by opening the skull posteriorly via the foramen magnum and opening the sagittal suture. The two halves of the vault were then reflected laterally revealing the two cerebral hemispheres. The nasal olfactory bulbs lie anterior to the frontal lobes within cavities in the bones above the nasal passages. A further cut anteriorly through the nasal bones was usually necessary to reveal them. The bulbs were lifted out with fresh forceps and snap frozen.

Exposure to ovalbumin by nebulisation

A transparent plastic container measuring approximately 40cm by 30cm and 30cm in height was used as a chamber for nebulisation (e.g. a plant propagator box). Mice were placed in

the container, the lid replaced and the entire container placed in a class 1 cabinet for safety. A Ventstream nebuliser (Medic-Aid, Pagham, W. Sussex, UK) was filled with either a 1% solution of ovalbumin in PBS or PBS alone and attached to the nebulisation container to an inlet drilled in the side of the container by elephant hosing. The nebuliser was driven by compressed air at a rate of 10 l/minute. Outlets were provided by two small holes drilled in the wall opposite the inlet and the fact that the lid did not make a close seal. Nebulisation occurred for 20 minutes a day for 10 days, the total volume per nebulisation was 12ml of liquid. After each nebulisation the containers were thoroughly cleaned with a 1% solution of virkon and dried before reuse.

Immunological Methods

Spleen cell bulk culture

Spleens were removed from the mice using careful aseptic technique. They were placed in R10F before being disrupted by compressing them through a cell strainer (Falcon). After washing in fresh R10F, the cells were placed in upright 25cm^2 tissue culture flasks (Corning) at a density of 1.5×10^7 in 10 mls. 3.75×10^6 cells (per flask) from an uninfected mouse were pelleted and incubated with RSV to provide stimulators. In these experiments all stimulators were infected by 8pfu RSV/cell for 2 hours at 37°C. During incubation the stimulators were gently resuspended every 20 minutes. The stimulators were added to the flasks and the total volume made up to 15ml with R10F and 2ME. The flasks were incubated at 37°C in 5% CO_2 for 5 or 6 days. The cells were then harvested and used as killers in CTL assays against RSV, vaccinia or peptide labelled targets.

Preparing Targets

P815 cells (ECACC, Porton Down, Wiltshire, UK) were grown in R10F medium. For infection with viruses (RS virus or vaccinia) the cells were incubated for 2 hours with 2pfu/cell then made up to 5×10^5 cells / ml and incubated overnight. For peptide targets the cells were placed in 1ml of RPMI containing the desired concentration of peptide for 2 hours (see experiments). 10^6 cells from each target were then centrifuged to a pellet and resuspended in 30µl of ^{51}Cr (1mCi/ml in PBS, Amersham, Little Chalfont, Bucks, UK) and incubated for 40 minutes at 37°C. The labelled P815s were then washed with 5ml of PBS, 5ml of RPMI and finally 5ml of RPMI (3 washes). P815 cells were then made up to 10^5 cells/ml. CTL from 5 day bulk cultures were made up to the appropriate concentration for maximum effector to target (E:T) ratios. Doubling dilutions were then performed in 96 well round bottomed tissue culture plates (Corning, New York, USA). After plating out the effectors, the targets were added (100µl/well). Targets were added to wells containing medium alone (no killers) to determine background release of ^{51}Cr. Remaining targets were then sonicated for at least 2 minutes (Transonic T310, Camlab) in 13ml conical tubes (Bibby Sterilin, Stone, Staffs, UK) and 100µl added to several wells containing medium alone to represent maximum lysis. The plates were then spun at 150g for 10 seconds and incubated for 3 hours at 37°C. After 3 hours the plates were centrifuged at 400g for 5 minutes and 50µl supernatants transferred to Spotwell 96 plates (Canberra Packard Ltd, Pangbourne, Berks, UK). Once dry the plates were read using a Matrix 96 plate reader (Hewlett Packard, Reading, Berks, UK). Results were generated using the equation: Specific Lysis (percent) is equal to:

(Sample (cpm) - background (cpm)) ÷ (Maximum lysis (cpm) - background (cpm))

Bioassay for cytokines IL-2 and IL-4

CTLL cells are an IL-2 and/or IL-4 dependant cell line and can be maintained in R10F and an exogenous source of cytokine to stimulate proliferation (114). 10% ConA stimulated rat spleen culture supernatant was used in these experiments as a source of exogenous cytokine. 48-72 hours after the last addition of cytokine, the CTLL cells were washed and suspended at 1×10^5 cells/ml in serum free RPMI. 50µl/well (5×10^3 cells) were incubated in a round bottomed 96 well plate with 100µl/well of medium alone or medium with antiIL-2 (S4B6) or antiIL-4 (11B11). 50µl/well of recombinant cytokines (IL-2 or IL-4 -) were then added to positive control wells and 50µl/well of the test samples. The plates were incubated at 37°C for 20 hours 0.5µCi/well of ^{3}H-Thymidine then added. After 4-6 hours the plates were harvested onto filters, dried and counted (using the Matrix 96 plate reader). Results were calculated as the mean of 3 or 4 replicates after the spontaneous counts of CTLL cells with medium alone had been subtracted.

ELISA

Protocol for detecting IgE, IgG1 or IgG2a to ovalbumin in mouse serum

96 well ELISA plates (Maxisorp, Nunc) were coated with 100µl/well 0.1M NaHCO3 pH 8.2 containing 5µg/ml of either rat anti-mouse IgE, rat anti-mouse IgG1 or rat anti-mouse IgG2a and incubated overnight at 4°C (antibodies from Serotec). After washing three times with PBS/0.05% Tween (BDH) the plates were blocked with 200µl/well 2% BSA in PBS for 1 hour at 37°C. After washing as above, 100µl of a 1/10 dilution of serum in 1% BSA in PBS was then added to each well and the plates incubated for 1 hour at 37°C.

Digoxigenin was conjugated to ovalbumin using a digoxigenin conjugation kit (Boehringer Mannheim, Bell Lane, Lewes, E. Sussex, UK). The ratio of conjugation was 1 ovalbumin to 40 digoxigenin. The plates were washed again as above and 100µl/well of digoxigenin conjugated ovalbumin added (diluted 1/50 in 1% BSA in PBS). The plates were then incubated for 1 hour at 37°C and then washed 3 times as before. 100µl/well of HRP conjugated sheep anti-digoxigenin Fabs (Boehringer Mannheim) diluted 1/3000 in 1% BSA in PBS was then added and the plates again incubated at 37°C for 1 hour. After washing, OPD substrate was added (0.034% in citrate-phosphate buffer) and left for 15 minutes for colour development, the reaction was stopped with 50µl 2M H_2SO_4 and the plates read at 490nm.

Protocol for detecting total IgE in mouse serum

ELISA plates (as above) were coated and blocked in the same way described above. Sera were then added at 1/40 dilution in 1% BSA in PBS 100µl/well and the plates incubated for 1 hour at 37°C. After washing three times (as above) 100µl/well sheep anti-mouse IgE HRP (The Binding Site Limited) was added at 1/500 dilution in 1% BSA in PBS. The plates were incubated again for 1 hour at 37°C. After washing the plates were developed with OPD substrate and read at 490nm. A standard curve was prepared from a known concentration of IgE (a kind gift of Dr Charlotte Hetzel) and a polynomial expression obtained using Minitab for Windows (figure 7.7). Using this polynomial expression quantities of IgE were calculated from the ODs obtained experimentally.

Anti-RSV ELISA

RSV antigen and control HEp-2 antigen were prepared according to established protocols

(273). In brief, approximately 10^7HEp-2 cells were infected with 0.1pfu/cell A2 RSV or left uninfected. After incubation for 48 hours at 37°C the medium was replaced with 40ml serum free medium. After 24 hours the cells were resuspended in a volume of 2ml and sonicated. The sonicated preparations from infected and uninfected cultures were then clarified by centrifugation at 10,000rpm, aliquotted and stored at -20°C.

96 well plates were coated with 100µl/well of RSV or control HEp-2 antigen. Plates were then dried overnight at 37°C in a dry incubator. Plates were blocked with 200µl per well 1% rabbit serum in PBS. Then sera to be tested were added to each well. After incubation for 1 hour at room temperature, plates were washed with PBS/0.05% Tween. 100µl/well HRP-rabbit anti-mouse Ig was then added, diluted 1/500 in blocker. The plate was then incubated again for 1 hour before washing and developing with OPD substrate as above. Specific anti RSV ODs were obtained by subtracting the response to HEp-2 (background) from the OD to RSV. The means of duplicate or triplicate wells were calculated.

Flow Cytometry

For cell surface staining alone, 1×10^6 cells were washed and pelleted in a 13ml conical tube on ice. Optimal conjugated antibody concentrations were determined by titration experiments. The cells were then treated with the appropriate concentrations in a total volume of 50µl for 30 minutes on ice in the dark. The cells were then fixed as below before flow cytometric (FCM) analysis using an Epics ELITE flow cytometer/cell sorter (Coulter, UK).

For intracellular cytokine staining splenocytes were stimulated for 4 hours at 37^0C with

50ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 500ng/ml ionomycin (Calbiochem). All cell incubations were performed in non-tissue culture plastic grade tubes to prevent adherence of cell populations during stimulation. 10μg/ml brefeldin A (Epicentre Technologies) was included for the last 2 hours of incubation to disaggregate the Golgi complex, thereby allowing intracellular cytokines to accumulate. Once stimulated, cells were harvested, washed in isoton (azide-free balanced electrolyte solution; Coulter Electronics Ltd., Luton, UK) and fixed in 2% formaldehyde in hypertonic PBS for 20 minutes at room temperature.

Fixed cells were then transferred to conical tubes and stained for intracellular cytokines. The cells were first permeabilised with 0.5% saponin diluted in PBS containing 1%BSA and 0.1% sodium azide (PBS/BSA/Azide) for 10 minutes at room temperature. Once permeabilised all subsequent washes and antibody dilutions were performed in this permeabilisation solution at room temperature unless otherwise stated. Cell suspensions were then treated with 5μg/ml of rat anti-IL-4 (11B11, IgG1) conjugated to FITC and 5μg/ml of PE-conjugated rat anti-mouse IFN-γ (AN18 PE) and incubated for 30 minutes at room temperature. After 2 washes, saponin was removed by washing with PBS/BSA/Azide to close permeabilised cells before the addition of the appropriate dilutions of either quantum red-conjugated rat anti-mouse CD4 (IgG2a, Sigma), rat anti-mouse CD8 (IgG2a, Sigma) or isotype matched control (to define background for FCM analysis). Conjugated anti-cytokine antibodies were a kind gift of Dr Anne O'Garra (DNAX). Except for cell sorting experiments all the data shown in this thesis is on cells found within the lymphocyte gate.

For sorting cells into myeloid and lymphoid populations, peripheral blood mononuclear cells

(PBMCs) from children's blood were first prepared by centrifugation for 20 minutes at 822g over 5ml Percoll (Pharmacia Biotech) or Ficoll Paque (Pharmacia Biotech) density gradients. Up to 5×10^6 cells were then stained (as above) with PE conjugated anti-CD13 (Dako Ltd). Cells were then sorted into myeloid and lymphoid population using the Coulter EPICS Elite flow cytometer/sorter according to size, granularity and CD13 staining.

Molecular Biology Techniques

RNA extraction

Two methods of RNA extraction were used to produce the results in this thesis 1) Proteinase K with phenol chloroform extraction and 2) QIAamp blood kit (QIAGEN). Several other methods were tested and used before these two methods were chosen including RNAzol B and RNeasy (QIAGEN). In chapter 3, RNA was extracted from nasopharyngeal aspirates in Southampton by members of Dr S.L. Johnston's laboratory using Tryzol (Gibco BRL). In my hands Proteinase K and phenol chloroform extractions, although time consuming, offered the best sensitivity when relatively cell free material was being tested, such as serum and CSF. However, Proteinase K and phenol chloroform was not adequate for blood and tissue specimens. The QIAamp blood kit method gave the best yield in cellular material such as tissue homogenates (up to 25mg of tissue or 10mg in the case of spleen) or whole blood. The QIAamp method was carried out according to manufacturer's instructions for blood. For tissue samples, 22-25mg (10mg for spleen) of the tissue was first quickly homogenised then made up to 200μL with PBS and snap frozen. Reagents recommended for blood extraction were then used to extract RNA. QIAGEN recommend a similar tissue extraction kit but in my hands the blood kit gave superior sensitivity.

Proteinase K / Phenol Chloroform RNA extraction (113)

To each 50µl sample the following reagents were added: 100μL Buffer (400mM tris HCl pH 7.5, 30mM EDTA, 600 mM NaCl), 210μL H_2O (RNAse and DNAse free), 40μL 10% SDS and 2μL Proteinase K (20mg/ml). The sample was incubated at 37°C for 40 minutes. Extraction with 400μL of phenol/chloroform was performed twice followed by re-extraction of the resulting supernatant with chloroform to remove any traces of phenol. 1μl of glycogen (20mg/ml) was added to the supernatant to act as a carrier and blocker and then 1.2ml of 100% ethanol. The mixture was placed at -70°C for 1 hour and centrifuged in a benchtop centrifuge at 12-14 000 rpm for 15 minutes to pellet the precipitated RNA. The pellet was washed once with 500ml 95% ethanol and again centrifuged to a pellet. After drying in air for 10 minutes the RNA was dissolved in 10μL of RNAse/DNAse free H_2O and store at -70°C until cDNA synthesis.

cDNA synthesis

10µl containing extracted RNA was mixed with an appropriate outer primer (e.g. 22k1 for viral vRNA or 22k2 for mRNA) in 10mM Hepes-HCl pH 6.9, 0.2mM EDTA with 4µM primer was heated to 90°C for 30 seconds then cooled to 4°C to anneal. 10µl of RT mix (reverse transcriptase) was then added containing 100mM Tris-HCl (pH 8.3), 150mM KCl, 6mM $MgCl_2$, 20mM DTT, 1mM dNTPs (each 1mM), 200U MMLV-RT (Life Technologies Ltd) and 40U RNAse inhibitor (RNAsin, Promega Corporation). The RT reaction was performed for 90 minutes at 37°C followed by 10 minutes at 70°C (enzyme denaturation). 1/10th of the resulting cDNA was used in the first round of PCR. Primer sequences are shown in figures 2.1 and 2.2.

Nested Polymerase Chain Reaction (PCR)

PCR reactions were carried out on the Perkin Elmer 9600 thermal cycler (Applied Biosystems / Roche). Unless larger volumes were required for a specific purpose, the PCR reactions were carried out in a 25µl volume. 2µl of cDNA was added to a solution containing 10mM Tris-HCl, 10mM KCl, pH 8.3, 2.5mM $MgCl_2$, 0.2mM each of dNTPs, approximately 2.75µM each of outer primers for N or M2 (N1 and N2 or 22k1 and 22k2) and 2.5U Stoffel fragment of Amplitaq (Applied Biosystems). After an initial 2 minute denaturation at 94°C, 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 30 seconds were performed. The reaction mixture for the second round was the same as the first except that inner primers (N3 and N4 or 22k3 and 22k4) were used. In the second round, 1µl of the first round mixture was transferred to the round two mixture and 30 cycles were carried out. Increasing the amount transferred or the number of cycles in the second round did not increase sensitivity but was associated with smearing of the lanes during visualisation on agarose gels which could sometimes obscure positive results. 10µl of the amplified product was analysed by running on a 2% agarose gel (1×TBE buffer). Bands were visualised by ethidium bromide staining and photographed using ultraviolet transillumination.

Sensitivity and specificity of PCR

Laboratory A2 strain RS virus was used as a positive control in all experiments. Virus, of known plaque forming units (pfu)/ml, was 10 fold serially diluted to 10pfu or 1pfu in 50µl human serum. Serum was chosen rather than PBS to try to approximate the nature of the materials from which RNA was to be extracted. 50µl aliquots were snap frozen and stored at -80°C until use. In RNA extractions from cell free material (such as CSF or serum) these

aliquots were used along with virus free serum as a negative control. In RNA extractions from cellular material, these 50µl aliquots were mixed with 150µl of negative tissue homogenates (final volume 200µl). One tenth of extracted RNA was used in cDNA synthesis and one tenth of that cDNA was used in PCR. 10pfu was therefore equivalent to 0.1pfu and 1pfu to 0.01pfu by the time of PCR. Throughout this thesis, controls referred to were derived in this way. Specificity was determined by cloning and sequencing positive isolates. All sequences were from RS virus.

Cloning of PCR products

(TA cloning kit, Invitrogen, De Schelp 26, NV Leek, The Netherlands)

3µl of PCR product was mixed with 6µl of H_2O. 1µl of this solution was mixed in a total volume of 10µl with ligation mix containing buffer, T4 ligase and the pCR vector (as supplied in kit). This solution was incubated overnight at 14°C. 10cm LB agar plates were prepared containing 50µg/ml ampicillin. TA cloning One Shot competent cells (E. Coli) were thawed on ice. 2µl of 0.5M β-mercaptoethanol was added to each aliquot of cells followed by 1µl of ligation mixture. The vials were incubated on ice for 30 minutes then transferred to a 42°C water bath for exactly 2 minutes. 450µl of prewarmed SOC medium (part of TA cloning kit) was then added to each vial of cells and the cells incubated for 1 hour in a rotary shaking incubator at 37°C for 1 hour. The LB agar plates were coated with 25µl of 40mg/ml Xgal in dimethylformamide and the cells plated out onto these plates. The plates were incubated at 37°C overnight. Colonies that contain the insert cloned DNA appear white (figure 2.3). Individual colonies were selected and grown overnight in 10ml LB medium containing 50µg/ml ampicillin at 37°C. After overnight growth the culture was

centrifuged at 822g for 20 minutes and the medium decanted. The DNA was then extracted from the pellet of cells using the Qiaprep Spin Plasmid Kit (QIAGEN). The concentration of extracted DNA was checked using a GeneQuant spectrophotometer (Pharmacia Biotech, St. Albans, UK).

Restriction Digest

DNA from purified clones was checked using restriction digest to make sure the correct sized insert was present. 5µl samples were incubated with 0.1µl EcoR1 (20000U/ml New England Biolabs, Hitchin, Herts, UK) and commercially supplied NEB buffer (50mM NaCl, 100mM Tris-HCl, 10mM $MgCl_2$, 0.025% Triton X-100, - New England Biolabs, Hitchin, Herts, UK) in a 20µl total volume at 37°C for 1 hour (see figure 5.1).

Sequencing of PCR products

7µl aliquots of DNA extracted from clones was sequenced using the USB Sequenase 2.0 kit (Amersham, Little Chalfont, Bucks, UK). All reactions were carried out according to the manufacturer's instruction with the following modifications:

Annealing

Annealing was performed by preparing a solution of 4µM of primer, 7µl of DNA and 2µl of sequenase 2.0 buffer (200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl) in a total volume of 10µl. This mixture was heated to 90°C for 5 minutes, cooled to 35°C over 5 minutes and then cooled to 4°C.

Labelling

The sequenase enzyme (13u/ml in 20mM KPO_4, 1mM DTT, 0.1mM EDTA, 50% glycerol) was prediluted by adding 25µl to 25µl pyrophosphatase (5u/ml in 10mM Tris-HCl, pH 7.5, 0.1mM EDTA, 50% glycerol) with 150µl of glycerol enzyme dilution buffer (20mM Tris-HCl, pH 7.5, 2mM DTT, 0.1mM EDTA, 50% glycerol). The labelling mixture contained 1µl of DTT (0.1M), 2µl of dGTP (7.5µM dGTP, 7.5µM dCTP , 7.5µM dTTP), 2µl of prediluted sequenase, 1µl of manganese buffer (0.15M Na Isocitrate, 0.1M $MnCl_2$) and 0.5µl of ^{35}S-dATP (10mCi/ml ICN Biochemicals Inc, Thame, Oxon, UK) in a total volume of 6.5µl. The labelling mixture was added to the annealing mixture for 10 minutes incubation on ice.

Termination

Termination tubes were prepared for ddATP, ddGTP, ddCTP and ddTTP (stock concentrations 80µM of each of the other dNTPs with 8µM of the relevant ddNTP in 50mM NaCl). During optimisation of this stage it was necessary to add extending mixture (180µM of each of the 4 dNTPs in 50mM NaCl) to ddTTP and ddATP tubes. This was in the ratio 1 ddNTP : 1.5 extending mix. 2.5µl of termination mix was added to each tube. 3.5µl of labelling mix was then added to each set of termination tubes. The termination reaction was performed for 5 minutes at room temperature before adding 4µl stop solution per tube. Sequenced DNA was stored at -20°C. All gels were run on a polyacrylamide gel within 3 days because of the manganese buffer (according to manufacturer's instructions).

Preparation and running of acrylamide gels

6% acrylamide gels were prepared from SequaGel solutions (National Diagnostics, Aylesbury, Bucks, UK). The gel was prepared using a glycerol tolerant buffer 1×TTE

(20×TTE contains 1.78M Tris Base, 0.57M Taurine). For 100ml of 6%, gel 80ml of Sequagel-6 (National Diagnostics) was mixed with 5ml 20×TTE and 15 ml H_2O. To initiate the polymerisation reaction 800µl of 10% ammonium persulphate (National Diagnostics) and 35µl of TEMED (National Diagnostics) were added. The gel was pre-run at 1500 volts for 45 minutes before loading samples. Samples were heated to 80°C for 5 minutes before loading onto the gel. The gel was run at 1500 volts until the first blue band was 1" from the bottom of the plate. The gel was fixed in methanol and acetic acid for 20 minutes, vacuum dried and exposed to a photographic film for up to 2 weeks. Control A2 R virus was used for comparison.

Primers were chosen according to the section to be sequenced. Primers used in sequencing were: 22k3, N3 or 22k primers referred to as Probes "A" and "B" complimentary to the middle of the amplified section:

Probe A	5'	GTA	TAG	ATA	CCT	TAT	CAG	AA	3'
Probe B	5'	TTC	TGA	TAA	GGT	ATC	TAT	AC	3'

Specific Buffers and reagents

ELISA wash buffer

PBS, pH 7.0, 0.05% Tween 20.

o-phenylenediamine (OPD) substrate solution

OPD was diluted to 0.034% in 25mM citric acid and 25mM di-sodium hydrogen phosphate., (pH 5.6). 0.03% H_2O_2 was added just before use.

Annealing Buffer

100mM Hepes-HCl (pH 6.9), 2mM EDTA.

1×TBE

9.68g/L Tris-HCl, 4.96g boric acid, 0.372g ethylenediamine-tetra-acetic acid (EDTA) was diluted in 1 litre of H_2O.

LB medium (1 litre)

10g bactotryptone (Difco Laboratories, W. Molesey, Surrey, UK), 5g bactoyeast (Difco Laboratories), 10g NaCl, was made up to 1 litre in H_2O. Sterility was maintained throughout by flaming spatulas with ethanol, using distilled water and immediately autoclaving the medium when it had been made up.

L-Agar

500ml LB medium with 7.5g agar (Difco Laboratories). Autoclaved to sterilise. 50μg/ml ampicillin added once less than 40°C but before setting. Poured into 10cm plates whilst cooling and bubbles flamed off using a Bunsen burner.

Peptides

Initial experiments were performed using peptide SYIGSINNI obtained from Genosys (Genosys Biotechnologies, Cambridge, UK). In experiments where peptide inhibition was investigated peptides SYIGSINNI, SYIGSINNN and TYQRTRALV were a kind gift of Dr Adrian Hill (Institute of Molecular Medicine, Oxford, UK). The method used to dissolve all peptides in these experiments was:

1mg added to 100µl of 50% acetic acid and resuspended until completely dissolved. 2.9ml of sterile 1M HEPES (pH checked to be between 7 and 7.5). Further dissolved in PBS to make a 10^{-4}M solution. Further dilutions made in RPMI.

Alum precipitation of proteins (This method was taken from the second edition of Practical Immunology by L. Hudson and F.C. Hay)

A known concentration of protein antigen in H_2O was made. For each 10ml of solution 4.5ml 1M $NaHCO_3$ was added. Then 10ml of 0.2M aluminium potassium sulphate was slowly added and the mixture left for 15 minutes. The mixture was centrifuged at 300g for 15 minutes, the precipitate washed 3 times with PBS and resuspended to the desired concentration in PBS.

AEC substrate

0.3ml of DMSO containing 1mg 3-amino 9-ethylcarbazole was mixed with 5ml 20mM sodium acetate pH 5.5 and 20µl of 30% H_2O_2.

Formaldehyde fixative in hypertonic saline

14ml of 10×PBS was mixed with 10ml of 40% formaldehyde and 76ml of H_2O. This makes up 2× concentrated stock which was mixed with cells to be fixed in an equal volume of PBS. After 20 minutes the fixative was washed out using PBS and the cells resuspended in PBS/BSA and azide.

5' **ATG TCA CGA AGG AAT CCT TGC** AAA TTT GAA ATT C**GA GGT CAT TGC**
Met Ser Arg Arg Asp Pro Cys Lys Phe Glu Ile Arg Gly His Cys

TTA AAT GGT AAG AGG TGT CAT TTT AGT CAT AAT TAT TTT GAA TGG CCA
Leu Asp Gly Lys Arg Cys His Phe Ser His Asp Tyr Phe Glu Trp Pro

CAT CCC GCA CTG CTT GTA AGA CAA AAC TTT ATG TTA AAC AGA ATA CTT
Pro His Ala Leu Leu Val Arg Gln Asp Phe Met Leu Asn Arg Iso Leu

AAG TCT ATG GAT AAA AGT ATA GAT ACC TTA TCA GAA ATA AGT GGA GCT
Lys Ser Met Asp Lys Ser Iso Asp Thr Leu Ser Glu Iso Ser Gly Ala

GCA GAG TTG GAC AGA ACA GAA GAG TAT GCT CTT GGT GTA GTT GGA GTG
Ala Glu Leu Asp Arg Thr Glu Glu Tyr Ala Leu Gly Val Val Gly Val

CTA GAG AGT TAT ATA GGA TCA ATA AAC AAT ATA ACT AA*A* *CAA* *TCA* *GCA*
Leu Glu Ser Tyr Iso Gly Ser Iso Asn Asn Iso Thr Lys Glu Ser Ala

TGT *GTT* *GCC* ATG AGC AAA CTC CTC ACT GAA CTC AAT AGT GAT GAT ATC
Cys Val Ala Met Ser Lys Leu Leu Thr Glu Leu Asn Ser Asp Asp Iso

AAA AA*G* *CTG* *AGG* *GAC* *AAT* *GAA* *GAG* *CTA* AAT TCA CCC AAG ATA AGA GTG
Lys Lys *Leu* *Arg* *Asp* *Asn* *Glu* *Glu* *Leu* Asn Ser Pro Lys Iso Arg Val

TAC AAT ACT G **3'**
Tyr Asn Thr

Base Order as cDNA
Derived Amino Acid

Primers used for M2 RT-PCR:

Primer 1	5'	ATG	TCA	CGA	AGG	AAT	CCT	TGC	3'
Compliments	5'	GCT	AGG	ATT	CCT	TCG	TGA	CAT	3'
Primer 2	5'	TAG	CTC	TTC	ATT	GTC	CCT	CAG C	3'
Compliments	5'	G	CTG	AGG	GAC	AAT	GAA	GAG CTA	3'
Primer 3	5'	GA	GGT	CAT	TGC	TTA	AAT	GG	3'
Compliments	5'	CC	ATT	TAA	GCA	ATG	ACC	TC	3'
Primer 4	5'	GC	AAC	ACA	TGC	TGA	TTG	T	3'
Complments	5'	A	CAA	TCA	GCA	TGT	GTT	GC	3'

Figure 2.1 Sequence of M2 (22K) used for RT-PCR derived from A2 strain RS virus. The underlined bases are either the sequences of second strand primers (bold) or the sequences complimentary to first strand primers (italic). From 5' to 3' the primers are 22k1, 22k3, 22k4 (complimentary sequence) and 22k2 (complimentary sequence). The virus RNA is naturally in an antisense (negative sense) form and therefore effectively transcribed from 3' to 5' in vivo. Primers 22k4 and 22k2 are in the sense form, 22k1 and 22k3 are antisense. The sequence is shown from position 7550 to position 7882.

5' AA**G** **ATG** **GCT** **CTT** **AGC** **AAA** **GTC** AAG TTG AAT GAT ACA CTC AAC AAA GAT
Met Ala Leu Ser Lys Val Lys Leu Asn Asp Thr Leu Asn Lys Asp

CAA CTT **CTG** **TCA** **TCC** **AGC** **AAA** **TAC** **AC**C ATC CAA CGG AGC ACA GGA GAT AGT
Gln Leu Leu Ser Ser Ser Lys Tyr Thr Ile Gln Arg Ser Thr Gly Asp Ser

ATT GAT ACT CCT AAT TAT GAT GTG CAG AAA CAC ATC AAT AAG TTA TGT GGC
Asn Asp Thr Pro Asn Tyr Asp Val Gln Lys His Ile Asn Lys Leu Cys Gly

ATG TTA TTA ATC ACA GAA GAT GCT AAT CAT AAA TTC ACT GGG TTA ATA GGT
Met Leu Leu Ile Thr Glu Asp Ala Asn His Lys Phe Thr Gly Leu Ile Gly

ATG TTA TAT GCG ATG TCT AGG TTA GGA AGA GAA GAC ACC ATA AAA ATA CTC
Met Leu Tyr Ala Met Ser Arg Leu Gly Arg Glu Asp Thr Ile Lys Ile Leu

AGA GAT GCG GGA TAT CAT GTA AAA GCA AAT GGA GTA GAT GTA ACA ACA CAT
Arg Asp Ala Gly Tyr His Val Lys Ala Asn Gly Val Asp Val Thr Thr His

CGT CAA GAC ATT AAT GGA AAA GAA ATG AAA TTT GAA GTG TTA ACA TTG GCA
Arg Gln Asp Ile Asn Gly Lys Glu Met Lys Phe Glu Val Leu Thr Leu Ala

AGC TTA ACA ACT GAA ATT CAA ATC AAC ATT GA*G* *ATA* *GAA* *TCT* *AGA* *AAA* *TCC*
Ser Leu Thr Thr Glu Ile Gln Ile Asn Ile Glu Ile Glu Ser Arg Lys Ser

TAC AAA AAA ATG CTA AAA GAA ATG GGA GAG GTA G*CT* *CCA* *GAA* *TAC* *AGG* *CAT*
Tyr Lys Lys Met Leu Lys Glu Met Gly Glu Val Ala Pro Glu Tyr Arg His

*G*AC TCT CCT GAT **3'**
Asp Ser Pro Asp

Base Order as cDNA
Derived Amino Acid

Primers used for N RT-PCR:

Primer 1	5'	G	ATG	GCT	CTT	AGC	AAA	GTC	3'
Compliments	5'	GAC	TTT	GCT	AAG	AGC	CAT	C	3'
Primer 2	5'	C	ATG	CCT	GTA	TTC	TGG	AG	3'
Compliments	5'	CT	CCA	GAA	TAC	AGG	CAT	G	3'
Primer 3	5'	CTG	TCA	TCC	AGC	AAA	TAC	AC	3'
Compliments	5'	GT	GTA	TTT	GCT	GGA	TGA	CAG	3'
Primer 4	5'	GTA	GGA	TTT	TCT	AGA	TTC	TAT C	3'
Complments	5'	G	ATA	GAA	TCT	AGA	AAA	TCC TAC	3'

Figure 2.2 Sequence of N used for RT-PCR derived from A2 strain RS virus. The underlined bases are either the sequences of second strand primers (bold) or the sequences complimentary to first strand primers (italic). From 5' to 3' the primers are N1, N3, N4 (complimentary sequence) and N2 (complimentary sequence). The virus RNA is naturally in an antisense (negative sense) form and therefore effectively transcribed from 3' to 5' in vivo. Primers N4 and N2 are in the sense form, N1 and N3 are antisense. The sequence is

shown from position 1069 to position 1558.

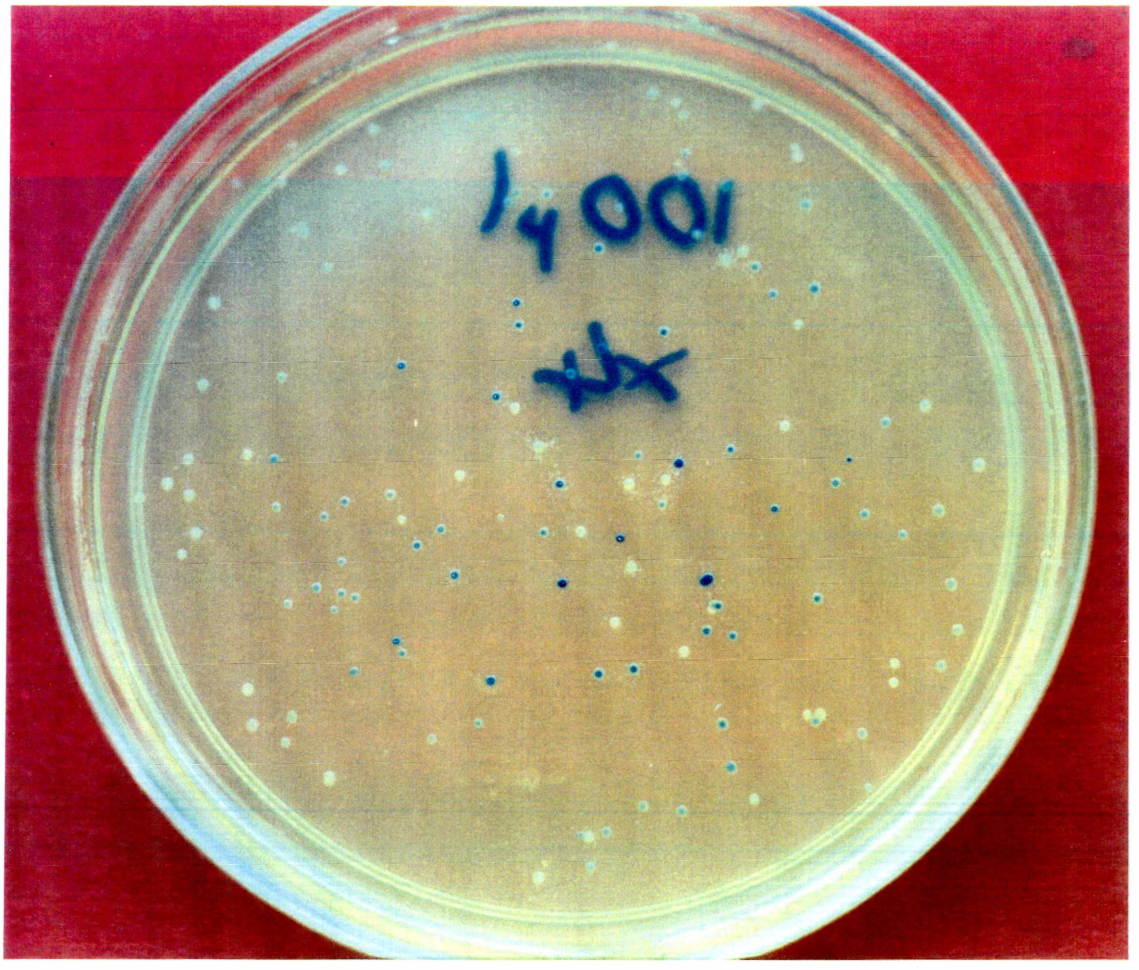


Figure 2.3 Appearance of colonies of E. Coli during cloning of PCR product. The plasmid (pCR II vector) is ligated to PCR product through the 3' A overhang generated as an artefact during PCR. After a ligation reaction has been performed E. Coli are transformed with the plasmid. The position of the insert disrupts the LacZα gene if ligation has been successful and prevents the blue colour generation that normally occurs. Therefore colonies that are white are selected for further expansion and miniprepping.

Chapter 3 RT-PCR studies of human specimens.

Hypothesis

RT-PCR has proved a very sensitive detection method for other RNA viruses. PCR can offer greater sensitivity than conventional methods of virus detection. By using PCR, the importance of viruses in a range of clinical settings can now be established where previously they were below the threshold of detection. Measles virus, a paramyxovirus closely related to RS virus, infects monocytes rather than lymphocytes. Domurat *et al* demonstrated that human monocytes could be infected *in vitro* with RS virus and showed viral antigen on the surface of circulating mononuclear cells in the blood of some children during infection. In children with HIV, RS virus is a frequent cause of serious respiratory illness (137) and is also important in adults with other immunodeficiencies (85). However the frequency of RS virus infection causing lower respiratory infection in adults with HIV has not been determined. In large community based studies in children, respiratory viruses are associated with the majority of acute exacerbations of asthma. Young infants with RSV often present with a clinical picture suggesting encephalopathy. Central nervous system infection is seen in other paramyxovirus infections.

To date, the location of the virus during and after primary RSV infection in children has not been addressed. In the following chapter a sensitive RT-PCR method for detecting viral RNA is developed specifically for use with clinical specimens. If RS virus enters a viraemic phase this method should be the most sensitive. In the following chapter, this technique is applied to blood, serum, circulating white blood cell including mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) from infants during primary infection. RT-PCR is also used to determine the frequency of RS virus in the bronchoalveolar lavage of adults with HIV undergoing investigation for respiratory illnesses. Finally the PCR technique was used to discover the frequency of RS virus in a series of children admitted with respiratory illnesses.

Chapter 3 RT-PCR studies of human specimens

Introduction

Initial infection with RS virus is by invasion of the mucosal epithelium of the conjunctiva or nose (132). The mechanism of spread to the lower respiratory tract is not clear. The virus is capable of cell to cell spread without appearing in extracellular fluid (330)and it has been assumed that virus spreads via the tracheal epithelium or through aspirated secretions (58). In animal studies (22,306) tracheal epithelium is only patchily affected so it seems less likely that this is the route *in vivo*. In clinical laboratories, detection of RS virus relies on immunofluorescence and virus culture. Immunfluorescence, using commercially available labelled monoclonal antibodies has been established to be an effective technique giving results in about 1 hour (64). Antigen detection may even be more sensitive than *in situ* hybridisation for viral RNA (63).

RT-PCR has proved a very sensitive detection method for other RNA viruses, including measles virus and hepatitis C virus (86,112). Johnston *et al* used RT-PCR for a rhinoviruses alongside conventional virus detection methods for a range of respiratory pathogens (190). In a large community based study in children, they showed that respiratory viruses were associated with the vast majority of acute exacerbations of asthma. PCR can offer greater sensitivity than conventional methods of virus detection. By using PCR, the importance of viruses in a range of clinical settings can now be established where previously they were below the threshold for detection.

Measles virus, a paramyxovirus closely related to RS virus, infects circulating leucocytes.

In 1937 it was demonstrated that transfer of leucocytes from an infected individual to a naïve individual could lead to the development of measles (280). More recently, RT-PCR has been used to determine the duration and frequency of peripheral mononuclear cells infected with measles. It has been found that monocytes rather than lymphocytes are usually infected. The frequency of infected cells is typically between 1 in 250 to 1 in 2500 by *in situ* hybridisation (86). Measles virus initially infects respiratory epithelium, seeds to draining lymph nodes and leads to viraemia by which several other mucosal sites become infected including the gastrointestinal tract, conjunctivae and skin. The rash associated with measles is associated with the peak of a secondary viraemia (86). Domurat *et al* demonstrated that human monocytes could be infected *in vitro* with RS virus and demonstrated viral antigen on the surface of circulating mononuclear cells in the blood of some children during infection (73). He suggested that viraemia may be a part of infections with RS virus.

RS virus reinfection occurs despite serum good levels of circulating antibody but usually reinfections do not cause severe disease. However, in immunocompromised individuals reinfection can be associated with serious morbidity and mortality (58,137,155). RS virus is shed for long periods, especially in those with defects in cell mediated immunity. Respiratory illnesses are a common presentation of the acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (243), most commonly pneumocystis carinii pneumonia (261). In children with HIV, RS virus is also a frequent cause of serious respiratory illness (137) and has been found to be important in adults with other immunodeficiencies (85). However the frequency of RS virus infection causing lower respiratory infection in adults with HIV has not been determined.

In this chapter, a nested RT-PCR was developed to detect RS virus in range of cell free and cellular tissues. In the first set of experiments this technique was applied to blood, serum, circulating white blood cell including mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) from infants during primary infection. In the second series of experiments, RT-PCR was used to determine the frequency of RS virus in the bronchoalveolar lavage of adults with HIV undergoing investigation for respiratory illnesses. Finally the PCR technique was used to discover the frequency of RS virus in a series of children admitted with respiratory illnesses.

Results:

a) RS virus genome can be detected in children's blood and PBMCs but not serum or cerebrospinal fluid (CSF) during primary infection.

Design: Children admitted to St. Mary's Hospital, Paddington, London during the winter RS virus epidemics in 1993/4 and 1994/5 were included in this study. Prospective ethical approval was sought from the Riverside ethics approval committee granted (EC2366 and EC2874). Children were included in the study if they were admitted during the winter months with a presentation consistent with RS virus infection. These were:

1. Acute bronchiolitis (defined as: a respiratory illness in a child of less than 18 months of age with signs of crackles and/or wheezes with a raised respiratory rate or evidence of hyperinflation on chest X ray).
2. Apnoeas.
3. Acute collapse (without another obvious cause, such as trauma)
4. Acute respiratory failure.

Up to 3ml of blood and approximately 100µl of CSF was taken at the same time as blood or CSF was taken for clinically indicated tests. In each case a parent or guardian had given signed consent. A full verbal explanation of the experiment was given. In addition the parents received a written summary. In total 34 children's blood samples were included in the study. In most cases blood was taken on day 1 (median day 1, range 1-4), usually within hours of admission. In addition 14 samples of CSF from 11 children were tested. St Mary's offers a paediatric intensive care facility and children are usually referred from other

hospitals. Therefore in many cases CSF was taken and stored as part of clinical investigation at the referring hospital. In these cases, after written consent was obtained the CSF was collected from the referring hospital and transported on dry ice.

2-3ml of blood was taken from each child and put into clinical bottles with EDTA anticoagulant (Becton Dickinson). In the first winter (1993-1994) 12 samples of blood were collected. In the second winter (1994-1995) 22 further samples were collected. The blood was separated into plasma and blood pellet by centrifugation at 4000rpm (1100xg) for 5 minutes in a benchtop centrifuge and frozen at -80 C until testing.

Density gradient separation

After the first season in addition to saving plasma and blood pellets the circulating leucocytes were isolated by density gradient separation. Initial experiments were performed to determine an optimal density for the gradient using Percoll (Pharmacia Biotech). A density of 1.086g/ml was found to give maximum leucocyte yield with a minimal red blood cell contamination using adult human blood. 6 children's blood specimens were separated by this method. Unfortunately, with the samples collected from sick infants red blood cell contamination was far higher and varied considerably from child to child. This variation would be expected to interfere with the planned flow cytometric sorting, so from that point Ficoll Paque (Pharmacia Biotech, density 1.077g/ml) was used. Blood was layered gently onto 5ml volumes of Percoll or Ficoll Paque and centrifuged at 822g for 20 minutes at room temperature. The cells removed from the interface were then washed once with 5ml of PBS counted and 200µl aliquots with 10^6 cells snap frozen until RNA extraction. The remaining cells were then sorted into myeloid and lymphoid populations by flow cytometry (EPICS

Elite cytometer, Coulter). Cell sorting was performed straight after density gradient separation which was begun immediately after blood was taken. In total each sample took between 3 and 5 hours to process.

Cell Sorting

Up to 5×10^6 cells, depending on total yield, were stained on ice with PE conjugated anti-CD13 at a final concentration of 20μg/ml (mouse anti-human CD13 Clone WM-47 IgG1 Dako Ltd, High Wycombe, Bucks, UK) for 20 minutes, washed with 10ml of PBS and fixed with 2% formaldehyde in hypertonic PBS for 20 minutes at room temperature. The cells were then sorted into myeloid and lymphoid populations by size and CD13 staining. The myeloid population was large and CD13 positive whereas the lymphoid population was smaller and CD13 negative. Purity was checked by flow cytometry and the purified populations were counted. 10^5 cells from each population was snap frozen in 200μl of PBS where possible. In some cases it was only possible to collect 10^4 cells or 5×10^4 cells in total especially from the myeloid population. The cells were stored at -80°C until RNA extraction.

RT-PCR

RNA extraction was performed using the Qiagen blood kit for blood, plasma and PBMCs. Proteinase K - phenol chloroform extraction was used for plasma and CSF. For blood pellets, PBMCs and serum cDNA synthesis was performed using positive sense (complimentary to cellular viral RNA) and negative sense oligonucleotide (complimentary to cellular mRNA transcripts of viral RNA) using primers both for M2 and N. For RNA extracted from CSF cDNA synthesis was performed using a positive sense oligonucleotide

(22k1). Nested PCR was performed using outer and inner primer pairs for either M2 or N. Positive PCR product was identified by visualisation on a 2% agarose gel. Aliquots of positive PCR product were used for ligation into the pCRII plasmid (TA Cloning Kit, Invitrogen). Super competent E. Coli (One Shot, TA Cloning Kit, Invitrogen) were transformed using ligated pCRII plasmids. Colonies of E. Coli showing disruption of the LacZα gene were selected and expanded in medium. DNA was extracted by miniprep and confirmation of the insert made by restriction digest and visualisation on a 2% agarose gel (figure 5.1). The cloned DNA was sequenced using the Sequenase 2 chain terminator method. Sequenced DNA was run on a 6% polyacrylamide gel.

Results: No child was positive for RS virus by PCR of serum (n=34). 4 children out of 34 were positive for RS virus by PCR of blood cells (see table below and figure 3.5). 3 out of the 4 children were positive for N and M2 genome by PCR. 2 of the positive children were from the first winter series (ZQ and LM). 2 of the children were from the second winter series. In the second winter series one child tested positive in blood pellet and PBMCs (DW). Unfortunately the blood pellet of the second child was discarded in error and only PBMCs could be tested (TO). 3 of the 4 children positive by PCR of blood cells were positive for RS virus by NPA (ZQ, LM, DW). One child TO, was negative by NPA but positive by PCR, frozen NPA material was requested from the clinical virology department to try to recheck it using RT-PCR. Unfortunately it could not be located. The positive samples were cloned and 3 clones sequenced (figure 3.6).

Table 3.1 PCR results using different first strand primers in cDNA synthesis:

	Primer used in cDNA synthesis			
Child	22k1	22k2	N1	N2
LM	positive	positive	positive	positive
ZQ	positive	positive	negative	negative
DW	negative	positive	not tested	positive
TO	positive	positive	not tested	positive

primers N1 and 22k1 are complimentary to viral negative sense RNA (vRNA)
primers N2 and 22k2 are complimentary to positive sense mRNA transcripts of vRNA.

CSF PCR

11 children with a mean age of 6 weeks (median 4.5 weeks) had CSF taken as part of this study. The usual presentation was with apnoeas or acute collapse for which there was no other obvious cause. One of these 11 children was positive by PCR of blood (LM). 7 of these 11 children were positive for RS virus by NPA. No child was positive for RS virus by PCR of CSF.

PCR of myeloid and lymphoid cells

14 samples of PBMCs were sorted into myeloid and lymphoid cells. Samples purities were checked by flow cytometry and were between 95% and 99.5% (figure 3.3 and 3.4). No positives were found in sorted cells from any child. 1 child was positive for RS virus by PCR of PBMCs (DW) but was not positive by PCR of 5×10^4 myeloid or 5×10^4 lymphoid cells.

b) RS virus detection by RT-PCR in BAL from adults with HIV.

Design: 80 adults seropositive for HIV were studied. All the patients had a diagnosis of

HIV-1 and underwent investigation for respiratory symptoms or abnormal chest radiograph between 1992 and 1994. At routine fibreoptic lavage was performed (250ml warmed sterile saline into right middle lobe) and 50ml aliquots centrifuged to obtain a cell pellet and supernatant. Specimens were stored at -20°C. RNA was extracted using the QIAamp blood kit (QIAGEN). RNA was eluted in water and 1/10th used in cDNA synthesis using a negative sense oligonucleotide (22k2, complimentary to cellular mRNA transcripts of viral RNA). Positive PCR product was identified by visualisation on a 2% agarose gel. Aliquots of positive PCR product were cloned (as above) and 3 clones sequenced using the Sequenase 2 chain terminator method as above.

Results: Of 80 patients tested 3 were positive for RSV mRNA transcripts. In each of three cases no other pathogen was isolated. Sequences derived from the positive subjects came from both A and B strains of RSV when compared to the published sequence and in all cases showed variation from controls and each other (figure 3.7). In each of the cases associated with RSV wheeze and cough were noted as a prominent presenting symptoms. The three positive cases were all male (ages 32 years, 44 years and 55 years). One of the three was noted to have sinusitis during the period of lower respiratory tract illness. In addition to wheeze and cough this individual also had marked shortness of breath and decreased peak expiratory flow reversible by beta agonist nebulisation. No other bacterial or viral pathogen was identified in any of the three BALs that were positive for RS virus.

c) RS virus by RT-PCR in NPA from children admitted with respiratory illnesses.

Design: RNA was extracted from NPA material from 80 children. The children were consecutive admissions for lower respiratory illnesses to the paediatric wards of

Southampton General Hospital from 27th August 1992 to 17th February 1993. RNA was extracted by Dr Ping Xie using Tryzol (Gibco BRL) in Dr Seb Johnston's laboratory in Southampton coded and sent to St Mary's on dry ice. 5µl aliquots of RNA were used in cDNA synthesis (using the method above) using a negative sense oligonucleotide (22k2, complimentary to cellular mRNA transcripts of viral RNA). 2µl of the 20µl cDNA was used in PCR. Positive PCR product was identified by visualisation on a 2% agarose gel. Positive samples were rechecked using a second RNA aliquot. Positive samples were subjected to Southern blotting using internal probe for A2 and a representative sample was cloned and sequenced (as above).

Results: 23 of the 80 children were positive for RS virus by RT-PCR of NPA and 57 were negative (figure 3.8). Southern blotting demonstrated control (A2) samples but the internal probe failed to detect any of the child samples. Sequencing of a representative isolate demonstrated that it was closer to the published B strain of RS virus and there were 4 sequence changes within the region of the probe. The mean age of the children was 2.4 years (median = 1.2 years, sem = 0.322 years, range 0-14.2 years). Of those positive for RS virus by PCR the mean age was significantly lower at 0.4 years (median 0.3 years, sem = 0.06 years, range 0-1.17 years). No difference was seen in the percent of children with wheeze or pneumonia between children positive or negative for RS virus by PCR. PCR detected 5 cases that were negative for RS virus by conventional methods (determined by routine clinical virology).

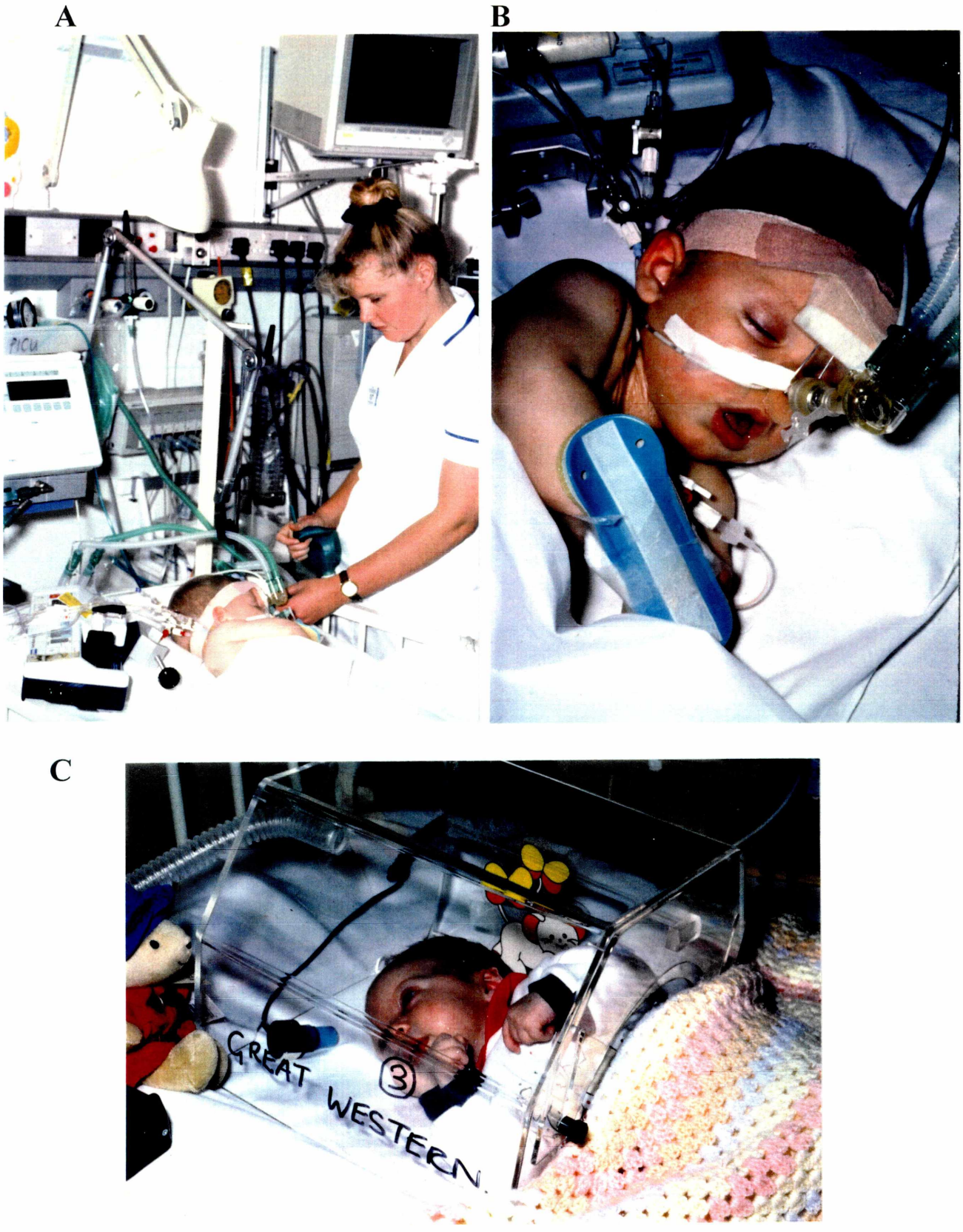


Figure 3.1 Children admitted with illnesses consistent with RS virus infection. Children receiving treatment for bronchiolitis, croup, apnoeas or acute respiratory failure were included in the study. A) a child in severe respiratory failure requiring positive pressure ventilation. B) The same child (close up). C) A child receiving headbox oxygen for respiratory failure. Nested RT-PCR was used to determine if RS virus was present in serum, blood cells and CSF.

	NPA Positive (n=24)	NPA Negative (n=10)	
	n (%)	n (%)	p
Male	14 (58)	6 (60)	ns
Premature (≤36weeks)	9 (38)	2 (20)	ns
Neonatal Ventilation	6 (25)	1 (10)	ns
Previous Disease (respiratory or cardiac)	7 (29)	4 (40)	ns
Age (median, range)	13 weeks (3-60)	8 weeks (2.5-43)	ns
Ventilated during admission (other than oxygen alone)	15 (63)	6 (60)	ns
Apnoeas	8 (33)	3 (30)	ns
Duration of illness before admission (median, range)	2 days (0-8)	2 days (1-10)	ns
PCR positive in blood	3 (12.5)	1 (10)	ns

Figure 3.2 Summary of clinical information from children's blood experiment.
Blood was taken from children during primary RS virus infection. RNA was extracted and RT-PCR performed. Clinical information was compiled and comparison made between the children that were positive or negative by routine clinical virology (immunofluoresence and / or virus culture for RS virus).

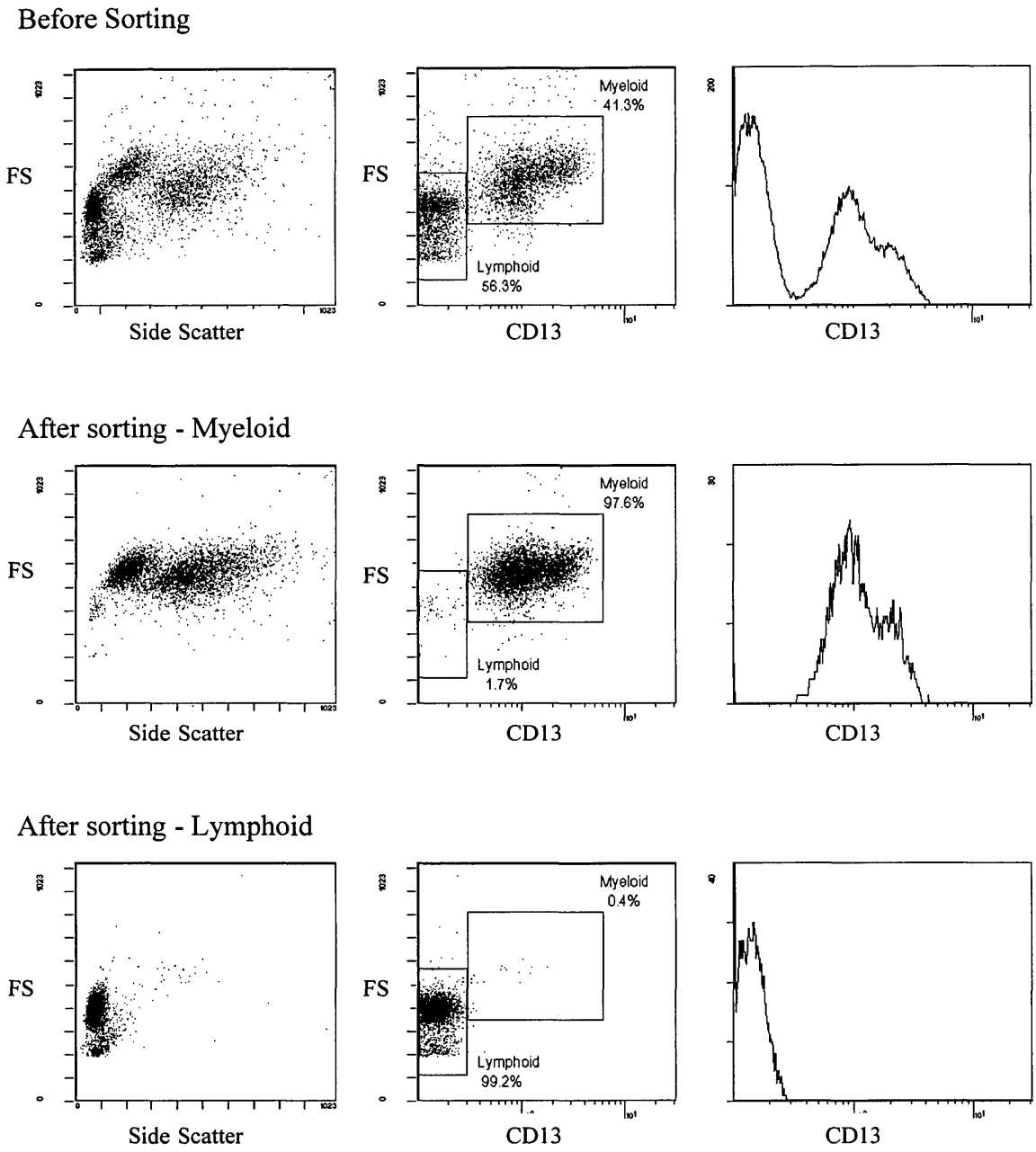


Figure 3.3 Flow cytometric sorting of white blood cells after Percoll separation
Peripheral blood was taken from 14 children admitted with a diagnosis consistent with RS virus infection. 2ml of blood from 6 (of the 14) children was separated by Percoll (density 1.086g/ml). Cells were sorted into myeloid and lymphoid by size and CD13 staining.

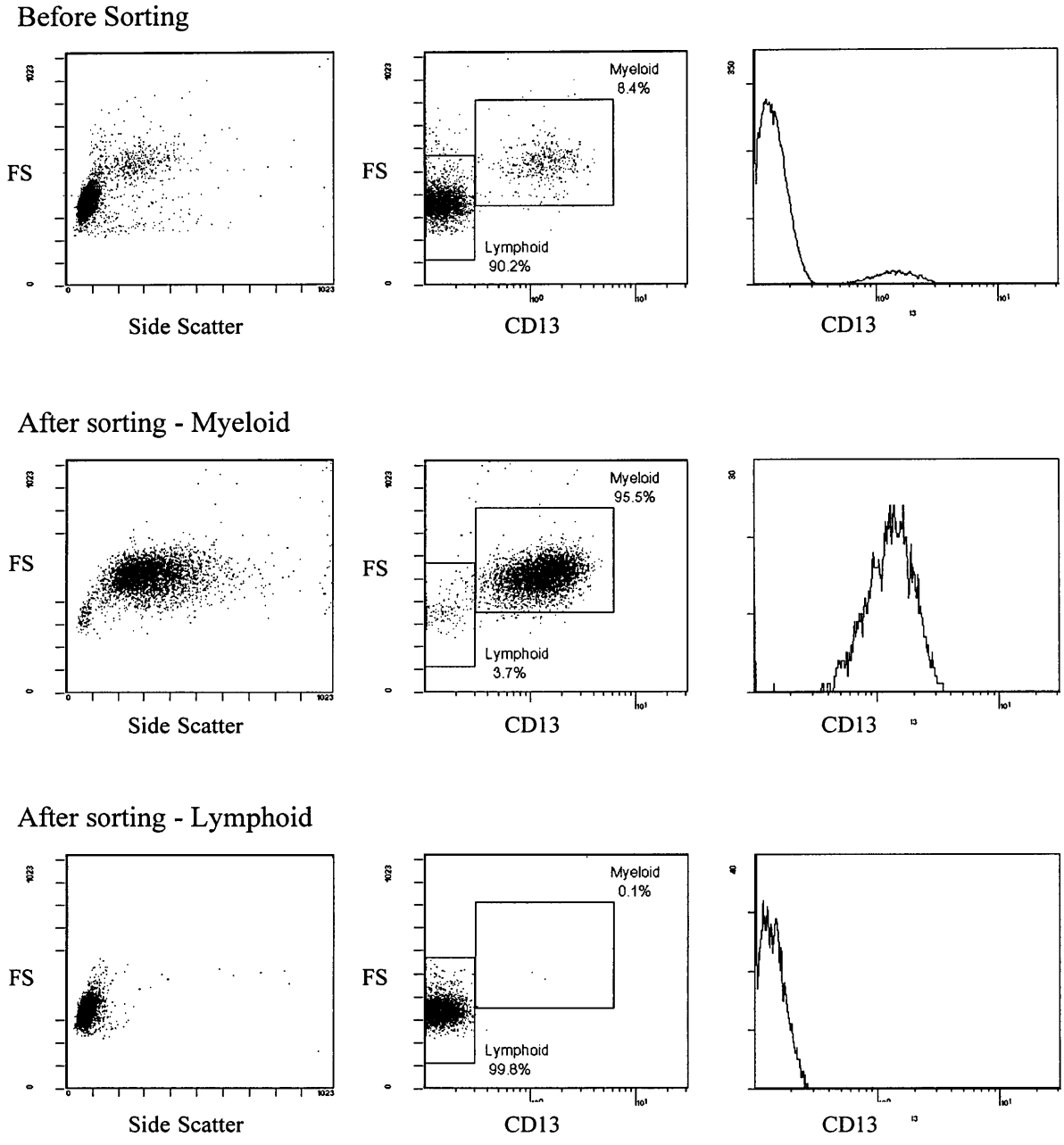


Figure 3.4 Flow cytometric sorting of white blood cells after Ficoll-Paque separation Peripheral blood was taken from 14 children admitted with a diagnosis consistent with RS virus infection. 2ml of blood from 8 (of the 14) children was separated by Ficoll Paque (density 1.077g/ml). Cells were sorted into myeloid and lymphoid by size and CD13 staining.

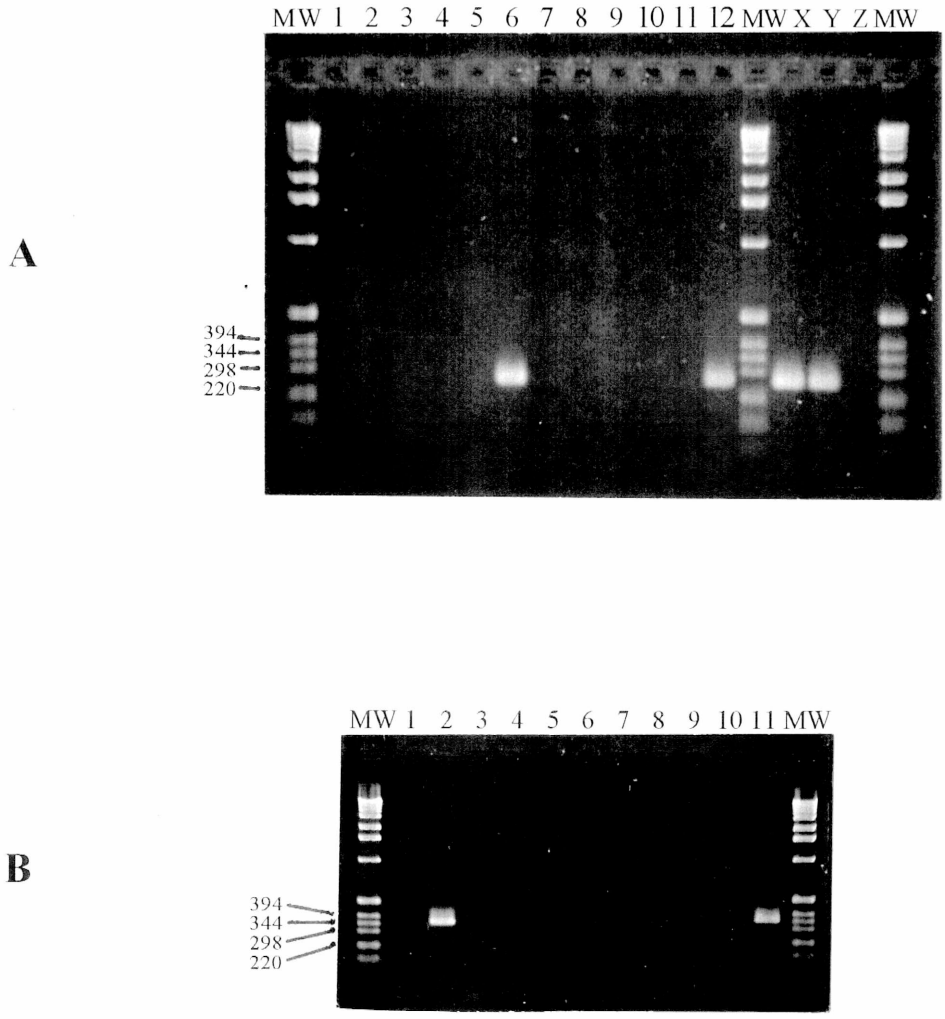


Figure 3.5 Nested RT-PCR for RS virus in children's blood and PBMCs.
Children admitted with an illness consistent with RS virus infection were bled. RNA was extracted and RT-PCR was performed using primers for M2 or N.
In group (A) the result with M2 primers is shown, RNA was extracted from blood pellets, 2 bands are visible (lanes 6 and 12).
In group (B) the result with primers for N is shown, in this case RNA was extracted from PBMCs, two 2 bands are visible (lanes 2 and 11).
Controls: X) positive equivalent to 0.01pfu RS virus and Y) positive equivalent to 0.001pfu RS virus Z) negative.
Using 1KB markers, PCR amplified products for M2 lie between 220 and 298bp consistent with an expected size of 260bp, and for N between 344 and 394bp consistent with an expected size of 351bp.

B Strain	5'	- - C	- - -	- - C	- - -	- - C	- - T	- - G	- - G	- - -	- T -	- - -	- - -		
A2 Strain	5'	GAT	AAA	AGT	ATA	GAT	ACC	TTA	TCA	GAA	AGA	AGT	GGA		
DW	5'	- - -	- - -	- - **C**	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		
TO	5'	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		
LM	5'	- - -	- - -	- - **C**	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		
ZQ	5'	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		

B Strain	- - -	- - T	- - A	C - -	- - T	- - -	- - -	- - -	- - A	- - -	- - -	- - -	- - A		
A2 Strain	GCT	GCA	GAG	TTG	GAC	AGA	ACA	GAA	GAG	TAT	GCT	CTT	GGT		
DW	- - -	- - -	- - -	- - -	- - -	- - -	- - **T**	- - -	- - -	- - -	- - **C**	- - **C**	- - -		
TO	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		
LM	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - **C**	- - **C**	- - -		
ZQ	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		

B Strain	A - -	- - -	- - -	- - -	- - -	- - -	- - -	- - C	- - -	- - -	- - -	- - -	- - -		
A2 Strain	GTA	GTT	GGA	GTG	CTA	GAG	AGT	TAT	ATA	GGA	TCA	ATA	AAC		
DW	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - **T**		
TO	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		
LM	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - **T**		
ZQ	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		

B Strain	- - C	- - -	- - A	- - -	- - -	- - -	- - -	
A2 Strain	AAT	ATA	ACT	AAA	CAA	TCA	GCA	3'
DW	- - -	- - -	- - -	- - -	- - -	- - -	- - -	3'
TO	- - -	- - -	- - -	- - -	- - -	- - -	- - -	3'
LM	- - -	- - -	- - -	- - -	- - -	- - -	- - -	3'
ZQ	- - -	- - -	- - -	- - -	- - -	- - -	- - -	3'

Figure 3.6 Sequences of positive PCR isolates from children's blood during acute infection. Nested RT-PCR for M2 of RS virus was performed on RNA extracted from 200µl aliquots of blood (pellets) or 10^6 PBMCs. 4 samples that were positive by PCR were cloned and sequenced. The sequences are shown above in comparison with A2 and B strains.

A

B Strain	T - A	- - A	- - G	- - G	- - -	- - -	- - C	- - -	- - -	- - -	- AG	- - -	- - -	
A2 Strain	CTG	CTT	GTA	AGA	CAA	AAC	TTT	ATG	TTA	AAC	AGA	ATA	CTT	
Patient A	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	
Patient B	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	
Patient C	- - -	- - -	- - -	- - -	- - -	- - -	- - G	- - -	- - -	- - -	- AG	- - -	- - C	

B Strain	- - -	- - A	- - -	- - C	- - -	- - C	- - -	- - C	- - T	- - G	- - G	- - -	- T -	
A2 Strain	AAG	TCT	ATG	GAT	AAA	AGT	ATA	GAT	ACC	TTA	TCA	GAA	AGA	
Patient A	- - -	- - -	- - -	- - -	- - -	- - C	- - -	- - -	- - T	- - -	- - -	- - -	- - -	
Patient B	- - -	- - -	- - -	- - -	- - -	- - C	- - C	- - -	- - T	- - -	- - -	- - -	- - -	
Patient C	- - -	- - A	- - -	- - C	- - -	- - C	- - -	- - C	- - T	- - -	- - -	- - -	- - -	

B Strain	- - -	- - -	- - -	- - T	- - A	C - -	- - T	- - -	- - -	- - -	- - A	- - -	- - -	
A2 Strain	AGT	GGA	GCT	GCA	GAG	TTG	GAC	AGA	ACA	GAA	GAG	TAT	GCT	
Patient A	- - -	- - -	- - -	- - -	- - -	C - -	- - -	- - -	- - T	- - -	- - -	- - -	- - C	
Patient B	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - C	
Patient C	- - -	- - -	- - -	- - -	- - -	C - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	

B Strain	- - -	- - A	A - -	- - -	- - -	- - -	- - -	- - -	- - -	- - C	- - -	- - -	- - 3'	
A2 Strain	CTT	GGT	GTA	GTT	GGA	GTG	CTA	GAG	AGT	TAT	ATA	GGA	TC3'	
Patient A	- - C	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - 3'	
Patient B	- - C	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - 3'	
Patient C	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - 3'	

B

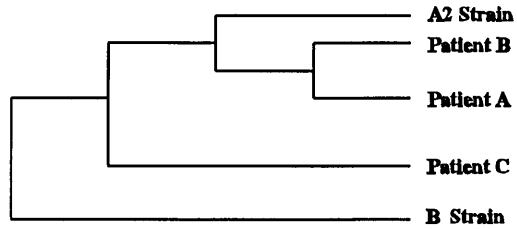


Figure 3.7 Sequences and relatedness of RS virus found in BAL from HIV positive adults. 80 adults with a known diagnosis of HIV had bronchoalveolar lavage (BAL) performed as part of investigation for unexplained respiratory illness. Nested RT-PCR for M2 of RS virus was performed on RNA extracted from 200µl aliquots of BAL. 3 samples that were positive by PCR were cloned and sequenced. The sequences are shown above (A). Relatedness of the sequences was determined using the Molecular Evolutionary Genetic Analysis v1.0 software (B). The length of the horizontal lines reflects sequence differences.

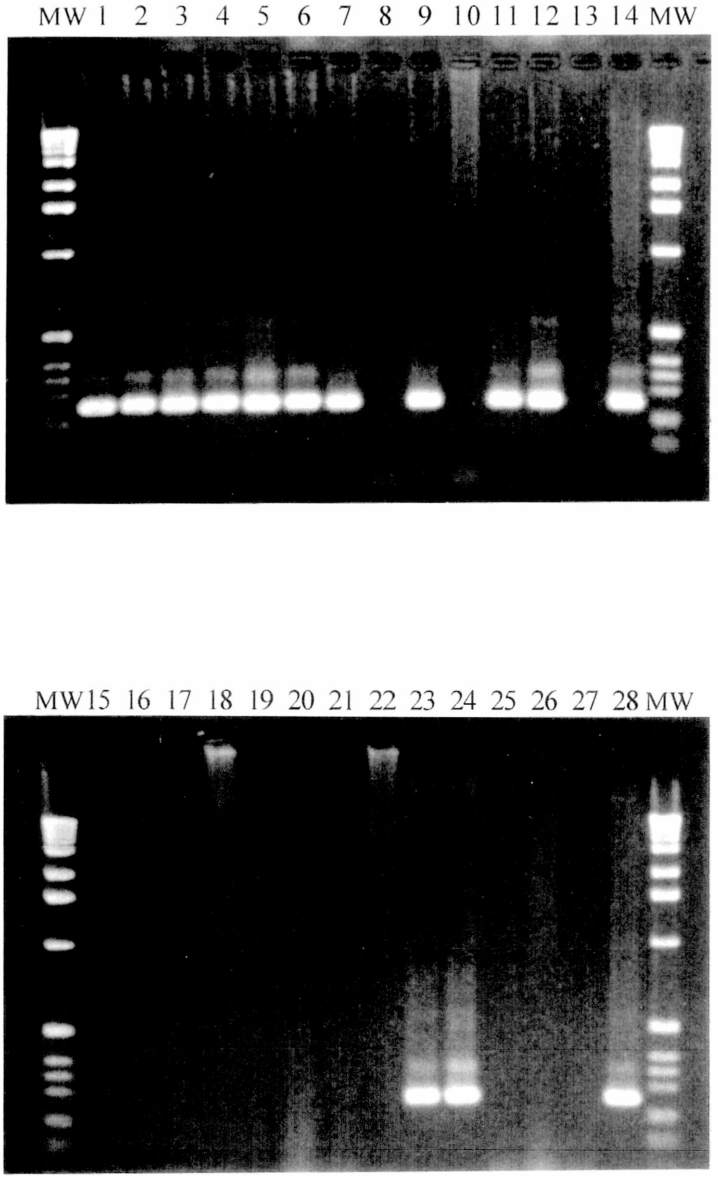


Figure 3.8 Nested RT-PCR for RS virus in NPA from children.
80 children admitted consecutively to hospital with illnesses consistent with lower respiratory tract infection had nasopharyngeal aspirates taken. RNA was extracted and RT-PCR was performed using primers for M2. The results from 28 consecutive children in the series are shown above.

	Conventional Detection Method			
	Immunofluoresence	**Virus culture**	**Negative**	Total
PCR positive	18	16	5	23
PCR negative	0	0	57	57
Total	18	16	62	80

	Result of NPA RT-PCR		
	RS virus positive	**RS virus negative**	
	Number (%)	Number (%)	p
Pneumonia	2 (8.7)	5 (8.8)	n.s.
Wheeze	10 (43.5)	37 (64.9)	n.s.
Bronchiolitis	11 (47.8)	3 (5.3)	$p<0.1$
Total	23 (100)	57 (100)	

Figure 3.9 RS virus in NPA by PCR - clinical and virological information
A series of 80 consecutive children that were admitted with respiratory illnesses had RNA extracted from NPA samples and RT-PCR was performed. A comparison between conventional methods of detection and PCR is shown above and clinical comparisons between those with pneumonia, wheezing or bronchiolitis is shown. The clinical information shown is taken from routine request forms for clinical virology.

Discussion:

The experiments described in this chapter indicate how valuable RT-PCR is as a tool with which to dissect the infectious cycle, not just of RS virus, but a range of viruses. Nested RT-PCR was more sensitive than conventional virological techniques like direct immunofluorescence and virus culture. It was demonstrated that RS virus can cause viraemia but that this viraemia is cell associated and found in PBMCs not in serum. No RS virus was found in the CSF during acute RS virus infection. This was despite the fact that the children who had lumbar puncture performed, did so because their clinical signs and symptoms suggested a potential neurological diagnosis, usually because of apnoeas or decreased level of responsiveness. These experiments also indicate the importance of RS virus in causing children to be admitted to hospital with respiratory illnesses. In the study of NPA material the samples were analysed "blinded" to the results from conventional studies and any clinical information. RS virus was found in 29% of all child admissions with respiratory diagnoses and in 61% (22/36) of children under 1 year of age. Although nested RT-PCR is at present too cumbersome and expensive to replace conventional methods of RS virus detection in the clinical setting, it will become feasible as technology improves. PCR virus detection is becoming more widely used in large scale epidemiological surveys. It also allows substrain typing to follow the evolution of RS virus.

In contrast to the NPA study RS virus is less common cause of respiratory illness in adults with HIV. Albertini *et al* (6) found RS virus in the BAL of 3 out of 15 children using conventional detection methods. In my hands only 3 out of 80 (3.7%) were found to have RS virus. One case report has previously described an HIV infected patient with pneumonia due to RS virus (258). As far as I am aware my study is the first to use PCR in a large series

of adults with HIV, to identify RS virus in BAL. Murphy *et al* (258) have shown that RS virus can be shed for long periods of time in children with HIV. This has public health implications that patients with HIV may wish to know about. Also, because of the possibilities of treatment with ribavirin, RS virus should be included in the differential diagnosis of pulmonary illness in adults with AIDS and other cell mediated immunodeficiencies . RSV is an important cause of respiratory disease in patients with HIV infections where no other pathogen is identified.

The finding of viraemia is also intriguing. For a few viruses infection is by direct inoculation and this may provide direct access immediately to the blood stream. In experimental bunyavirus infection for example with sufficient inoculum passive viraemia can then lead to CNS infection and fatal encephalitis (354). For most viruses, however, there is primary replication at the site of entry in particular mucosal surfaces before spread via local lymph nodes to the thoracic duct and systemic circulation e.g. measles virus and polio virus. Viraemia occurs in a number of human viral infections, some examples of this are given in table 3.2 below:

Table 3.2 Examples of viruses known to cause viraemia (adapted from Tyler *et al* (355)):

Method of spread	Examples
Free in Plasma	Togaviruses, picornaviruses
Red cell associated	Colorado tick fever virus
Lymphocyte associated	EBV, CMV, HIV, LCMV, measles, mumps, rubella
Monocyte/macrophage associated	Polioviruses, LCMV, CMV, HIV, measles
Neutrophil associated	Influenza

For many examples viraemia is difficult to detect and at a low level. The amount and duration of virus in the blood stream will be determined by the dynamic relationship between the amount of virus entering the blood stream and the efficiency of host clearance. Experiments in mice with vaccinia indicate that 99.99% is cleared within 1 hour after i.v. injection (354). Coating of virus with antibody or complement will facilitate this clearance. Primary infections with RS virus usually occur when passively acquired maternal antibody is still present in the circulation. The absence of free serum RS virus in my experiments is probably not surprising given this hostile environment. The ability to replicate in cells such as monocytes and lymphocytes may confer an advantage in that these non specific defence mechanisms may be avoided. RS virus has been shown to be able to infect pulmonary macrophages and monocytes derived from cord blood. Domurat *et al* showed in vitro Viraemia occurs in other paramyxoviruses and orthomyxoviruses. Measles virus, like RS virus, enters by the respiratory tract and establishes initial replication there for the first 2 to 4 days after infection. There is then spread via local lymphatic tissues leading to a cell associated viraemia. The primary cell infected by measles is the monocyte but lymphocytes have also been shown to be infectable *in vitro* (86) and may be infected *in vivo* (277). Measles spreads by viraemia to other organs such as skin, causing the rash. RS virus is sometimes associated with a rash but it is uncommon (24,355). Viraemia is thought to be uncommon in parainfluenza virus infection, but has been reported both in man (130)and in a hamster model (184). Viraemia has also been found with influenza virus (196) and mumps virus (278) in which viruria also occurs (357). PCR has been used determine the infecting strain after dissemination of mumps to the CNS and CSF (32). CD13 is human aminopeptidase N and mediates cytomegalovirus infection of cells. It is expressed on all human myeloid cells and for this reason was chosen to separate lymphoid from myeloid

populations. No positive samples were detected although one of the children was positive for RS virus in whole blood and PBMCs (DW). This could have been for a number of reasons. The most likely reason, in my view, is that the virus was too scarce to be detected. in only 5×10^4 cells. However the amount of cells available was limited by the amount of blood it was permissible to take. It is also possible that the time taken to sort the cells or cell fixation interfered with RNA extraction (30).

In younger infants, infected with RS virus during the first 2 months of life, apnoeas or acute collapse are often seen. These presentations can closely resemble encephalopathic illnesses and children frequently have a lumbar puncture performed. Using a lamb model Lindgren *et al* showed that reflex apnoea to chemoreceptor stimulation was significantly augmented by RS virus infection although the response to hypoxia and hypercapnia were unchanged (219). RS virus infections are rarely associated with frank neurological illness (131,248,362), usually the situation resolves with observation and supportive measures alone. However, Wallace *et al* described a series of 78 children admitted to a children's hospital with fits or febrile illnesses. 53% were found to have viral illnesses. More severe fits, permanent neurological damage and prolonged EEG changes were more common where a virus was detected. Temporary and permanent neurological sequelae were documented in children with RS virus infection alone including: ataxia facial palsy and hemiplegia (362). Parainfluenza virus has been isolated from CSF in association with both Guillain-Barre syndrome and aseptic meningitis (62,313). Specific anti-RS virus antibodies have been detected in CSF but the virus has not (41). In my study no children were found to have RS virus in CSF by PCR. In the lung and in culture most RS virus is cell associated, so this result does not prove that RS virus does not directly infect the CNS. It does show that PCR

of CSF for RS virus is not likely to be useful clinically. One interesting phenomenon limited the availability of samples that were collected. As the study progressed less and less CSF samples were taken from children admitted to St Mary's with apnoeas or acute collapse. In the second RS virus season all the samples were collected by liaising with referring hospitals. Through education and awareness of the clinical pattern junior clinicians learnt to recognise features that distinguished RS virus associated illness and early NPA immunofluorescence was sought.

Summary

In this chapter it was shown that a cell associated viraemia occurs in children with primary RS virus infection. Separated PBMCs can be shown to be positive for RS virus by nested RT-PCR. Serum and CSF did not contain viral genome. In HIV adults RS virus can be associated with pulmonary disease. However only 3 of a series of 80 adults, investigated for unexplained pulmonary illness, were found to have RS virus by PCR. The importance of RS virus in causing childhood admissions with respiratory illnesses was shown using RT-PCR of nasopharyngeal samples.

Chapter 4 Viral persistence in the mouse model

Hypothesis

Persistence of RS virus after initial infection is an important issue that has never been addressed. Sendai virus, a natural parainfluenza virus infection of mice, has been shown to persist in the olfactory bulb. Measles virus has also been shown to persistent in humans. If RS virus persists in the mouse model it is likley to be in a very low copy number. The RT-PCR method is therefore an ideal technique for addressing this question.

The ability of virus to persist in an immunocompetant host depends on avoidance of immune mediators. As cytotoxic T cells are clearly important for clearance of RS virus during primary infections it is possible that viral persistence occurs through mutation in the dominant CTL epitope. In H-2^d expressing BALB/c mice this epitope is located within the M2 protein. The following studies are designed to examine viral persistence, its likley location and whether it is present despite memory CTL activity to the M2 epitope in both normal and cytokine depleted mice.

Chapter 4 Viral persistence in the mouse model

Introduction

In the past, the hallmark of virus infection was often cell death, tissue destruction and lymphocytic infiltration (268). However it is now known that not all viruses are dramatically cytopathic and that some viruses remain in the host long after the primary illness is over. Sigurdsson (331) carried out key studies in visna virus infected sheep. He introduced the concept that there may be a very long period between infection and a clinical illness. Later Gajdusek and Gibbs suggested a similar concept for the human diseases Kuru and Creutzfeldt Jakob disease (108). Although no viral agent has been associated with these particular diseases, this work lead to the search for persistent virus infections in a number of human illnesses for which the aetiology was not determined. It is now known that many viruses persist after primary infection and with the evolution of more sensitive methods of detection, such as the polymerase chain reaction, it has become easier to detect persistent pathogens. Until these methods became available the study of the natural history of many virus infections was often limited to the period of acute infection.

One of the key points Sigurdsson noted was that a pathogen that kills its host may not survive to complete its infectious cycle. This has become a cornerstone for the way viral infections are viewed today . Therefore a successful pathogen might be viewed as one that replicates but allows host survival, favouring hosts that are not adversely affected by infection. It follows that both viral and host factors contribute to viral persistence (148).

A number of general concepts have been suggested from studies of persistent virus

infections. Firstly persistent virus infections are usually poorly or not cytopathic, although in cell culture some persistent viruses may abrogate "luxury" functions whilst vital cell functions are preserved (growth, levels of RNA, vital enzymes) (267). An example of this is lymphocytic choriomeningitis virus (LCMV); in culture in neuroblastoma cells LCMV stops acetyl choline production and degradation but allows cell growth and infected cells are not distinguishable morphologically from uninfected ones.

Ahmed *et al* (5) have suggested three broad mechanisms that should be fulfilled for virus persistence. Firstly a virus should infect without being cytopathic, secondly there must be a method to maintain the viral genome and thirdly the virus must avoid detection and elimination. The ability to infect a host cell without leading to the death of the cell would clearly be advantageous if a virus is to become persistent but in some cases viruses that are highly lytic to many cell types may cause no injury to specific types of cell. In many cases these cell types may be semipermissive to replication and allow little viral gene expression allowing a latent infection. An example of this is herpes simplex virus (HSV) that is generally a cytolytic virus but infects sensory neurons which are nonpermissive and latency can be established (5). Through this latency HSV infects an immunologically privileged site as well as the restricted expression of viral genes HSV. Herpes viruses are some of the most extensively studied persistent viruses and have been shown to have a number of strategies to avoid immunity. There is also evidence that during lytic infection immediate early proteins act to interfere with TAP dependant transport of peptides into the ER (101,158) decreasing the likelihood of T cell mediated lysis.

Maintaining the viral genome is also important if the virus is to survive and if the infected

cell is replicating the genome may be diluted if it is not replicated also. DNA is generally more stable within cells and DNA viruses may be more likely to persist. However RNAses are abundant both intracellularly and extracellularly and cellular RNA is not generally long lived. RNA viruses may have to continue to replicate at a low level to maintain infection. In the case of retroviruses like HIV an additional step occurs by integration of the genome into the host cell DNA.

Mechanisms by which viruses evade the immune response have also been found. These include antigenic variation such as in HIV and hepatitis B virus which will be discussed later in this thesis. Measles virus may have a number of mechanisms to avoid immunity, it may suppress class 1 MHC expression in infected cells (303) and class 2 MHC upregulation by IFN-γ (215). Some viruses may even interfere with cytokine signalling. When the gene for IL-10 was first cloned it was found to have a close homology with a gene of unknown function in Epstein-Barr virus: BCRF1 (169). The product of BCRF1 has many of the natural activities of IL-10 including the ability to stimulate the proliferation of B cells (the cells in which EBV persists).

Immunisation has focused on the production of antibody responses to pathogens. This approach has been very successful for a number of pathogens. However, persistent virus infections are usually cell associated and several million antibody molecules are needed to destroy virally infected cells (281,333). Cell surface viral protein expression is often absent in persistent virus infections and therefore antibody mediated clearance of infected cells is less likely to be effective. As discussed in chapter 1 cytotoxic T lymphocytes require only very low numbers of MHC bound peptide molecules for activation. The CTL response to

virus might be expected therefore to be the most efficient means of eliminating low levels of virus and preventing persistence.

It has been suggested that respiratory syncytial virus could be persistent (296). There are several pieces of evidence indicating the possibility of persistence. RSV is only poorly cytopathic *in vitro* and *in vivo*. It causes no interruption of host cell protein synthesis and no detectable interference with cell function (58). The effects of severe RSV bronchiolitis are long lasting despite no detectable alteration in lung anatomy and the absence of viral antigen. Bronchial hyperresponsiveness is common after bronchiolitis and wheezing with subsequent respiratory virus infection (290,366). Measurable bronchial hyperresponsiveness can be present 10 years later (299). Other closely related paramyxoviruses have been found to be persistent in certain cases. Measles virus, for example, can persist in neurological tissue and is associated with the devastating neurological condition subacute sclerosing panencephalitis (SSPE). Interestingly SSPE has declined markedly with the widespread use of the vaccine against measles in the USA. This provides hope that any illnesses associated with other persistent paramyxoviruses could be prevented if effective vaccination was available. Persistent measles virus has also been associated with other conditions including autoimmune illnesses such as chronic active hepatitis and systemic lupus erythematosus (15,308).

In these studies I have investigated the persistence of viral genome in the mouse model; a finding initially discovered in a study of mice deficient of IL-3. The nested RT-PCR was used to determine the duration of RSV in lung and bronchoalveolar lavage. The same technique was used to examine spleen, brain and olfactory lobe to determine whether

infection by RSV occurred and whether it was persistent.

T lymphocytes are the major source of IL-3, a pleiotropic cytokine. It can also be produced by mast cells after stimulation via the IgE receptor (288,374). Haemopoietic cells are derived from a common pluripotential stem cell which gives rise to both myeloid and lymphoid lineages. IL-3 supports proliferation of myeloid lineages at many stages of maturation (174). The murine IL-3 gene has been mapped to chromosome 11 (175) in close proximity to the gene for GM-CSF. Until recently the role of IL-3 in lymphocyte responses has been unclear. It has been determined that IL-3 may have a number of subtle functions on T cells often in association with other cytokines. There is now evidence that in some situations IL-3 may be able to stimulate specific primary CTL (235), support proliferation of T helper clones (54) and lower the threshold for mast cell degranulation (323). In particular IL-3 may be involved in T_H2 responses with a role in proliferation of eosinophils (371) and mast cells as well as being a cofactor with IL-4 for the growth of CD4 positive T cells (252). The role of IL-3 in acute virus infection has not been examined. Mice deficient in IL-3 were offered to my laboratory to determine whether any effect on virus clearance was present. In this chapter data are presented about primary infection with RS virus in normal and IL-3 deficient mice. Nested RT-PCR is used to detect viral genome in bronchoalveolar lavage and lung homogenate because I had demonstrated that it offered significantly greater sensitivity than virus culture or antigen detection. In the experiments below I received help with animal procedures from Mr Andreas Georgiou who also assisted me with the cytospins. All of the presented data other than the cytospins were performed solely by me.

Results:

a) Mice deficient of IL-3 show the same cellular efflux into the lung as normal mice.

Design: 129/Sv (H-2^b) mice were kindly provided by Dr V.L.J. Tybulewicz. The IL-3 gene was disrupted in 129/Sv embryonic stem (ES) cells by homologous recombination after transfection with omega-type constructs flanked with a herpes simplex thymidine kinase gene. These cells were used to produce a mouse strain deficient of IL-3 (which was confirmed by Dr Tybulewicz). Normal and knockout mice were infected intranasally with 5×10^5 pfu per mouse A2 RS virus. Cellular efflux into the lung was determined by bronchoalveolar lavage, cytospins and flow cytometry. Splenocytes were stained for CD4 (Fitc conjugated rat IgG2a antimouse, at 1.25µg/ml, Sigma) and CD8 (PE conjugated rat IgG2a antimouse, at 1.25µg/ml, Sigma) and LECAM-1 (Mel 14) (first layer: biotin conjugated rat IgG2a antimouse CD62L at 5µg/ml, Pharmingen, second layer: streptavidin conjugated Quantum Red at 1/75 concentration, Sigma). Flow cytometric analysis was performed to determine if IL-3 deficient mice have different proportions of naïve and "memory" T cells in the spleen.

Results: There were no significant differences between normal and IL-3 deficient mice in the proportions of lymphocytes, polymorphs, macrophages or eosinophils in the bronchoalveolar lavage (figure 4.1). The lymphocytic efflux into the BAL showed a similar time course in IL-3 knockout as normal mice peaking at day 10 before resolving. Spleens from normal and knockout mice contained similar proportions of CD4 and CD8 lymphocytes (figure 4.2) and no differences were seen in the proportion of $CD4^+$/LECAM-1^+,

$CD4^+$/LECAM-1^-, $CD8^+$/LECAM-1^+ and $CD8^+$/LECAM-1^- cell subsets.

b) Clearance of RS virus from the bronchoalveolar lavage but not the lung both in normal mice and mice deficient of IL-3.

Design: 200μl aliquots of bronchoalveolar lavage fluid from normal mice and mice deficient of IL-3 were collected during acute primary infection and stored at -80°C until testing. Lungs were removed after BAL was performed and snap frozen. Lungs were sliced into 22mg-25mg portions homogenised, made up to 200μl with sterile PBS and again snap frozen. Total RNA extraction was extracted from BAL and lung homogenate using the QIAgen blood kit. cDNA synthesis was performed followed by nested RT-PCR for M2.

Results: RS virus was detected by RT-PCR of bronchoalveolar lavage at day 4 and day 10 but not at day 17 and day 24. RS virus was detected at all time points in lung homogenate. At day 24 half of the mice were still positive for RS virus by RT-PCR in lung homogenate but none were positive by RT-PCR of BAL. There were similar proportions of mice positive for RS virus by RT-PCR whether they had IL-3 or not. Clearance of RS virus from the BAL occurred at the same time in both normal and IL-3 knockout mice (Figures 4.3 and 4.4).

c) RS virus is cleared from the bronchoalveolar lavage of BALB/c mice after day 14.

Design: 8 to 12 week old BALB/c mice were infected i.n. with 5×10^5 pfu A2 RS virus. Bronchoalveolar lavage was performed on days 3, 5, 7, 10, 14 and 28. Total RNA was extracted with the QIAamp blood kit method and cDNA synthesis and nested PCR was performed..

Results: Viral genome was detected at days 3, 5, 7, 10 and 14 but not at day 28 (figure 4.5a).

d) RS virus is not found in spleen after primary infection.

Design: BALB/c mice were infected as in c) and spleens were removed at days 3 and 5 after infection and snap frozen. They were homogenised, made up to 200μl with sterile PBS and again snap frozen. RNA was extracted using the QIAgen blood kit method, cDNA synthesis and nested PCR were performed.

Results: No spleens were found to contain RS virus genome (figure 4.5b).

e) RS virus can be detected 100 days after infection in BALB/c mice.

Design: BALB/c mice aged 8-12 weeks were infected i.n. with A2 RS virus (5×10^5 pfu per mouse). Groups of mice were infected three times with 2 weeks between infection or once only. The mice were housed in filtered top cages and given autoclaved diet and water. After 100 days the mice were anaesthetised using intraperitoneal pentobarbital and exsanguinated. The lungs, olfactory bulbs, brains and spleens were removed and snap frozen. Splenocytes were used in CTL assays below. Lung, brain and olfactory bulbs were homogenised and made up to 200μl with sterile PBS and again snap frozen. RNA extraction was performed using the QIAgen blood kit. cDNA synthesis was performed and nested RT-PCR for M2.

Results: RS virus genome was detected 100 days after infection in the lung of previously infected mice but not controls (figure 4.6). Although mice infected three times were found to have viral genome slightly more frequently than mice infected once this was not found in

all experiments. In the first experiment 3 out of 4 mice infected three times were positive by RT-PCR whereas only 1 out of 4 mice infected once was positive. In the second experiment 2 out of 6 mice in each group was positive by RT-PCR. Brain tissue was divided into olfactory bulbs and other brain tissue. Olfactory bulbs were tested by RT-PCR on one occasion and 1 mouse out of 6 previously infected was positive for viral RNA compared with none in the uninfected control group. No positives were found in other brain tissue (figure 4.9).

f) Sequences from mice with viral genome contained the dominant epitope for CTL recognition in H-2^d mice.

Design: Mice were infected once and three times with RS virus as above. The lungs were removed 100 days after the last infection (as above), homogenised and nested RT-PCR for M2 performed. Positive PCR samples were cloned and expanded in E. Coli. Individual cloned DNA was purified for sequencing. At least 10 clones from each sample were sequenced.

Results: All the samples found to be positive for viral genome, 100 days after infection, contained M2 genes with the correct sequence for the dominant epitope for CTL recognition of RS virus M2 protein by mice with K^d MHC class I molecules.

g) BALB/c mice infected with RSV have persistent CTL memory responses and specific antibody 100 days after infection.

Design: Spleens were removed from BALB/c mice infected 100 days before once , three times or not at all (as in e) above). 5 day bulk cultures of splenocytes were prepared using

syngeneic spleen cells infected with A2 RS virus as stimulators (as in materials and methods). CTL were determined by specific lysis of ^{51}chromium labelled P815 target cells incubated with M2 (82-90) peptide (SYIGSINNI) or medium alone. Serum antibody against RS virus was also determined by ELISA (as in materials and methods).

Results: Splenocytes from BALB/c mice previously infected with RS virus lysed target cells incubated with the peptide SYIGSINNI (M2 82-90 the dominant epitope for class I recognition of M2 in H-2^{d} mice). Uninfected mice did not generate SYIGSINNI specific CTL. There was no difference in lytic activity between mice primed once and mice primed three times. The presence of memory CTL specific for RS virus M2 peptide did not correlate with viral RNA persistence in the lungs (figure 4.7). Mice had specific anti RS virus antibody 100 days after infection (figure 4.8).

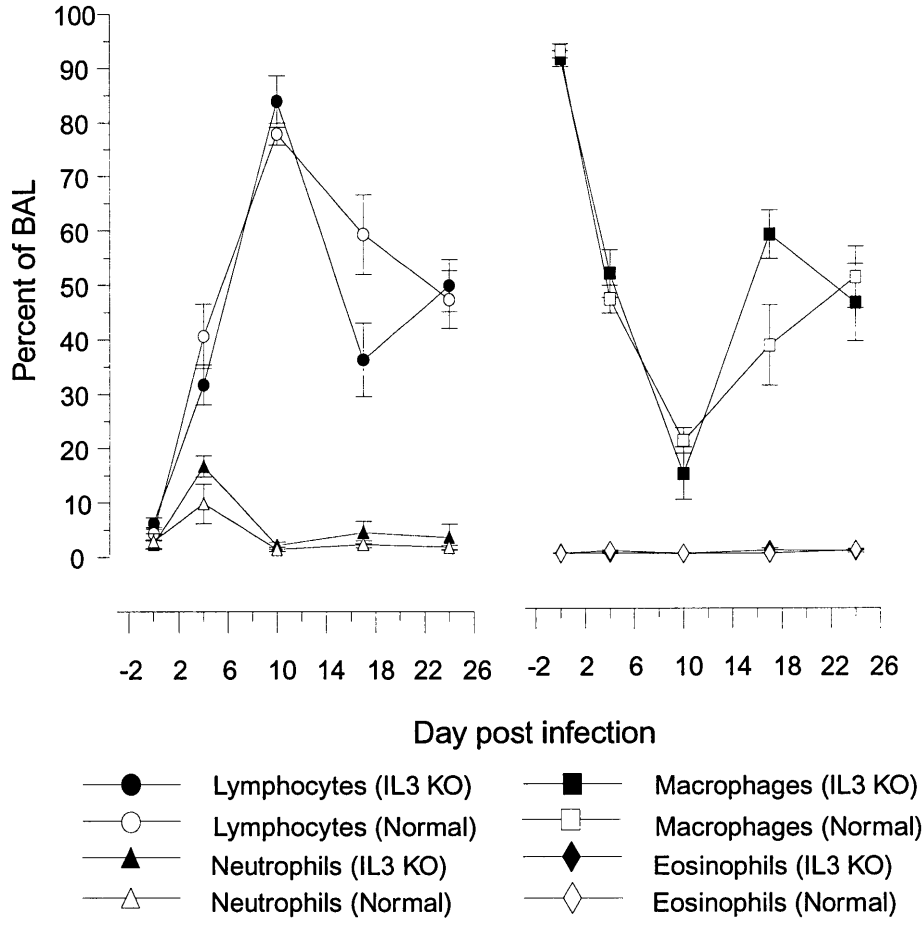


Figure 4.1 Timecourse of cellular efflux into the alveolar space in IL-3 deficient mice. Cellular efflux in to the alveolar space (assessed by BAL) in normal 129/Sv mice and 129/Sv mice deficient in IL-3. 2-3 month old mice were infected i.n. with A2 strain RSV. Cellular efflux was determined, at the times after infection shown, by bronchoalveolar lavage and differential counts from Giemsa stained cytospins.

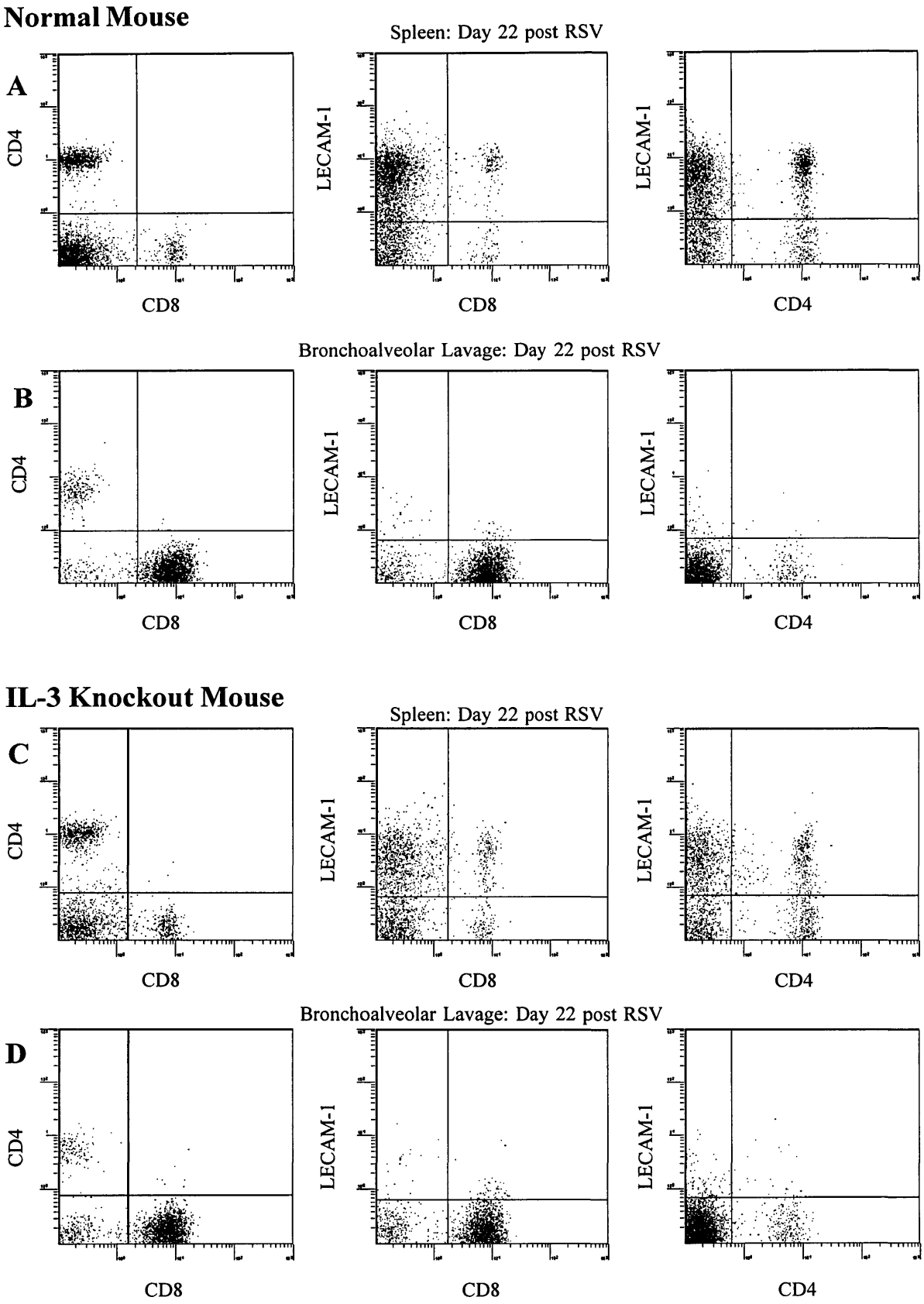


Figure 4.2 Representative flow cytometric data from BAL and spleens in normal and IL-3 deficient 129/Sv after infection with RS virus.

A Normal mouse spleen
B Normal mouse BAL day 22 post RSV
C IL3 KO mouse spleen
D IL3 KO mouse BAL day 22 post RSV

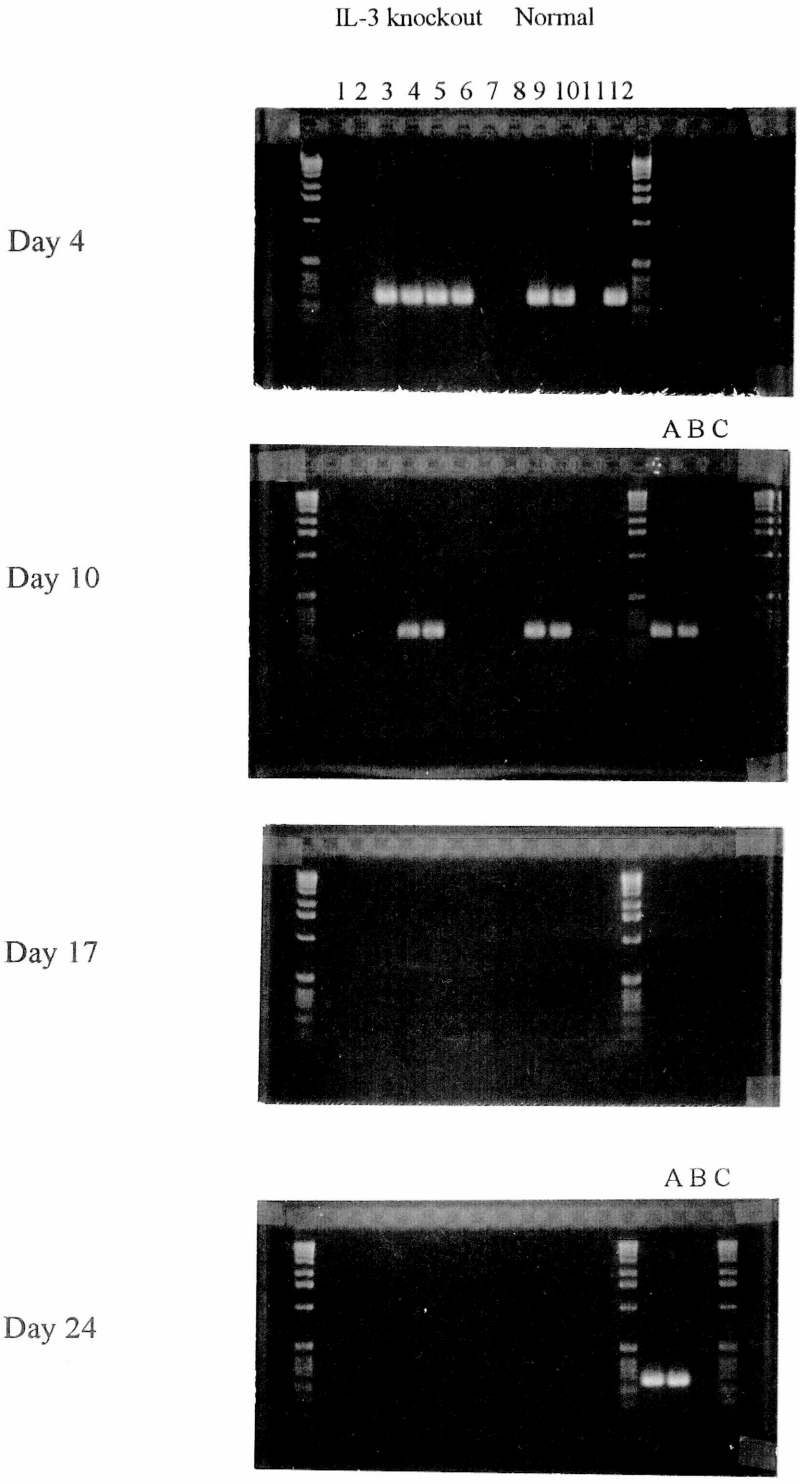


Figure 4.3 Nested RT-PCR for M2 genome of RS virus in bronchoalveolar lavage of 129/Sv mice infected with A2 RS virus. Gels are shown for days 4, 10, 17 and 24 after infection. On the left (lanes 1-6) are mice deficient of IL-3, on the right (lanes 7-12) are normal mice. Six mice per time point are shown. In each group the first 2 mice (1,2, 7 and 8) were mock infected, the following 4 mice were infected with RS virus. Controls: A) positive equivalent to 0.01pfu RS virus and B) positive equivalent to 0.001pfu RS virus C) negative. Controls shown for day 10 were also for day 4. Controls shown for day 24 were also for day 17. Using 1KB markers, PCR amplified products lie between 220 and 298bp consistent with an expected size of 260bp.

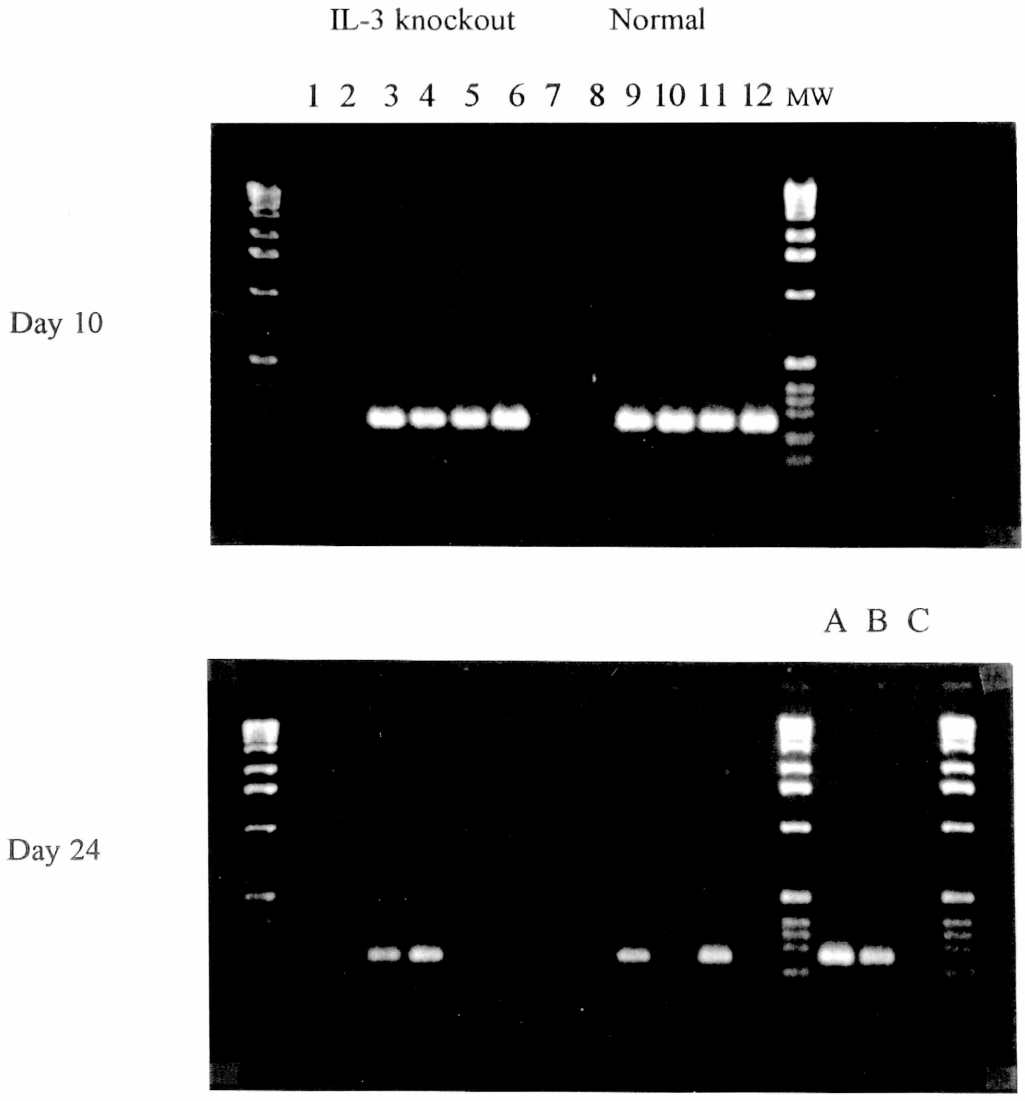


Figure 4.4 Nested RT-PCR for M2 genome of RS virus in lung homogenates of 129/Sv mice infected with A2 RS virus. Gels are shown for days 10 and 24 after infection. On the left (lanes 1-6) are mice deficient of IL-3, on the right (lanes 7-12) are normal mice. Each lane at each time point represents an individual mouse. In each group the first 2 mice (1,2, 7 and 8) were mock infected, the following 4 mice were infected with RS virus. Controls: A) positive equivalent to 0.01pfu RS virus and B) positive equivalent to 0.001pfu RS virus C) negative. Using 1KB markers, PCR amplified products lie between 220 and 298bp consistent with an expected size of 260bp.

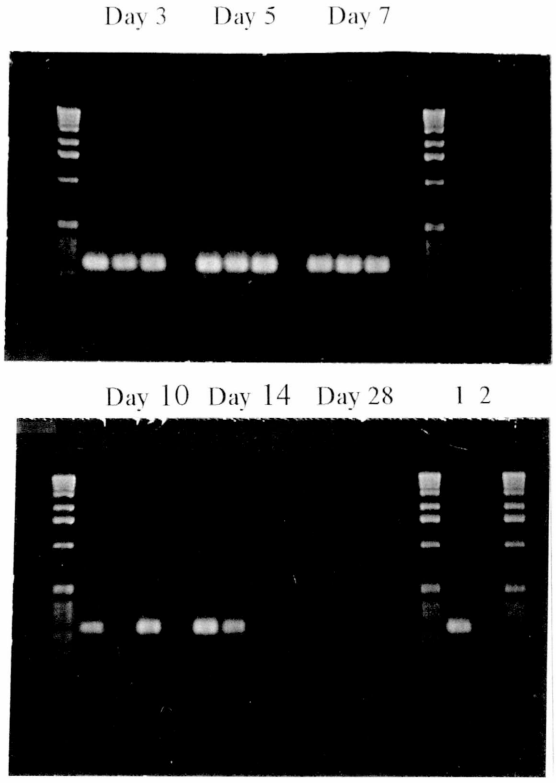


Figure 4.5a Nested RT-PCR for M2 genome of RS virus in bronchoalveolar lavage of BALB/c mice infected with A2 RS virus. BAL was performed on days 3, 5, 7, 10 ,14 and 28 after infection (three mice per group). Every fourth lane is the result for an uninfected mouse. Controls: 1) positive equivalent to 0.01pfu RS virus and 2) negative. (1KB markers)

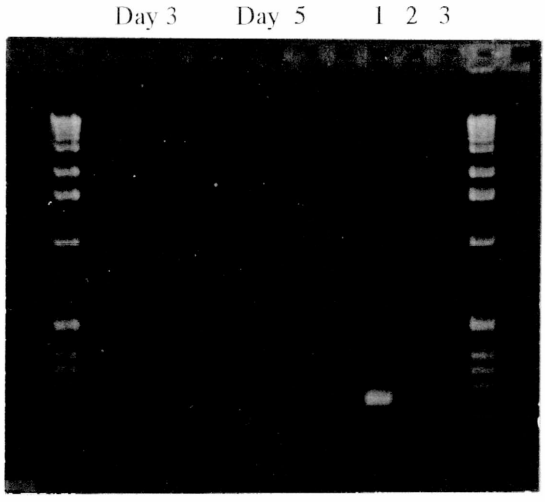


Figure 4.5b Nested RT-PCR for M2 genome of RS virus in spleens of BALB/c mice infected with A2 RS virus. Spleens were removed on days 3 and 5 after infection (three mice per group). Every fourth lane is the result for an uninfected mouse. Controls: 1) positive equivalent to 0.01pfu RS virus and 2) positive equivalent to 0.001pfu RS virus 3)negative. (1KB markers)

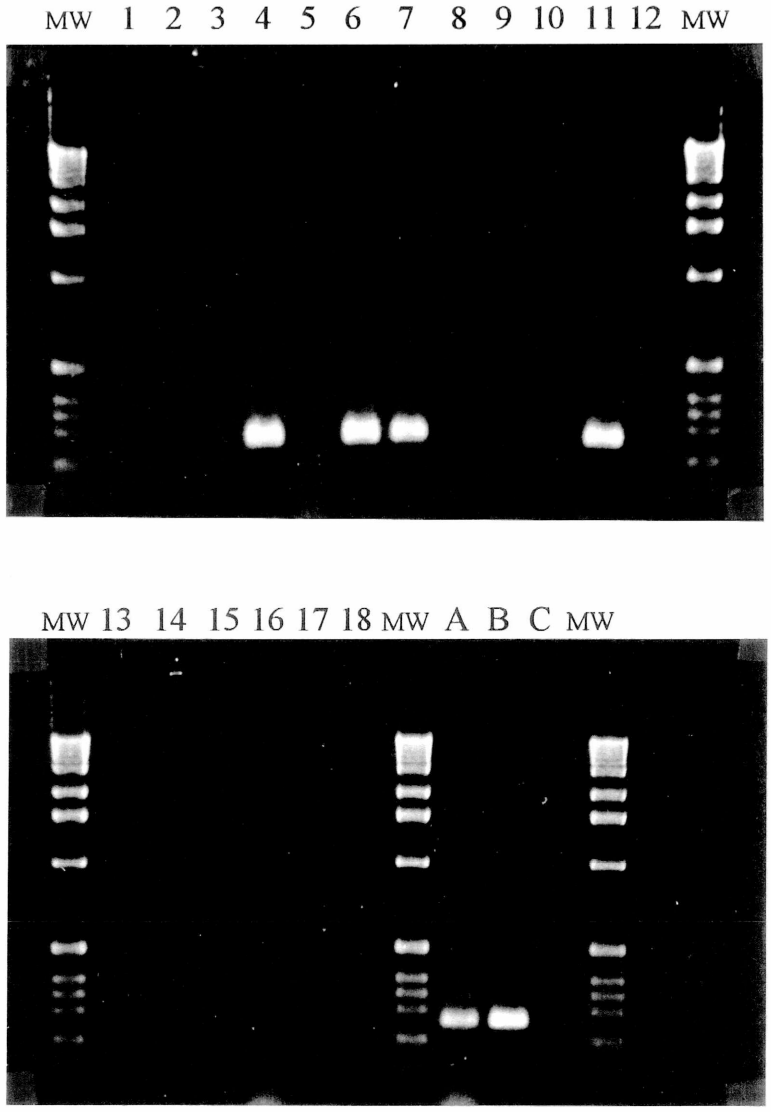


Figure 4.6 Nested RT-PCR for M2 genome of RS virus in lung homogenates of BALB/c mice. Mice were infected once, three times or not at all. Then 90 days after last infection with A2 RS virus the lungs were removed and RT-PCR performed. Lanes 1-6 are mice infected once, lanes 7-12 are mice infected three times and lanes 13-18 are uninfected mice. Each lane represents an individual mouse. Controls: A) positive equivalent to 0.01pfu RS virus and B) positive equivalent to 0.001pfu RS virus C) negative. Using 1KB markers, PCR amplified products lie between 220 and 298bp consistent with an expected size of 260bp.

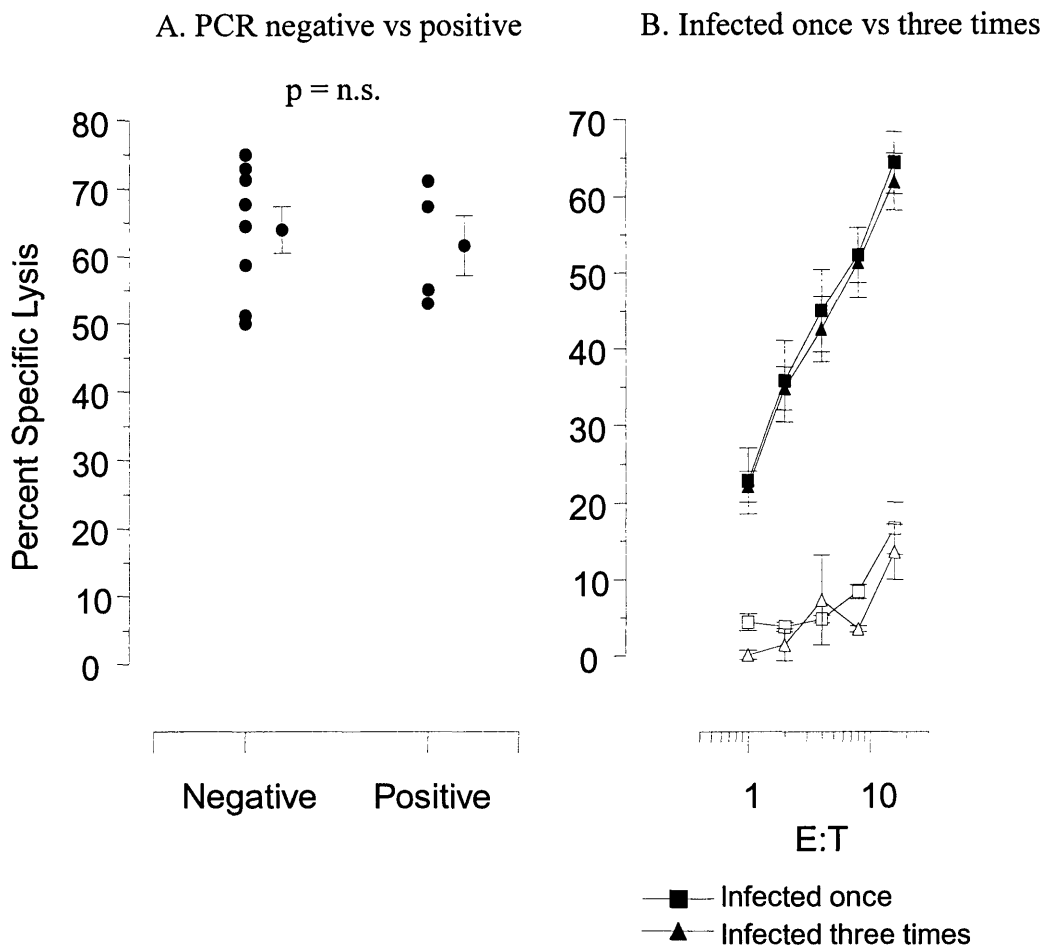


Figure 4.7 Memory CTL 100 days after RS virus infection in BALB/c mice.
BALB/c mice were infected i.n. with A2 RS virus once or three times. One hundred days after the last infection RS virus persistence was tested by nested RT-PCR. Splenocytes were stimulated in bulk cultures for 5 days with A2 RSV. CTL killing was assessed by chromium-51 release assay. P815 target cells were incubated with peptide (SYIGSINNI) at 10^{-8} M and labelled with ^{51}chromium. In the left panel percent specific lysis is shown at an E:T ratio of 16:1 for mice found to be positive or negative for RS virus in lung. Lytic activity is is compared between mice infected once with RS virus and three times with RS virus (right panel). Lytic activity is shown for targets incubated with peptide (filled symbols), versus no peptide (open symbols).

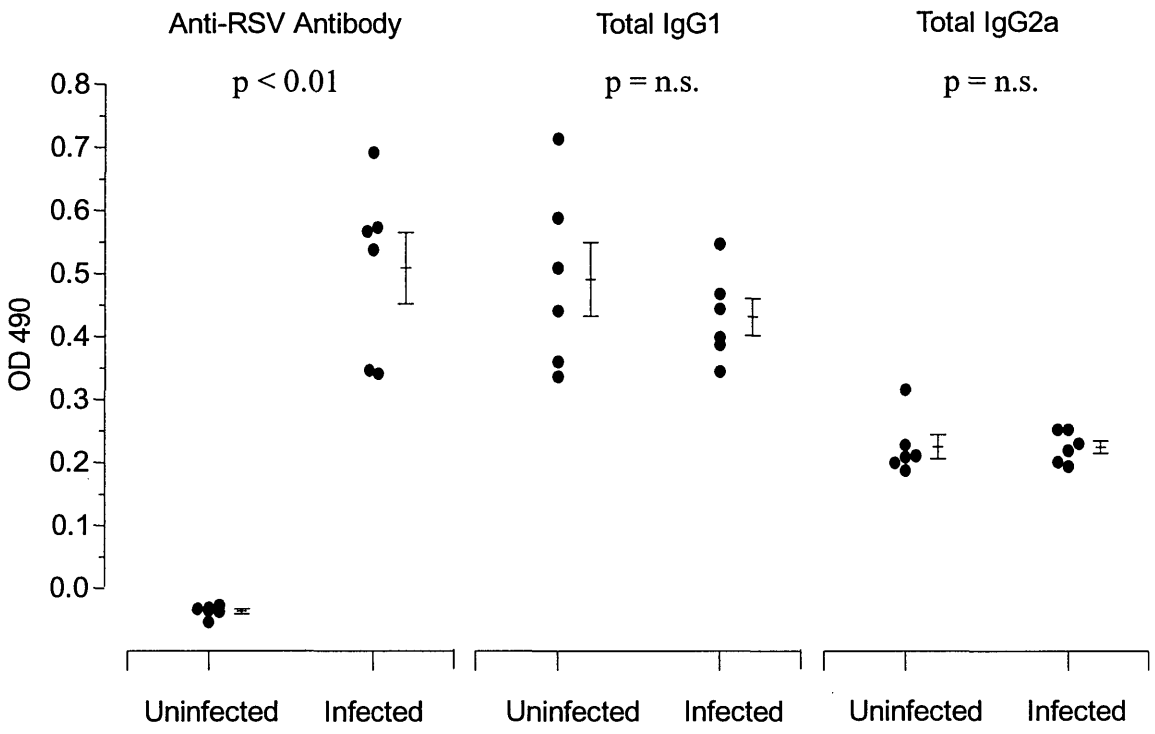


Figure 4.8 Antibody (ELISA) levels 100 days after RSV infection. BALB/c mice were infected i.n. with A2 RSV. One hundred days after the last infection the mice were sacrificed and tested by nested RT-PCR for RSV. Specific anti-RSV antibody (left panel), total serum IgG middle panel) and total serum IgG2a (right panel) are shown for infected and uninfected mice .

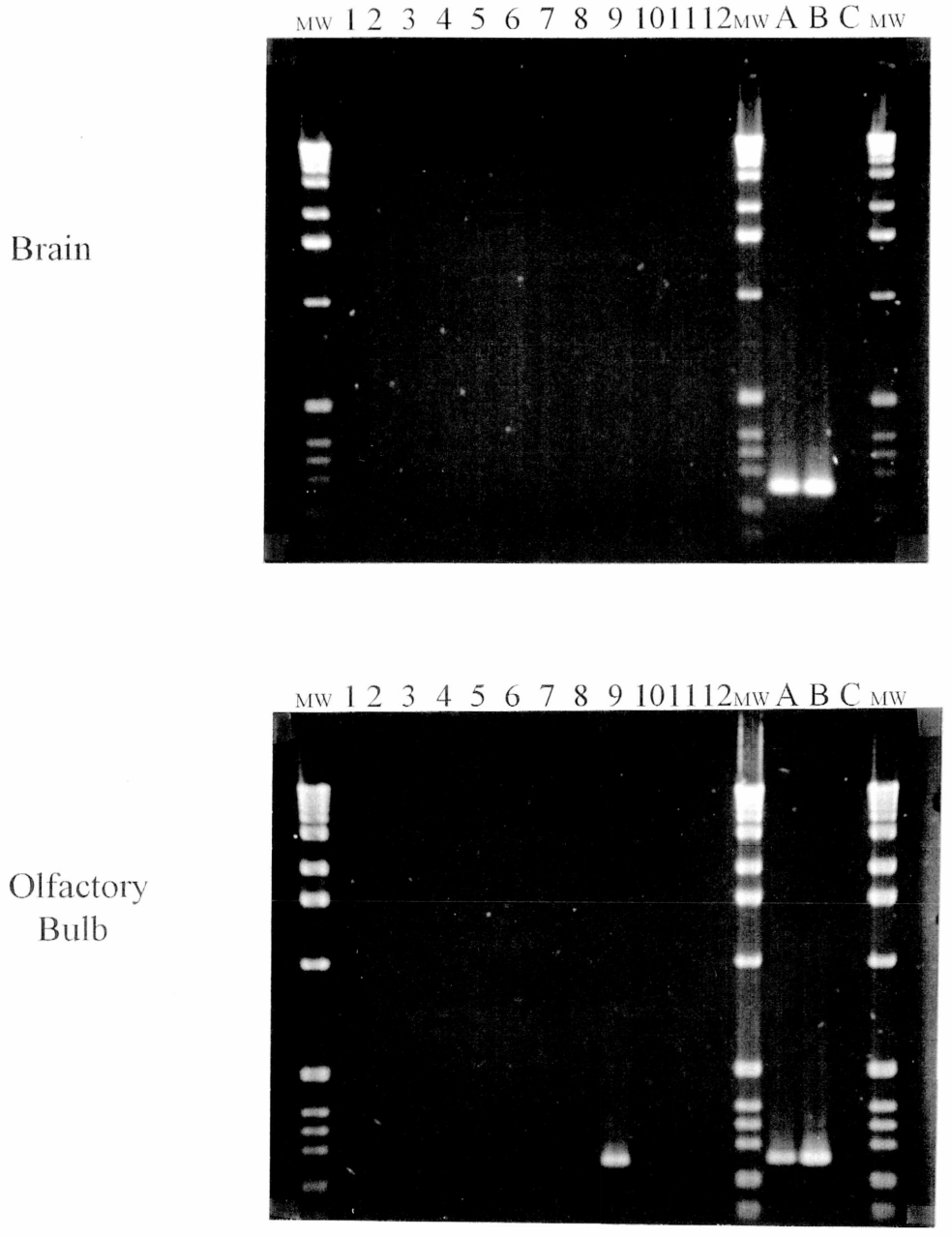


Figure 4.9 Nested RT-PCR for M2 genome of RS virus in brain (other than olfactory bulb) and olfactory bulb of BALB/c mice. Mice were infected once with A2 RS virus. Then 108 days after infection the brains and olfactory bulbs were removed, homogenised and RT-PCR performed. Lanes 1-6 are uninfected mice, lanes 7-12 are mice infected 108 days previously. Each lane represents an individual mouse. Controls: A) positive equivalent to 0.01pfu RS virus and B) positive equivalent to 0.001pfu RS virus C) negative. Using 1KB markers, PCR amplified products lie between 220 and 298bp consistent with an expected size of 260bp.

Discussion:

IL-3 and RS virus

Very few studies of viral infections have been undertaken using mice with specific cytokine deficiencies. Although the technique for generating knockout mice is relatively recent, many cytokine deficient mouse strains do now exist including nitric oxide, IL-2 (337), IL-4 (34), IL-6 (157), IL-7 (359), IL-10 (206) and IL-12 (225). In some knockout mouse strains the absence of cytokine has been associated with an abnormal phenotype or disease for example mice deficient of IL-7 are lymphopenic and IL-10 knockout mice develop enterocolitis (206). No phenotypic differences had been determined in the 129/Sv mice provided for us. There had been no evidence of any alteration in blood baseline cell counts, haemoglobin, growth or any suggestion of disease.

Mice deficient in the beta subunits of IL-3 receptor have been used to examine the response to Nippostrongylus brasiliensis. The beta c subunit of the IL-3 receptor is also part of the receptors for GM-CSF and IL-5, whereas there is a subunit found only in the IL-3 receptor (beta IL-3). Removal of the beta IL-3 subunit made no difference to lung pathology whereas the mice deficient in beta c had pathology with lymphocytic infiltration and low levels of eosinophils in blood and bone marrow (265). In the studies presented in this chapter I have shown that RS virus is cleared from the BAL equally well in normal and IL-3 deficient mice. The cellular efflux into the BAL followed a similar pattern and time course in normal and knockout mice. Also the percentages of both CD4 and CD8 positive T cells in the BAL after virus clearance were unchanged by removal of IL-3.

Other single cytokine knockouts such as IL-4 have been shown to have only marginal differences in their responses to infection. IL-4 knockout BALB/c mice remain susceptible to Leishmania major (266) and Leishmania donovani infection but are less susceptible to Leishmania mexicana (324). Whereas γ-IFN receptor deficient mice from genetically resistant strains to Leishmania major become susceptible to infection but do not mount a exaggerated T_H2 response (341). They are also more susceptible to infection with pseudorabies virus (325). Although there were no differences found in the pathology associated with RS virus infection in normal and IL-3 knockout mice, there was one striking and unexpected finding: at the end of the experiment (day 24 after infection) half of the mice were still positive for RS virus in lung homogenate. This finding led directly to the studies in BALB/c mice. Further studies in IL-3 deficient mice were not performed because viral clearance from the bronchoalveolar lavage appeared to follow the same time course with no significant effect on lymphocyte efflux into the BAL. This indicates that IL-3 is not likely to play a particular role in the development of the immune response to RS virus. If IL-3 does play a part in the anti viral response it is likely that many of the functions of IL-3 can also be subserved by cytokines which have similar activities such as GM-CSF and IL-5.

Detection of RS virus in the lung

Data presented in this chapter suggest that RS virus although cleared from bronchoalveolar lavage fluid, persists in the lungs of mice after primary infection. Nested RT-PCR was used to detect RS virus M2 genome. This region of the genome was selected because it is very highly conserved (57). The sensitivity of using M2 primers was compared with N primers. On each occasion primers for M2 were equal to or better than amplification using N primers. Therefore M2 primers were used in these experiments. Positive sense and negative sense

first strand primers for N and M2 were tested in cDNA synthesis. Interestingly, no significant difference in sensitivity between positive and negative sense was found. It might have been anticipated that a first strand primer complimentary for a positive mRNA transcript would have some advantage in that many mRNA transcripts would be expected to be produced from each viral RNA (negative sense) strand. However this was not found to be detectable using these primers. Nested RT-PCR is used because it offers extreme sensitivity and great care must be taken to avoid contamination. In all of these experiments uninfected mice were used as controls as well as negative serum to ensure contamination was not present either from transmission in the animal facility, contamination during harvesting or contamination while performing RT-PCR. In no experiment was any uninfected control animal ever found to be positive for RS virus by PCR.

RNA extraction proved to be the most difficult part of the RT-PCR to optimise. Although many methods of RNA extraction deal efficiently with cell free fluids. The cellular debris in tissue homogenates proved to be inhibitory in many methods. Methods using guanidinium RNA extraction such as RNAzol B gave poor results as did proteinase K digestion with phenol chloroform re-extractions. Initial experiments using the QIAGEN blood kit method, for DNA and RNA, proved successful for blood. This method uses a proteinase and guanidinium extraction step followed by affinity column purification. However the QIAGEN tissue extraction method was found to be relatively insensitive. By homogenising lung tissue and making up to a volume compatible with the QIAGEN blood kit excellent sensitivities were possible. These sensitivities could not be matched using the specific RNA kit QIAGEN makes.

RS virus persists

Persistence of RS virus has been suspected by previous investigators but never before shown. There is evidence that Paget's disease of bone, a chronic disease associated with pain, fractures and deformity, may be caused by a paramyxovirus and possibly RS virus (296). Osteoclasts in Paget's disease often contain cytoplasmic inclusion bodies not seen in other types of bone disease. Electron microscopic studies indicated that these inclusion bodies contain filaments consistent with the dimensions of the nucleocapsid of pneumoviruses rather than other paramyxoviruses. Mills *et al* (241)were able to show RSV antigen in the osteoclasts from patients with Paget's disease and in cells cultured from such lesions. In 1985 Pringle *et al.* carried out a survey of patients with Paget's disease to determine the level of serum antibody both to RSV and parainfluenza virus type 3 (PIV3) (296). Although the authors could draw no conclusions about whether RSV was associated with Paget's disease, they found that control patients had fluctuating levels of antibody to RSV whereas those with Paget's disease had constant levels. They commented that this finding was reminiscent of other slow viruses and persistent viruses and interpreted this as a possible indicator of persistence by RSV.

Sendai virus, a natural parainfluenza virus infection of mice, has been demonstrated to be persistent in the olfactory bulb. Mori *et al* detected viral genome in olfactory bulbs but not other brain tissue using nested RT-PCR 168 days after primary infection (246). They demonstrated viral antigen using immunohistochemistry up to 7 days post infection. In my experiments it was clear that the olfactory bulbs lie in very close proximity to the nasal mucosa. Although viral genome was found 108 days after primary infection there was a possibility that during dissection a small contamination of the sample with olfactory mucosa

might take place. If this experiment was to be repeated it would be preferable to address this question using in situ hybridisation or in situ PCR. It would also be of value to examine olfactory bulbs using immunohistochemistry during acute infection. From my experiment I conclude that it is likely that RS virus can persist in olfactory bulb and this needs further investigation.

RS virus was detected in the BAL of BALB/c mice at 14 days by RT-PCR after 14 days it was undetectable. Using conventional methods virus is not found after day 8 (123,344) in normal mice. This difference probably reflects the superior sensitivity of nested RT-PCR. In the lung RS virus was detectable even after 100 days in a proportion of the mice. Since I started my experiments in the mouse model, Hegele and Hogg (149) published data from a guinea pig model of RS virus infection. They found RS viral genome by RT-PCR and protein by immunohistochemistry 60 days after infection. It was not possible in these studies to grow virus from the lung tissue. They also noted increased polymorph infiltrate in bronchial walls with no increase in mast cell numbers or bronchus associated lymphoid tissue (BALT) hyperplasia. These results and my results are in contrast to data from influenza infections (80,179) where viral antigen has been found to persist but not viral genome.
In our experiments it has been possible to show that viral genome is present despite the presence of humoral and cellular immunity. There is evidence that B cell memory requires the presence of antigen (124,171). This is probably through presentation by follicular dendritic cells of trapped antigenic fragments. However views differ on whether cellular immunity also requires antigen persistence. On one side Gray and Matzinger have shown that transfer of T cells without antigen results in absent specific CTL responses in 16 weeks (125). However, Mullbacher suggested that CTL memory to influenza in recipients of

splenocytes from immune animals can be found 25 weeks after transfer (253) in the absence of antigen. Lymphocytic choriomeningitis virus (LCMV) normally induces protective CTL responses that are also associated with immunopathology (55,382). However, Moskophidis *et al* used a strain of LCMV that is not associated with immunopathology and which becomes persistent to show that after infection with high doses of virus, CTL responses may be lost (249). This phenomenon of loss of protective CTL after vigorous early stimulation was described as 'exhaustion' of CTL. This data also suggest that continuous induction of CTL responses requires persistent antigen and that by early exhaustion of CTL through over stimulation with a very high infectious dose may allow persistence. In the experiments in this chapter I have shown that CTL responses remain 90 to 100 days after infection and that the number of priming infections does not alter the specific lysis seen. Mice that were shown to have persistent viral genome had similar levels of specific CTL as mice that were negative by PCR. There was no evidence of exhaustion of CTL responses.

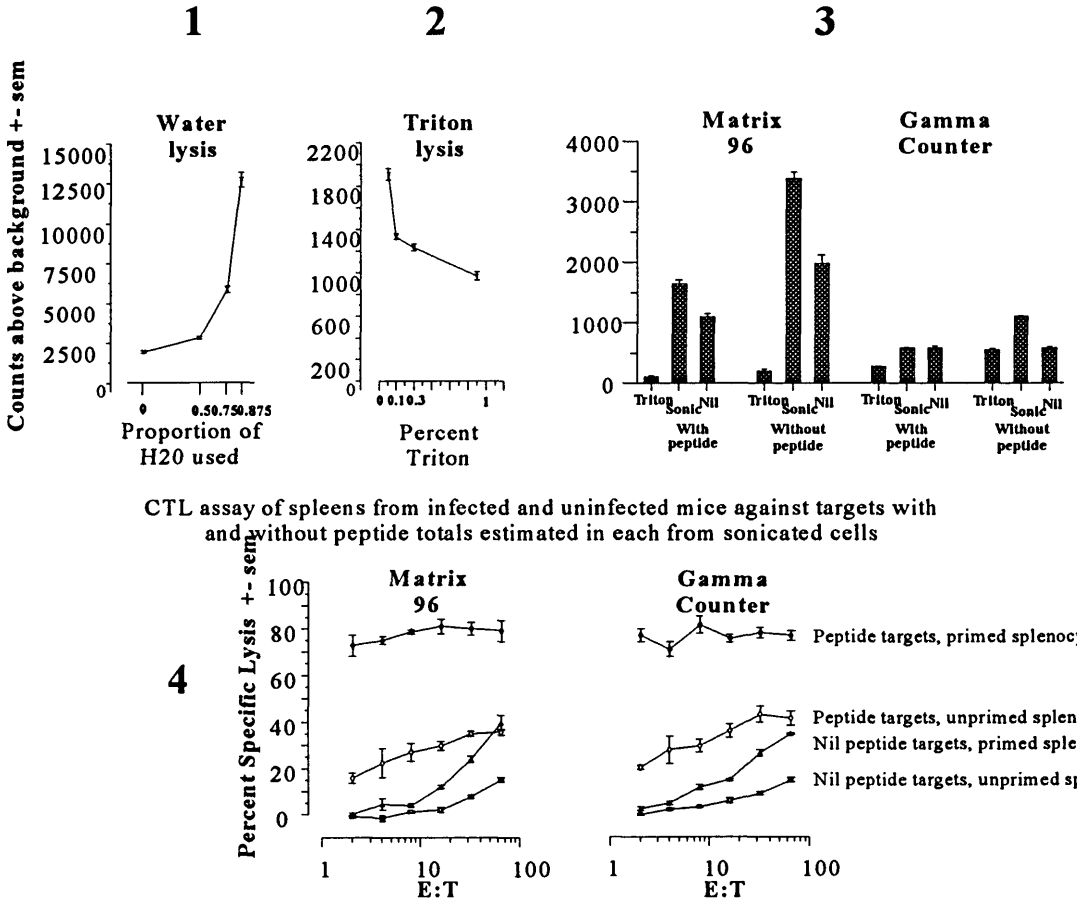
Summary

In summary, it was noted in experiments using IL-3 deficient mice that RS virus persists in the lung after acute infection. Further studies in BALB/c mice showed that viral genome could still be found 100 days after the last RS virus infection. This persistence was despite vigorous CTL memory responses and circulating antibody. Cloned PCR product from the lungs of mice with persistent RS virus, showed that the M2 gene still contained the dominant epitope for CTL recognition in the vast majority of clones from all the animals studied.

Chapter 5 Studies of cytotoxic responses after infection.

Hypothesis

The previous chapter showed that virus does indeed persist in the mouse model despite memory CTLs. This persistence could occur through mutation at the site of the dominant epitope for CTL recognition (M2). Sequence analysis of the M2 protein is therefore performed in the following chapter. In addition, peptides of any mutated epitopes are investigated for their ability to inhibit binding of normal M2.



The graphs above show the results of optimisation experiments that determine how artifacts can be introduced into CTL assays if insufficient lysis of targets for maximal chromium release occurs. Graphs 1, 2 and 3 show counts above background for "total" lysis with different methods for aliquots of 10^4 ^{51}Cr labelled P815 cells. Lysis agents added to the wells generating total counts data could either quench (triton) or remove quenching (water) from the counts altering the apparent result (1, 2 and 3). The manufacturer informs me that quenching can be due to a certain amount of beta radiation that is detected by the Matrix 96 in addition to gamma radiation. Figure 4 shows the data from a CTL assay in which sonication was used and counts collected using the gamma counter and the Matrix 96 in the same experiment. Using comparison with gamma counts it was found that sonication gave results equal in both the gamma counter and the Matrix 96 counter. This method of lysis was used from that time in all experiments. The data shown in figures 5.4 and 5.5 conveys that the assay using peptide was comparable to previous methods using whole virus and that a dose response curve exists. These conclusions are not dependant on the absolute level of total lysis therefore the experiments were not repeated and are included here as background information. Further experimental background is given in the introduction that follows.

Chapter 5 Studies of cytotoxic responses after infection

Introduction

De Magristis *et al* (67)showed that variants of an antigenic peptide could antagonise the activation of $CD4^+$ T cell clones to the original peptide. This was not due to differences in avidity of binding to MHC but involved binding to the TCR. APC's first incubated with the stimulatory peptide could be inhibited by next exposing them to a slightly altered peptide. The peptides which antagonised one T cell clone were not inhibitory for others. This indicated that some form of specific recognition of the altered peptide must occur by TCR for inhibition to occur. One explanation for this is that the mutant peptide is capable of binding to the TCR but causes no conformational change or perhaps a different conformational change not consistent with triggering the activation pathway.

Similar effects have recently been shown for $CD8^+$ T cells; potent antagonists could competitively inhibit the killing of target cells pulsed with wild-type peptide (180). Cytotoxic T-lymphocytes (CTL) have an important role in clearing RS virus. Specific $CD8^+$ CTL alone, passively transferred into naïve irradiated BALB/c mice, can eliminate RS virus from the lung (39). Specific $CD4^+$ T helper (T_H) lymphocytes also clear RS virus either alone or in combination with CTL (8,39). For H-2^d mice (BALB/c or DBA/2) that express K^d, Openshaw *et al* showed that the M2 protein of RS virus is the major target for CTL (276). Cells from spleens of mice who had recovered from RS virus infection were stimulated with RS virus *in vitro* and the resulting CTL lysed target cells infected with recombinant vaccinia virus (rVV) containing a cDNA for M2 protein. After prior sensitisation using M2-rVV, mice infected with RS virus eliminated virus more quickly than

controls (264). This protection was found to be due to T lymphocytes that were almost exclusively $CD8^+$ CTL.

Alwan *et al*, working in this laboratory, primed mice with rVV expressing different RS virus proteins. Splenocyte cultures were generated using irradiated feeders infected with whole RS virus. Interestingly, just changing the protein to which the mice were exposed changed the proportion of CD4+ and CD8+ T cells in the culture. T-cell lines from mice primed with the G protein rVV produced 90% $CD4^+$ cells and 8% $CD8^+$ cells. The supernatants from these cultures contained IL-3, IL-4 and IL-5 suggesting a T_H2 profile. Spleen lymphocytes from mice primed with the F protein rVV were 62% $CD4^+$ and 35% $CD8^+$,and produced IL-2 and IL-3 in antigen specific lines, suggesting a T_H1 profile. However, splenic T cells from mice primed with M2-rVV failed to grow in response to RS virus, in the absence of exogenous cytokines. When cytokines were added to maintain the culture (supernatants from ConA stimulated rat splenocyte cultures) the cells proliferated and were overwhelmingly $CD8^+$ (8,9).

In other viral diseases, such as those caused by lymphocytic choriomeningitis virus (LCMV) (287), hepatitis B virus (79) and human immunodeficiency virus (HIV) (286), it has been suggested that changes in virus sequence may occur as a result of pressure from CTL. In human HIV studies Phillips *et al* showed that changes in amino acid sequences tend to cluster at or near known sites of CTL recognition. Over time, as a result of these changes, there may be loss of CTL recognition of epitopes in certain MHC class I haplotypes (286), for example HLA B8. At other sites and with different MHC proteins, CTL recognition may not cause escape by viral mutation. This appears to be so in the patients with HLA B27.

Conservation of virus T-cell epitopes could indicate that the region must be conserved to ensure virus survival. Therefore these epitopes would be expected to elicit sustained CTL responses (286). It will be important in the design of any future vaccines to understand the mechanisms of change or conservation of virus sequence. It has been suggested that, there may be an advantage to a virus if avid CTL binding occurs but proliferation is not induced (199). The effect of not inducing proliferation would be expected to be especially beneficial, where it occurred with other virus escape or immune modulation mechanisms. There is little sequence data available about the M2 gene, although one A sequence and one B strain sequence have been published (57). It has a major open reading frame (ORF) which codes for a 22 kilodalton protein. The sequence also contains a second ORF that codes for a smaller 9.0kd protein. This smaller protein has been synthesized in vitro but has not been demonstrated in vivo. Falk *et al.* have suggested a consensus sequence for K^d restricted CTL epitopes (88). Kulkarni *et al.* have shown that one of the four predicted epitopes, is the immunodominant CTL epitope for K^d (SYIGSINNI at location 82-90 in the peptide sequence) and that this epitope is shared by both A and B RS virus subgroups (208).

It was shown in the last chapter that RS virus persists in the lung of mice 100 days after infection. In the experiments in this chapter it was determined whether this persistent RS virus still contained the dominant epitope for CTL recognition. An altered sequence cloned from persistent RS virus in the lung was tested to establish whether or not it could lead to the escape from or antagonism of CTL responses.

Results:

a) Mutant virus sequences may occur at the site of immunodominant epitopes after RS virus infection in mice.

Design: 8 to 12 week old BALB/c mice were infected i.n. with 5×10^5 pfu A2 RS virus. Mice were infected once, three times or not at all. 90 to 100 days after the last infection the mice were given a lethal intraperitoneal injection of pentobarbitone and exsanguinated through the femoral vessels. The lungs were removed and snap frozen. 22 to 25mg sections of lung were homogenised and resuspended in a volume of 200µl in PBS. RNA extraction was performed using the Qiagen blood kit. cDNA synthesis was performed using a negative sense oligonucleotide (complimentary to cellular mRNA transcripts of viral RNA). Nested PCR was performed using outer and inner primers for M2. Positive PCR product was identified by visualisation on a 2% agarose gel. Aliquots of positive PCR product were used for ligation into the pCRII plasmid (TA Cloning Kit, Invitrogen). Supercompetant E. Coli (One Shot, TA Cloning Kit, Invitrogen) were transformed using ligated pCRII plasmids. Colonies of E. Coli showing disruption of the LacZα gene were selected and expanded in medium. DNA was extracted by miniprep and confirmation of the insert made by restriction digest and visualisation on a 2% agarose gel (figure 5.1). After annealing to a primer close to the site of the dominant epitope for K^d (figure 5.2), the DNA was sequenced using the Sequenase 2 chain terminator method. Sequenced DNA was run on a 6% polyacrylamide gel.

Results: PCR products from four mice that were shown to have persistent RS virus genome were cloned and sequenced. 12 clones were tested for each mouse. 36 clearly readable sequences were obtained. From these sequences one clone from one mouse showed an alteration at the site of the dominant epitope for K^d recognition. This clone was sequenced on three separate occasions to confirm no error had taken place during the sequencing procedure and the same result was obtained. The sequence alteration was a double deletion; a loss of a T and an A at the 3' end of the epitope when given in its coding sense. This double deletion would cause an alteration of the normal isoleucine at position 9 of the epitope to an asparagine (figure 5.3). This alteration changes the sequence of the epitope from SYIGSINNI to SYIGSINNN.

b) Specific CTL lysis of target cells incubated with SYIGSINNI peptide

Design: 8 to 12 week old female BALB/c mice were infected pernasally with 5×10^5 pfu A2 RS virus. After at least three weeks the mice were killed by cervical dislocation and the spleens harvested in an aseptic manner. Bulk cultures were made using stimulators infected with 2pfu/cell RS virus at a stimulator to effector ratio of 1:4. The bulk cultures were harvested after 5 days for use in the CTL assay. P815 cells, a mouse cell line bearing K^d class 1 MHC molecules, were used as targets. Targets were incubated overnight (16 hours) with vaccinias when targets infected with vaccinias were used. Targets were incubated for 2 hours when peptide targets were used. In both cases target labelling was with ^{51}chromium for at least 40 minutes followed by washing to remove excess radioisotope. CTL prepared from the 5 day bulk cultures were set up for 3 hours with labelled targets after which supernatants from the assay were tested for release of ^{51}chromium by lysis of targets.

Percent specific lysis was calculated as given in the methods chapter except that in the experiments for figures 5.4 and 5.5 maximum release was determined with hypotonic lysis using water.

Results: CTL from RS virus infected mice lysed targets incubated with SYIGSINNI peptide more potently than targets incubated with recombinant vaccinia virus encoding M2 (figure 5.4). For vaccinia targets spontaneous release was approximately 20% of the maximum release, whereas for peptide targets it was approximately 3%. Maximum specific lysis was found to be at peptide concentrations of 10^{-9} to 10^{-8}M (figure 5.5). In this experiment it was found that maximum lysis exceeded 100%. This is clearly not possible. A series of experiments (not given here) determined that this was an artefact caused by incomplete lysis of the cells used to determine the totals. This gave an underestimate of the true maximum lysis. Sonication was used to determine maximum lysis for all other experiments after it was shown to be optimal. The data is shown here despite the problems with the level of lysis because it still demonstrates clearly the dose response relationship for the peptide SYIGSINNI.

c) The mutant epitope is a partial agonist for CTL lysis.

Design: CTLs were derived from BALB/c mice in a similar way to b). Peptides SYIGSINNI, SYIGSINNN and TYQRTRALV were kindly made for me by Dr Adrian Hill. TYQRTRALV (a K^d restricted influenza NP CTL epitope) was used as a negative control. CTL were tested in a three hour assay of lysis of ^{51}chromium labelled targets incubated with different concentrations of peptide.

Results: Targets incubated with TYQRTRALV were not lysed efficiently by CTL from RS virus mice. Targets incubated with SYIGSINNN were lysed by CTL from RS virus infected mice. The lysis of targets incubated with SYIGSINNN was less potent than targets incubated with SYIGSINNI (figure 5.6).

d) The mutated peptide SYIGSINNN fails to antagonise the lysis of targets incubated with SYIGSINNI.

Design: CTLs were derived from BALB/c mice in a similar way to b). Targets were first labelled with ^{51}chromium, washed and then incubated with a suboptimal concentration (10^{-10}M or 10^{-11}M) of the native SYIGSINNI peptide (agonist) for 90 minutes. A second peptide (SYIGSINNN or TYQRTRALV) was incubated with the targets either simultaneously or during a second 90 minute incubation. This second peptide was called the "antagonist". When the peptides were added the agonist concentration (SYIGSINNI) was at a known concentration (as above), the antagonist peptides were added at several concentrations: from 10000 fold less to 1000 fold more. Where the antagonist was added after incubation with the agonist, the target cells were washed once in medium before the second incubation. The targets were exposed to CTL at several effector to target ratios in a three hour assay of lysis.

Results: Lysis of targets incubated with SYIGSINNI was not inhibited by the presence of SYIGSINNN at any concentration. No antagonism was seen whether the second peptide was added at the same time as the native peptide or if a second incubation was performed after washing (figure 5.7). In figure 5.7 these results are expressed for two different E:T ratios

by comparison with the lysis seen in the absence of a second peptide. No inhibition of lysis was seen.

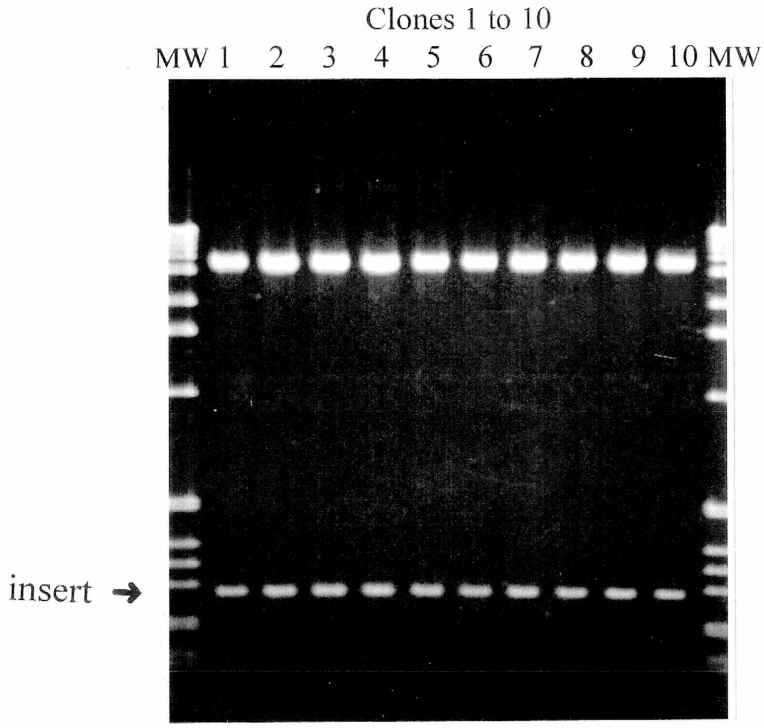


Figure 5.1 Restriction digest with EcoR1
PCR product was ligated into the pCR vector using T4 ligase. Supercompetant E. Coli were transformed using the plasmid. Clones were expanded overnight in medium and purified. Restriction digest using EcoR1 in NEB buffer was performed to confirm that an insert was present. An EcoR1 cleavage site lies close to each end of the insertion site. The correct band appears between 220 and 298bp, consistent with the expected size of the PCR product.

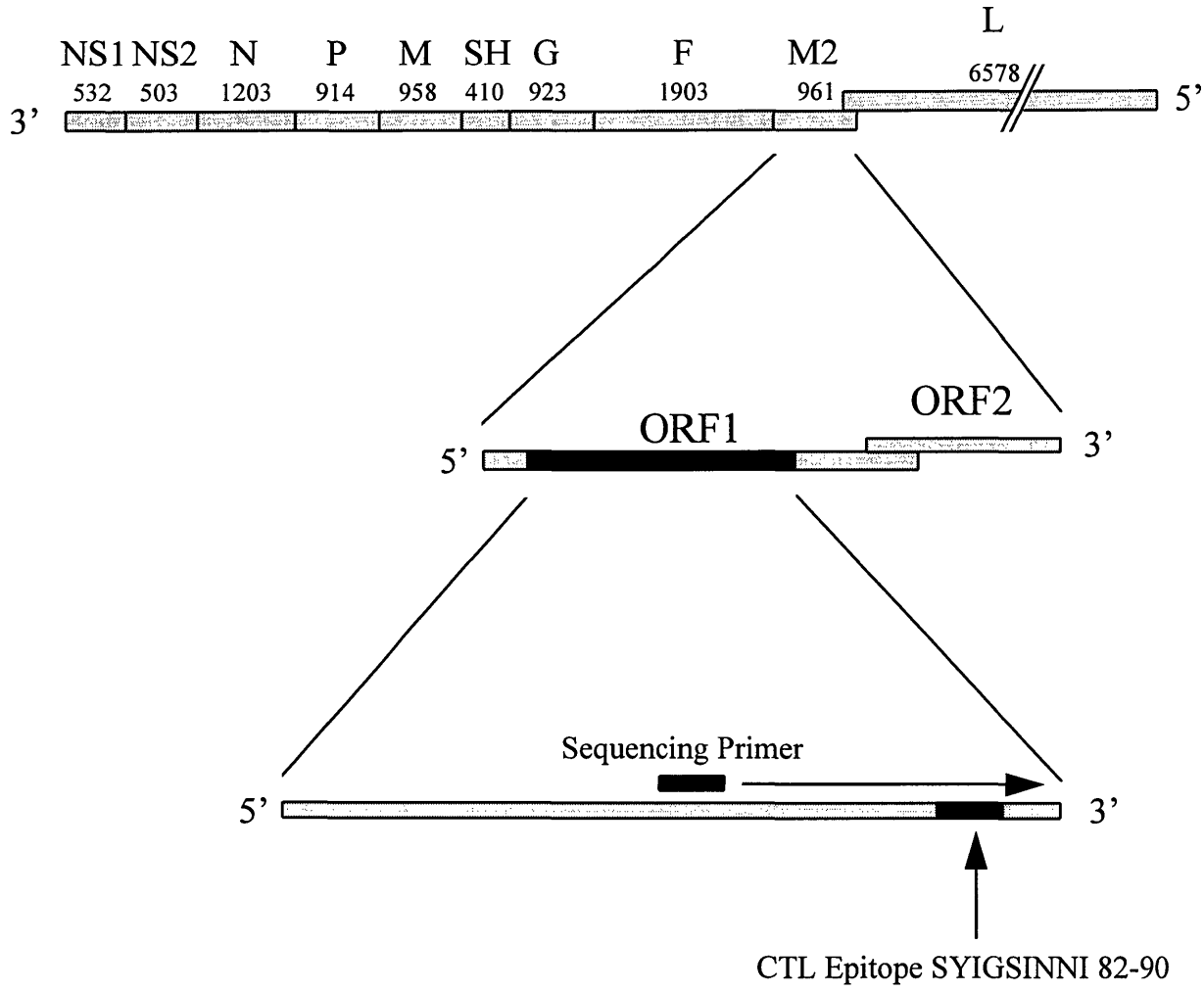


Figure 5.2 Sequencing region for SYIGSINNI (M2 82-90)
The genetic map for RS virus is shown (top) with genes identified by protein (nucleotide length below). This is shown in the negative sense (viral RNA). The segment amplified by primers for M2 is shown below the gene sequence, with the overlap between open reading frame 1 (ORF1) and the second open reading frame (ORF2). This is shown in the positive (coding mRNA) sense. At the bottom is the amplified segment using nested primers for M2 with the approximate position of the dominant epitope for K^d CTL (SYIGSINNI).

Known epitope

5'	GTG	CTA	GAG	**AGT**	**TAT**	**ATA**	**GGA**	**TCA**	**ATA**	**AAC**	**AAT**	**ATA**	ACT	AAA	3'	Nucleotide
	Val	Leu	Glu	**Ser**	**Tyr**	**Iso**	**Gly**	**Ser**	**Iso**	**Asn**	**Asn**	**Iso**	Thr	Lys		Amino acid
				1	2	3	4	5	6	7	8	9				Epitope position

Altered Epitope

5'	GTG	CTA	GAG	**AGT**	**TAT**	**ATA**	**GGA**	**TCA**	**ATA**	**AAC**	**AAT**	**AAC**	TAA	ACA	3'	Nucleotide
	Val	Leu	Glu	**Ser**	**Tyr**	**Iso**	**Gly**	**Ser**	**Iso**	**Asn**	**Asn**	**Asn**	Stop	Pro		Amino acid
				1	2	3	4	5	6	7	8	9				Epitope position

Figure 5.3 Sequence of the mutated clone - showing deletion of two bases
A single clone was shown to contain a mutation at the site of the known epitope for K^d CTL recognition of M2. This mutation was a double deletion (indicated above) and altered the derived peptide sequence from SYIGSINNI to SYIGSINNN (shown below). This peptide was then made and tested to determine first, whether it was able to be a target for CTL lysis and then whether it antagonised recognition of the native sequence SYIGSINNI.

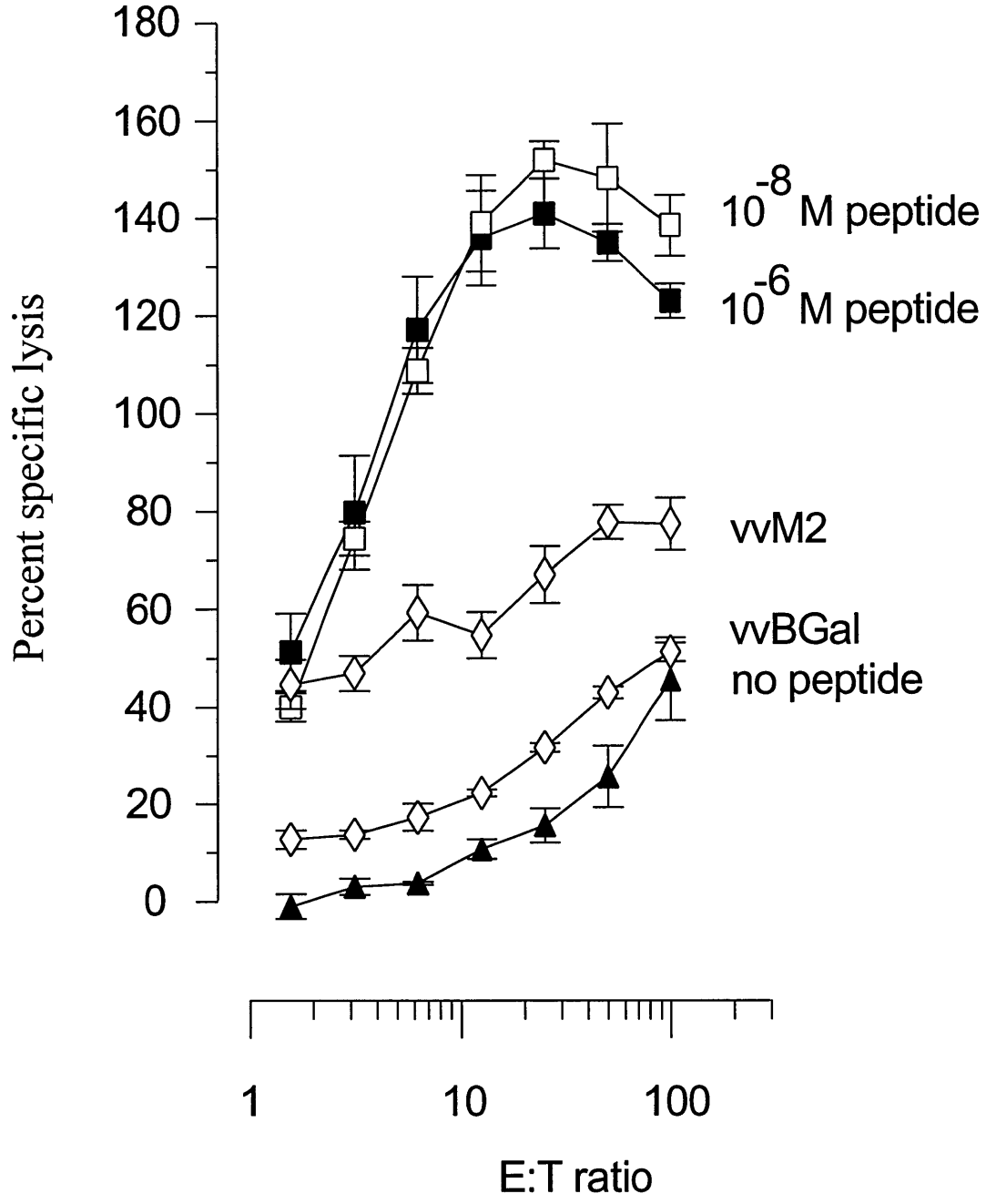


Figure 5.4 CTL lysis of P815 targets incubated with peptide or vaccinia recombinants. 8-12week old BALB/c mice were infected i.n. with A2 RS virus. After three weeks the spleens were removed and splenocytes were cultured with A2 RS virus for 5 days. Targets labelled with ^{51}chromium were incubated with peptide (SYIGSINNI) at different concentrations or vaccinia recombinants encoding either M2 or β-Gal. CTL were tested in a 3 hour assay for lysis of these different targets. Unfortunately, in this experiment totals estimates were incorrect due to an artefact (see results).

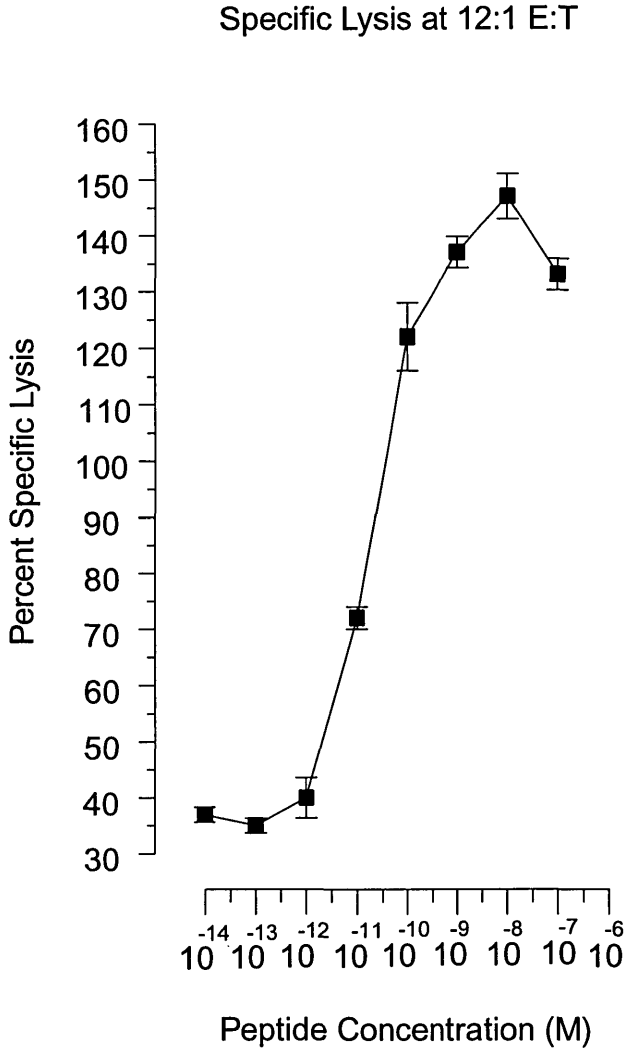


Figure 5.5 CTL lysis of P815 targets with peptide SYIGSINNI at different concentrations. 8-12week old BALB/c mice were infected i.n. with A2 RS virus. After three weeks the spleens were removed and splenocytes were cultured with A2 RS virus for 5 days. Targets labelled with ^{51}chromium were incubated with peptide (SYIGSINNI) at different concentrations. CTL laysis was tested in a 3 hour assay. The results at a representative effector to target ratio of 12:1 are shown.

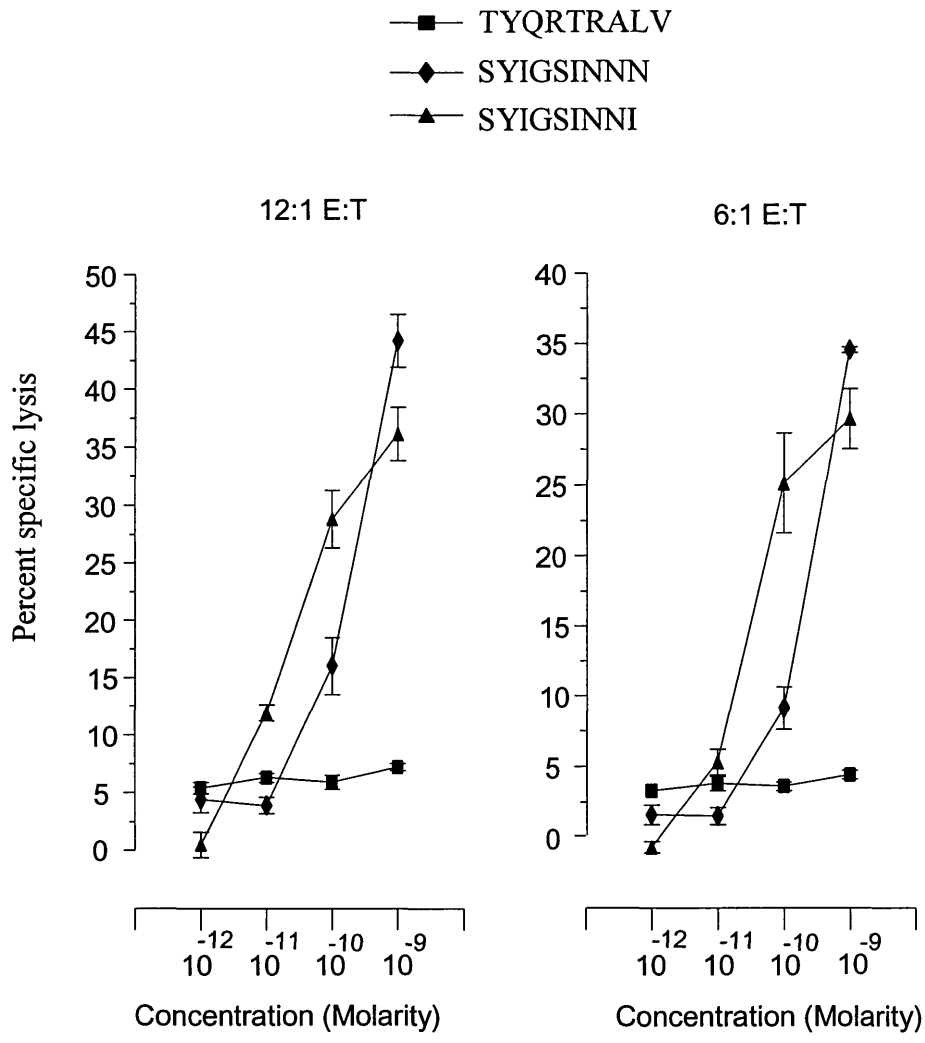


Figure 5.6 CTL lysis of targets with SYIGSINNI, SYIGSINNN or TYQRTRALV. 8-12week old BALB/c mice were infected i.n. with A2 RS virus. After three weeks the spleens were removed and splenocytes were cultured with A2 RS virus for 5 days. Targets labelled with ^{51}chromium were incubated with peptides SYIGSINNI, SYIGSINNN or TYQRTRALV at different concentrations CTL were tested in a 3 hour assay for lysis of these different targets. Data is shown at two representative effector to target ratios of 12:1 and 6:1.

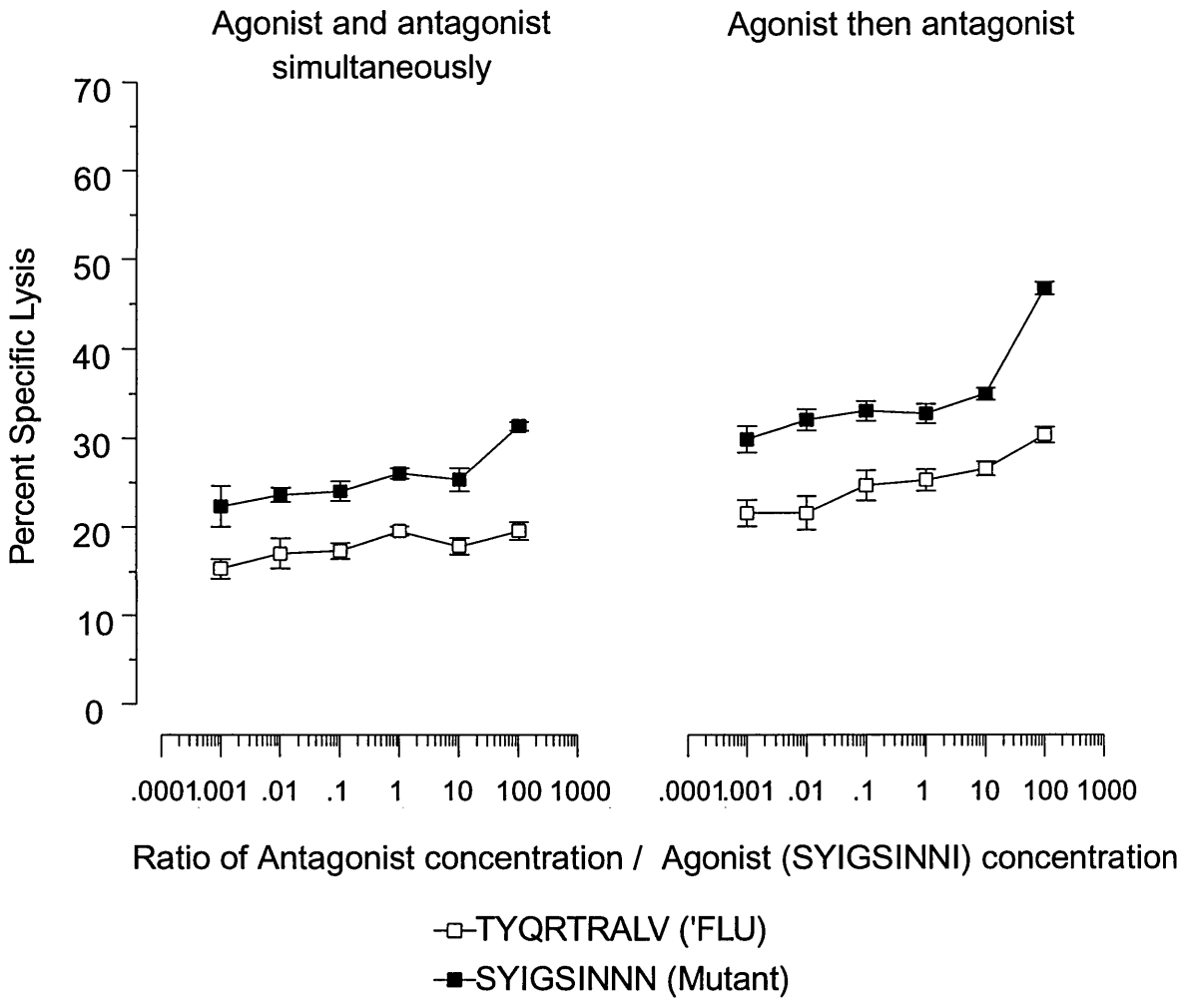


Figure 5.7 Percent lysis with targets exposed to "agonist" and "antagonist" peptides. Splenocytes from RS virus primed mice were cultured with A2 RS virus in 5 day bulk cultures. Targets labelled with ^{51}chromium were incubated with peptide SYIGSINNI at suboptimal concentrations (for the data shown 10^{-10}M). Peptides SYIGSINNN or TYQRTRALV were added to the targets at different concentrations either at the same time as SYIGSINNI (left panel) or after washing and a second incubation (right panel). Each incubation was 90 minutes. CTL were tested in a 3 hour assay for lysis of these different targets. Data is shown at a representative effector to target ratio of 16:1.

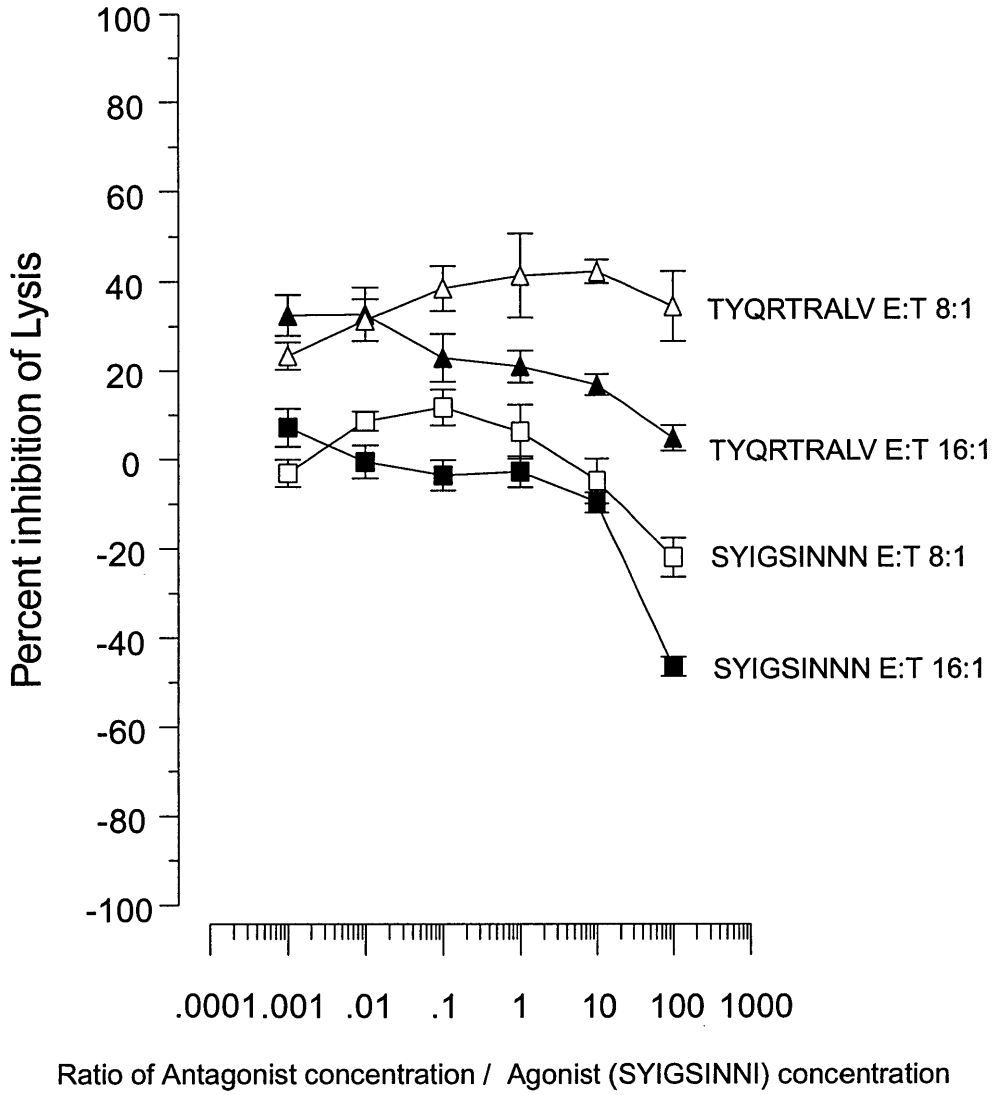


Figure 5.8 Inhibition of lysis: targets exposed to "agonist" and "antagonist" peptides. Splenocytes from RS virus primed mice were cultured with A2 RS virus in 5 day bulk cultures. Targets labelled with ^{51}chromium were incubated with peptide SYIGSINNI at suboptimal concentrations (for the data shown 10^{-10}M). Peptides SYIGSINNN or TYQRTRALV were added to the targets at different concentrations either at the same time as SYIGSINNI (left panel) or after washing and a second incubation (right panel). Each incubation was 90 minutes. CTL were tested in a 3 hour assay for lysis of these different targets. Data is shown at representative effector to target ratios of 8:1 and 16:1 as a percent of the inhibition of lysis. +100% would be no specific lysis and 0% is the same lysis as with SYIGSINNI alone.

Discussion:

This chapter summarises a series of experiments to determine the significance of an altered nucleotide sequence derived from a mouse with persistent RS viral genome 100 days after primary infection. MHC class 1 molecules present peptides derived from cellular proteins to $CD8^+$ class 1 restricted T cells (350,381). It is now possible to isolate and determine the nature of presented peptides from complete cells or purified MHC molecules (315). Viral antigens tested always elute a single sharp peak from reversed HPLC columns indicating that one defined peptide is being made by the cell (88). The type of peptide presented is determined by the type of MHC molecule present on the cell which is encoded by the genome of the individual (345). Falk *et al* have eluted peptides from cells infected with influenza virus and identified the nonapeptide TYQRTRALV from the nucleoprotein (position 147-155) to be the naturally processed epitope (88,89). I chose TYQRTRALV as a control therefore, because it was shown to be the dominant nucleoprotein epitope for influenza infected $H\text{-}2^d$ mice. By comparison of natural and other peptides known to contain CTL epitopes, a consensus sequence of peptides presented by K^d was identified. This sequence is shown below:

Table 5.1: Consensus sequence for K^d (from Falk *et al* (89))

Positions	1	2	3	4	5	6	7	8	9
									V
			Q						**I**
Residues	*	**Y**	L	*	V	A	*	*	**T**
			V		N	G			**A**
			*		*	*			**L**

* indicates variable residue. Bold type and or increased size indicates the importance of the amino acid residue.

It is striking that a tyrosine (Y) at position 2 is common to all peptides associated with K^d. This was consistent with studies by Maryanski *et al* who showed that all the residues between position 2 and position 9 could be replaced by proline without interfering with binding to K^d (231). Position 9 also seems to be an important determinant of binding for MHC molecules of this type. Falk *et al* comment that the presentation of relatively rare allele specific peptide motifs could be a way to ensure that not too many peptides of a single abundant protein are presented at the expense of peptides from less abundant proteins. They have estimated the chance of a Tyr residue followed by either a Val, Ile, Thr, Ala or Leu seven positions later to be 1 in 80 in random nonapeptides (88). From the consensus sequence above it would be predicted that the mutant epitope found in my studies would not be able to bind to K^d. However, it is shown to be able to cause specific lysis in my experiments although it would seem less efficient lysis than the native sequence.

Openshaw *et al* have previously identified that M2 is the major target for K^d restricted CTL in mice after infection (276). Following this Kulkarni *et al* found that of seven RS virus proteins that do not induce neutralising antibodies (M, M2, SH, NS1, NS2, N and P) in H-2^d mice, only M2 was associated with resistance to infection when mice were first primed with vaccinia recombinants containing single proteins of RS virus then challenged with whole virus (207). This protection was through CTL and was absent in other strains (H-2^b and H-2^k). Using vaccinias encoding M2 epitopes predicted by the consensus sequence Kulkarni *et al* then showed that the predominant CTL population induced by RS virus infection in BALB/c mice recognised peptide SYIGSINNI (position 82-90 of M2) (208). This was true for both A and B strains. Using this peptide my experiments showed that the peptide SYIGSINNI was associated with specific lysis of P815 targets by splenocytes from mice

infected with RS virus. The optimal concentration for lysis was 10^{-9} to 10^{-8}M but lysis was seen even at 10^{-11}M. This is consistent with the 10 to 100pM levels previously shown to be physiological (26,88,172) for natural peptide epitopes.

In lymphocytic choriomeningitis virus infection, Aebischer *et al* showed that relevant point mutations occur frequently and that they are selectable in vitro by CTLs (4). Phillips *et al* then showed that individuals infected with human immunodeficiency virus (HIV) evolved mutations over time which were not recognised by CTL (286). In my studies with RS virus only one mutation at the site of the CTL epitope was found and therefore in order to alter the recognition of the virus it would not be sufficient merely to fail to be recognised but would have to be actively antagonistic. Such antagonism has been previously shown for both HIV and hepatitis B virus (HBV). Klenerman *et al* isolated variant sequences from adults with HIV and showed inhibition of lysis by the derived peptide of targets prepulsed with suboptimal concentrations of the agonist peptide (199). Bertoletti *et al* showed similar findings for HBV, showing inhibition of lysis was most pronounced when the target cells were first incubated with the agonist peptide, then washed and incubated with the antagonist (26). In both cases antagonism was demonstrable at antagonist to agonist ratios of 0.1 to 1 and remained at several higher antagonist levels. The antagonists in both these experiments were partial agonists for CTL recognition and at higher concentrations induced specific lysis. In my experiments the mutant peptide (SYIGSINNN) appeared to be a partial agonist for CTL recognition but no antagonism was found. The significance of this mutant is not clear. The possibility exists that it represents an error during PCR amplification. However the Thermus aquaticus DNA polymerase (Taq) has a low error rate producing deletion or frameshift error at a frequency of about 1/40,000 (18,347). The genetically engineered

Stoffel fragment of Taq has better proofreading ability and lower error rate probably a half that of normal Taq (personal communication - Applied Biosystems). For this reason it would seem unlikely to have been a technical artefact. However mutations in transcription in vivo occur frequently with RNA viruses and this mutant may reflect this.

Summary

In summary this series of experiments demonstrate that mutation at the site of the epitope for K^d binding in M2 is rare, occurring in 1 clone out of 36. The cloned sequences came from 4 separate immunocompetent mice shown to have persistent RS virus in the lung, 100 days after infection, by nested RT-PCR. The peptide derived from this mutation was associated with decreased CTL lysis of targets at low concentrations. Although in other viruses epitopes associated with antagonism have also been partial agonists, no evidence of antagonism was found for this mutation.

Chapter 6 Effect of immunosuppresion after infection

Hypothesis

In a previous chapter, after a single infection with RS virus, genome was still detectable 100 days later in lung by nested RT-PCR. CD4 and CD8 positive T cells are important in clearing RS virus and many other viral infections. Immunocompromised people suffer from recurrent viral infections and RS virus infections can be prolonged. In other animal models of viral infection, removal of cell mediated immunity using radiation, chemotherapy or depleting antibodies results in reactivation of latent virus. In the following chapter, the role of T cells in controlling latent virus is addressed by using depleting antibodies to remove CD4+ and CD8+ T cells. Though persistent genome was detected in a previous chapter it is not known whether this virus is still in an infectious form. Removal of cell mediated immunity may allow latent virus to grow to a level that is detectable by conventional culture techniques.

Chapter 6 Effect of immunosuppression after infection

Introduction

For a virus to become persistent in an immunocompetent host it must: retain genetic material within host cells, be non-lytic and avoid host immunity. In many cases viruses are able to persist only at very low levels and in specific cell types (335). RNA viruses are particularly prone to mutation and persistent RNA virus infections are often associated with defective viral genomes that may have been selected by the host environment or a vigorous immune response (301). Defective viral RNAs may be amplified using RT-PCR but may not indicate that infectious virus is present. In chapter 4 I showed that RS virus genome could be detected in lung homogenates long after it was cleared from the bronchoalveolar lavage. 100 days after a single infection with RS virus, genome was still detectable in lung by nested RT-PCR.

Pringle *et al* showed that BS-C-1 cells could become persistently infected *in vitro* with temperature sensitive mutants of RS virus at nonpermissive temperatures (295). The persistent virus was capable of forming plaques and persistently infected cells contained abundant RS virus antigen internally but little at the surface. It was suggested that the virus was partly defective in maturation and infectious virus could not be extracted to infect fresh BS-C-1 cells. Mutated measles viruses also persist both *in vitro* and *in vivo* (45,46) and hypermutation has been found *in vitro* in persistent human parainfluenza virus infection (259). Canine distemper virus (CDV), a single stranded negative sense RNA virus like measles, parainfluenza and RS virus, can also become persistent *in vivo* and *in vitro. In vivo*, CDV persistence can be associated with a progressive demyelinating disease of dogs

(60). In contrast to measles and parainfluenza viruses CDV persistence is not associated with defective virus production in primary dog brain cultures (383).

In the experiments that follow mice were infected with RS virus and immunosuppressed using monoclonal antibodies against T cell surface antigens (CD4 and CD8). As suggested by prolonged virus shedding in infants with defects in cell mediated immunity (reviewed in (131)), T cells may be the most effective arm of the immune system in controlling RS virus. In persistent infection, cell mediated immunity may be important in keeping virus at a low level. It was hypothesised that abolishing the T cell response would allow RS virus to replicate at a higher level within the lung and that if enough virus was present, detection could be by conventional virus culture. This would indicate whether RS virus was persisting in a complete infectious form or as a defective virus.

Results:

a) Immunosuppression of mice after primary infection

Design: Three groups of 8-12 week old female BALB/c mice were used. Group 1 were mock infected and immunosuppressed 120 days later (depleted/uninfected). Group 2 were infected once pernasally with 5×10^6pfu of A2 strain RS virus then immunosuppressed 120 days later (depleted/infected). Group 3 were infected once pernasally with 5×10^6pfu A2 strain RS virus but not immunosuppressed (undepleted/infected). Immunosuppression was achieved using T cell depleting antibodies kindly provided Dr S. Cobbold (William Dunn School of Pathology, Oxford). Mice were kept in filter top cages with autoclaved bedding and received autoclaved feed and water. During the period of immunosuppression the cages were transferred to a filter cabinet unit for extra protection. T cell depletion was achieved by i.v. and i.p. injection of antiCD4 and antiCD8 according to the schedule given in table 6.1 below:

Table 6.1: Schedule of immunosuppression with T cell depleting antibodies

Day	Dose	Route
0	1mg antiCD4+1mg antiCD8	i.v.
4	1mg antiCD4+1mg antiCD8	i.v.
11	1mg antiCD4+1mg antiCD8	i.p.
18	1mg antiCD4+1mg antiCD8	i.p.
25	1mg antiCD4+1mg antiCD8	i.p.
30	1mg antiCD4+1mg antiCD8	i.p.

Antibodies were at a concentration of 10mg/ml anti-CD4 and 10mg/ml anti-CD8 and were

stored at -20°C. The antibodies used were equal mixtures of two synergistic antibodies for CD4 (YTS 191.1 (53), YTA 3.1 (300)) and two for CD8 (YTS 169.4 (53), YTS 156.7 (301)). Before use aliquots of anti-CD4 and anti-CD8 were thawed and mixed together in equal volumes. 200µl of the mixed antibodies was injected i.v. via the tail veins or i.p. according to the schedule. This amount was estimated to be approximately twice the amount normally required to produce immunosuppression (Dr S. Cobbold - personal communication). During the induction of immunosuppression the mice were observed and weighed. 5 days before the end of the experiment representative mice chosen at random were bled via the tail vein to confirm T cell depletion had occurred. Approximately 200µl of blood was put into pre-heparinised 1.5ml eppendorf tubes. The blood cells were stained for CD4 and CD8 and flow cytometric analysis of lymphocyte subsets performed. At the end of the experiment splenocytes from three randomly chosen mice were stained for CD4 (Fitc conjugated rat IgG2a antimouse, at 1.25µg/ml, Sigma) and CD8 (PE conjugated rat IgG2a antimouse, at 1.25µg/ml, Sigma) or CD3 (PE conjugated rat IgG2b antimouse, at 1.25µg/ml, Sigma) and B220 (first layer: biotin conjugated rat IgG2a antimouse, at 0.5µg/ml, Sigma, second layer: streptavidin conjugated Quantum Red at 1/75 concentration, Sigma). Flow cytometric analysis was again performed to check that treatment had depleted T lymphocytes from the spleen as well as the circulation. The data shown is for cells within the lymphocyte gate.

Results: During the induction of immunosuppression the mice appeared well with no fur ruffling, obvious respiratory illness or discomfort. There was no significant weight loss compared with control mice (figure 6.1). Flow cytometric analysis of peripheral blood showed that effective T cell depletion had occurred (figure 6.2). When splenocytes were

analysed by flow cytometry a residual $CD3^+$ population remained (figure 6.3). This represented approximately 4% in uninfected T cell depleted mice and 10% in previously infected T cell depleted mice (figure 6.4) compared with about 32% in undepleted mice. Analysis of CD4 and CD8 subsets showed that although most CD8 cells had been effectively removed (less than 2% compared with 10% in undepleted mice), there remained a small but significant population of CD4 cells (figure 6.5). This result was expressed as a proportion of the lymphoid population (figure 6.6). CD4 cells were markedly less depleted in mice previously infected with RS virus than in uninfected mice. The mice were treated at the same time with exactly the same antibody depletion, and it may be that this small residual population was proliferating in response to emerging RS virus making complete depletion harder to attain in a solid organ like the spleen.

b) CTL responses are abolished in immunosuppressed mice but serum antibody to RS virus remains.

Design: After immunosuppression the mice were given a lethal i.p. injection of pentobarbitone, the femoral vessels were exposed and transected and the mice exsanguinated. The blood was collected and the serum obtained. Specific antibody to RS virus was determined by ELISA. Spleens were removed and a single cell suspension of splenocytes made. Bulk cultures were made from 1.5×10^7 splenocytes from three representative spleens from each group. For each bulk culture normal uninfected BALB/c mouse splenocytes were infected with 2pfu/cell of A2 RS virus for 90 minutes and used as stimulators at a ratio of 1 stimulator to 4 responders. Bulk cultures were harvested 5 days later and used in a routine CTL assay. Targets were made using ^{51}chromium labelled P815 cells incubated with or without peptide SYIGSINNI.

Results: In mice that were T cell depleted after infection, antibody to RS virus remained at a similar level to control mice not given depleting antibodies after infection (figure 6.7). Uninfected mice had no detectable anti-RS virus antibody. CTL responses in mice that were T cell depleted after infection were abolished compared with mice that did not have depleting antibodies (figure 6.8). Cell numbers in bulk cultures from immunosuppressed mice were much lower than from mice that had not received depleting antibodies. This is the reason why effector to target ratios in the CTL assays shown are relatively low.

c) RS virus was grown from homogenates of lung 150 days after primary infection.

Design: Mice immunosuppressed using monoclonal antibodies to CD4 and CD8 as described in a) were humanely killed as described in b). The lungs were removed aseptically. The right upper lobe and right lower lobe were placed in RPMI medium at room temperature. The lung samples from each mouse were homogenised using a manual glass tissue homogeniser and made up to a volume of 500µl with serum free RPMI with penicillin, streptomycin, 2ME and glutamine. Positive and negative control wells were by serial dilution of A2 RS virus to 1000pfu, 100pfu 10pfu and 0pfu in a volume of 300µl serum free medium as above. 300µl was placed on a monolayer of HEp-2 cells in a 2ml tissue culture well and incubated for 2 hours at 37°C. The volume was made up to 2ml using RPMI containing 10% FCS, penicillin, streptomycin, 2ME and glutamine. The cultures were incubated at 37°C for 48 hours. The supernatants were removed gently using a micropipette and 500µl of serum free medium with antibiotics added. The cells were harvested using a scraper and vigorously resuspended several times using a 1000µl micropipette. The 500µl contents of each well was then sonicated in a 13ml conical tube for 1 minute. 100µl aliquots were placed onto fresh HEp-2 monolayers in a 96 well plate (flat bottomed) and incubated

2 hours at 37°C. Then each well was made up to 200µl with medium containing 10% FCS, penicillin, streptomycin, 2ME and glutamine. After 24 hours incubation at 37°C the plates were washed twice with PBS before fixation and staining using the protocol of microplaque assay for infective virus. The microplaque assay was read by a colleague who was blinded to which group was which.

Results: Four out of six mice that were infected 150 days before and then immunosuppressed were found to have RS virus by microplaque assay. The amount of virus was equivalent to approximately 10pfu of control virus in 3 out of these 4. In one out of the four positive samples the amount of virus was closer to 100pfu than to 10pfu. 1 mouse out of 6 mice previously infected but not immunosuppressed was weakly positive for RS virus equivalent to less than or equal to 10pfu of control virus. No mice were found to have RS virus in the uninfected group.

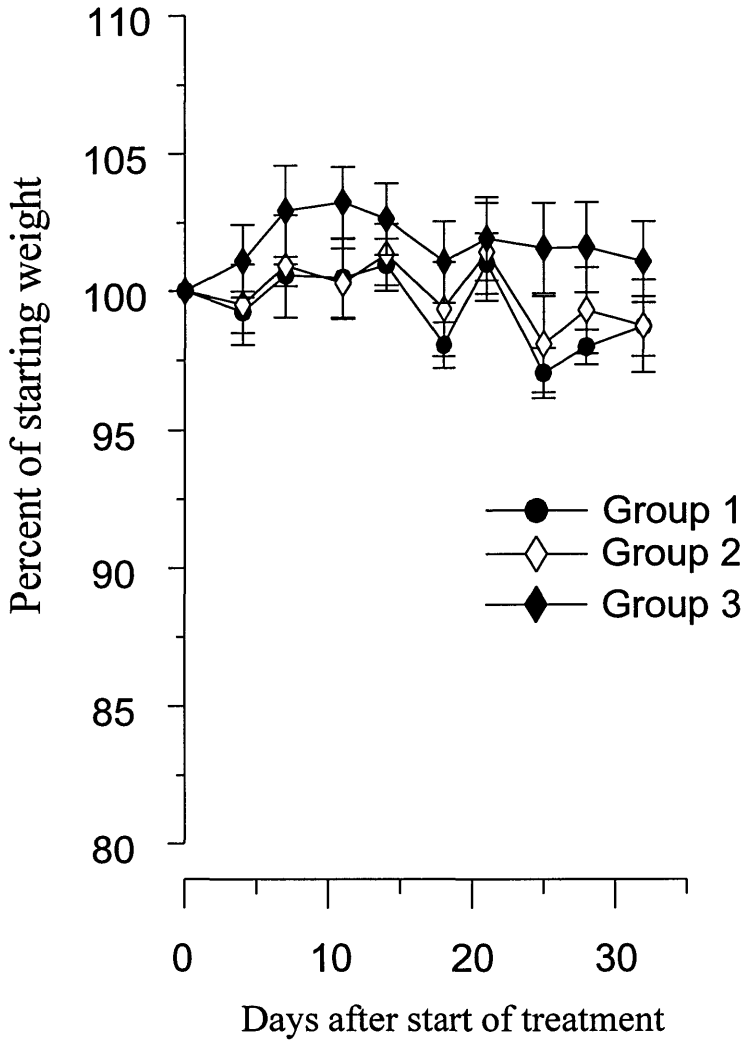


Figure 6.1 Weight loss after depletion of CD4 cells and CD8 cells with antibodies 8-12 week old BALB/c mice were infected intranasally with RS virus or left uninfected (controls). 120 days after infection CD4 and CD8 lymphocytes were depleted using monoclonal antibodies (as in methods). Group 1 were uninfected and immunosuppressed. Group 2 were infected and immunosuppressed. Group 3 were infected but not immunosuppressed.

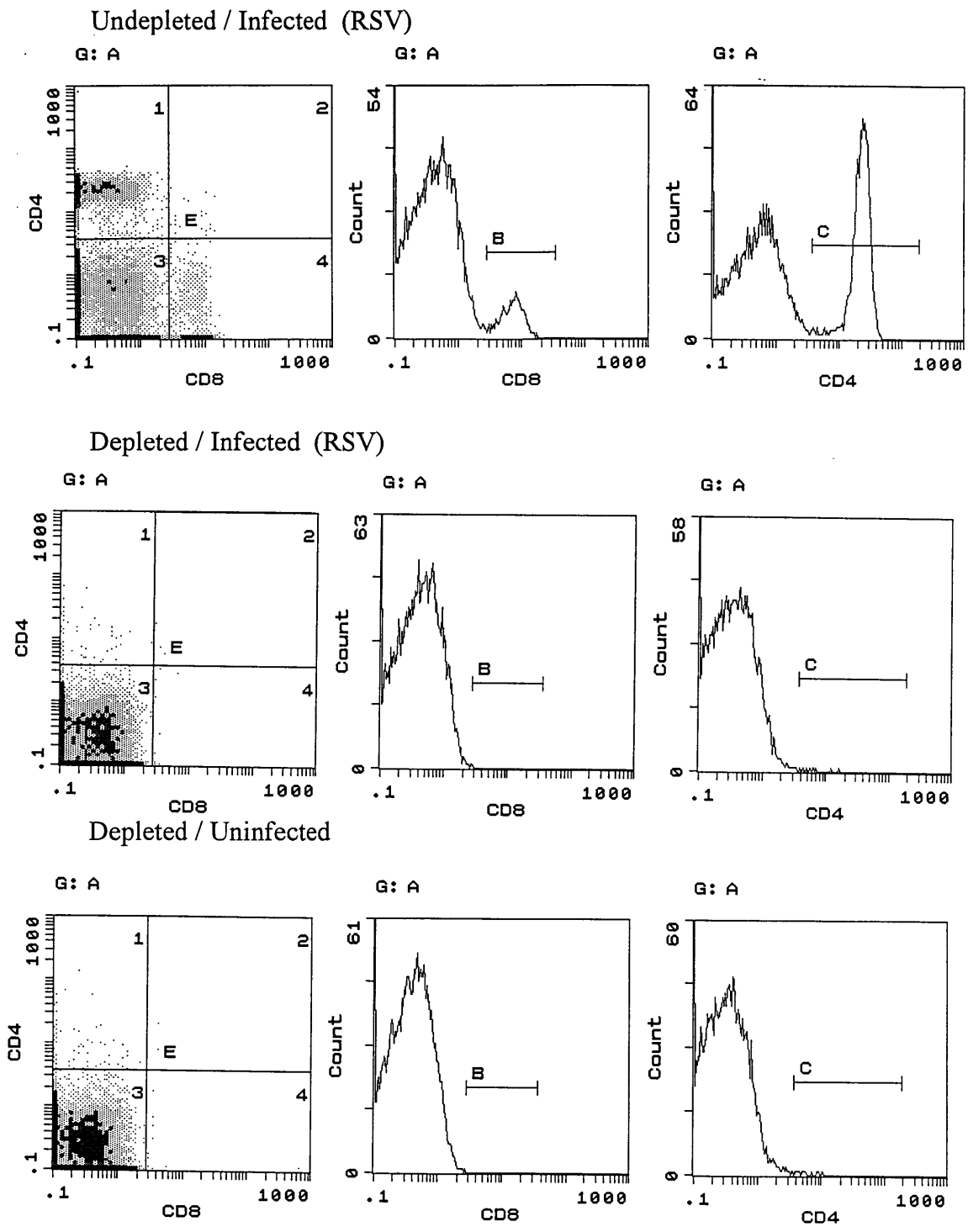


Figure 6.2 Flow cytometric analysis of peripheral blood after CD4/8 depletion
8-10week old BALB/c mice were infected with RS virus or not infected (controls). 120 days later T cell depletion was performed using monoclonal antibodies. 5 days before the end of the experiment, immunosuppression was checked by bleeding representative mice (via the tail vein). Flow cytometric analysis of lymphocytes was performed for CD4 and CD8 cells. The results for undepleted/infected mice (top), depleted/infected mice (middle) and depleted/uninfected mice (bottom) are shown.

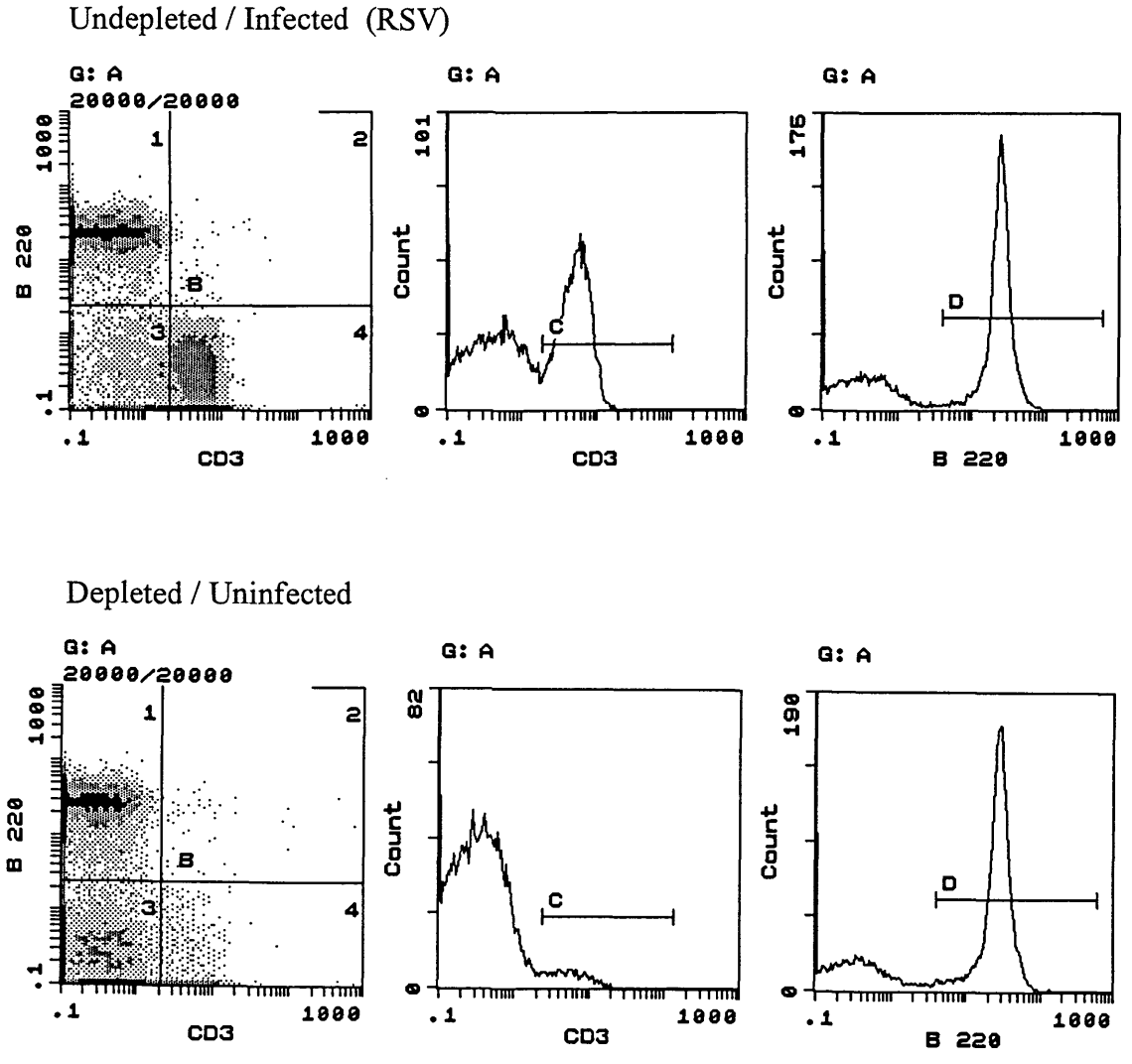


Figure 6.3 Flow cytometric analysis of splenocytes after CD4/8 depletion
8-10week old BALB/c mice were infected with RS virus or not infected (controls). 120 days later T cell depletion was performed using monoclonal antibodies. At the end of the depletion period, splenocytes were analysed by flow cytometry for CD3, CD4, CD8 and B220 (B cells). The results of CD3/B220 staining for undepleted/infected and depleted uninfected mice is shown above.

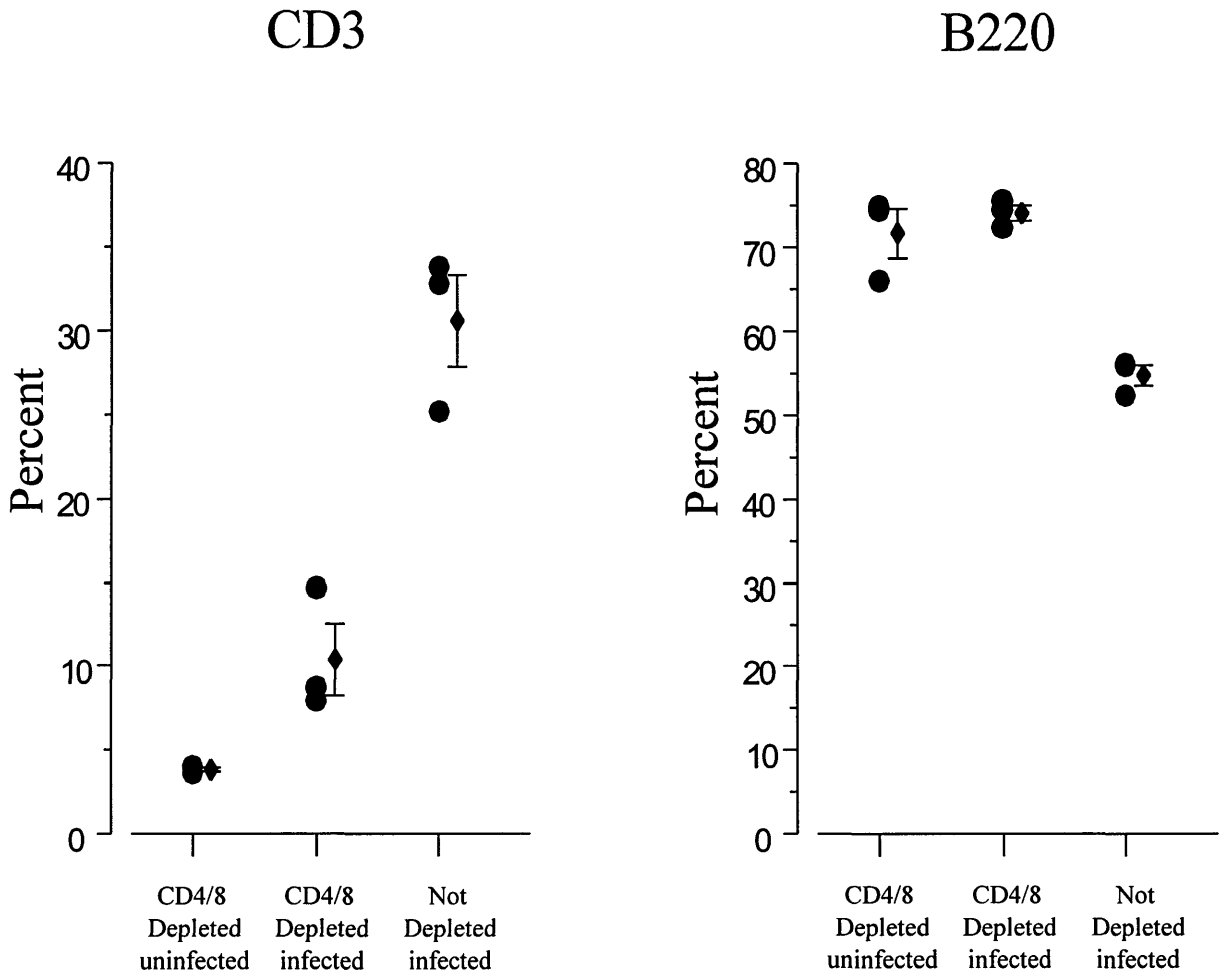


Figure 6.4 Percentages of CD3 positive and B220 positive cells in spleen after depletion. 8-12 week old BALB/c mice were infected intranasally with RS virus or left uninfected (controls). 120 days after infection CD4 and CD8 lymphocytes were depleted using monoclonal antibodies (as in methods). Splenocytes stained with fluorochrome conjugatedantibodies (as in chapter 2) were analysed by flow cytometry. The percentage of CD3 and B220 positive cells is shown above.

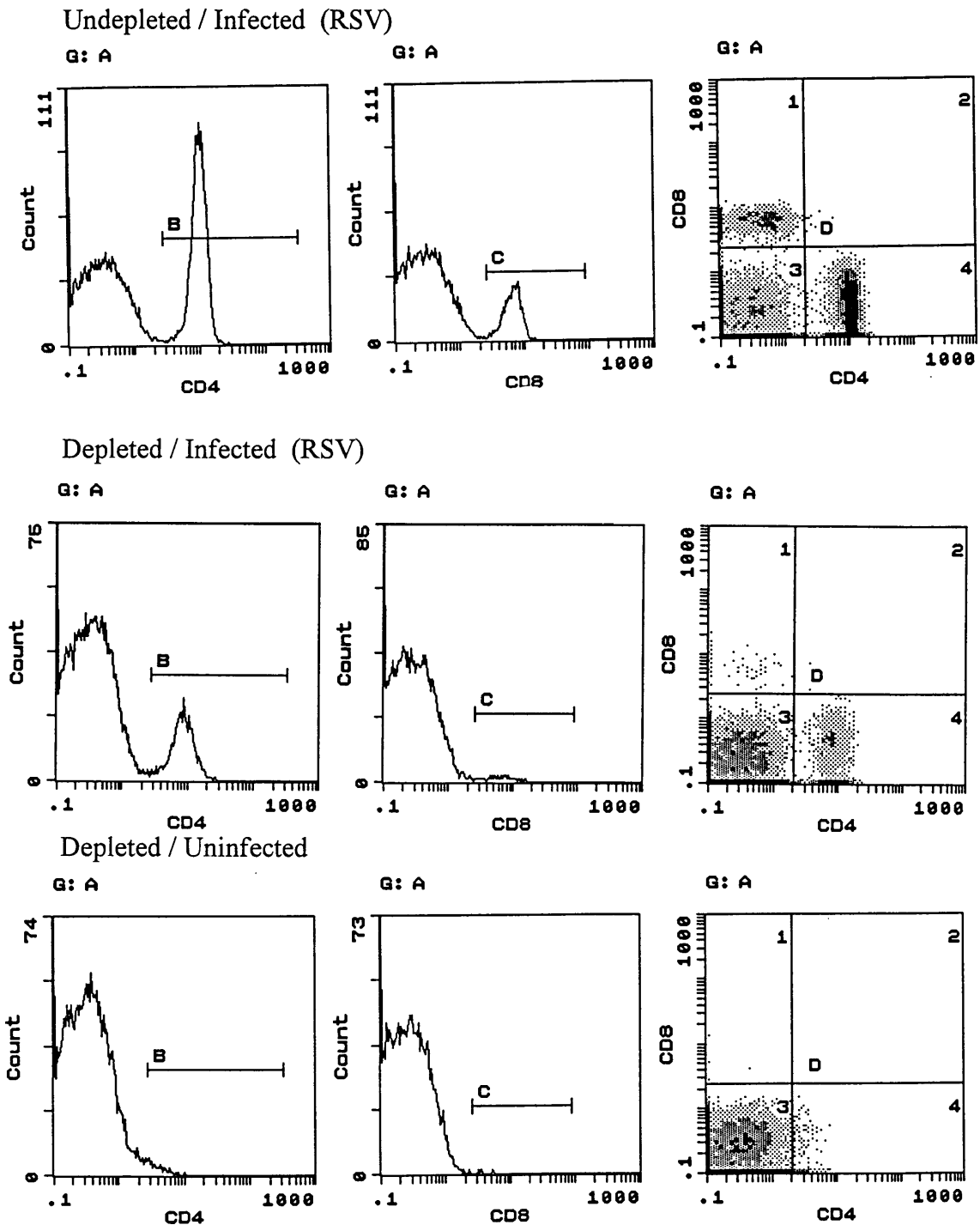


Figure 6.5 Flow cytometric analysis of splenocytes after CD4/8 depletion
8-10week old BALB/c mice were infected with RS virus or not infected (controls). 120 days later T cell depletion was performed using monoclonal antibodies. At the end of the depletion period, splenocytes were analysed by flow cytometry for CD3, CD4, CD8 and B220 (B cells). The results of CD4/CD8 staining for undepleted/infected, depleted/infected and depleted uninfected mice is shown above.

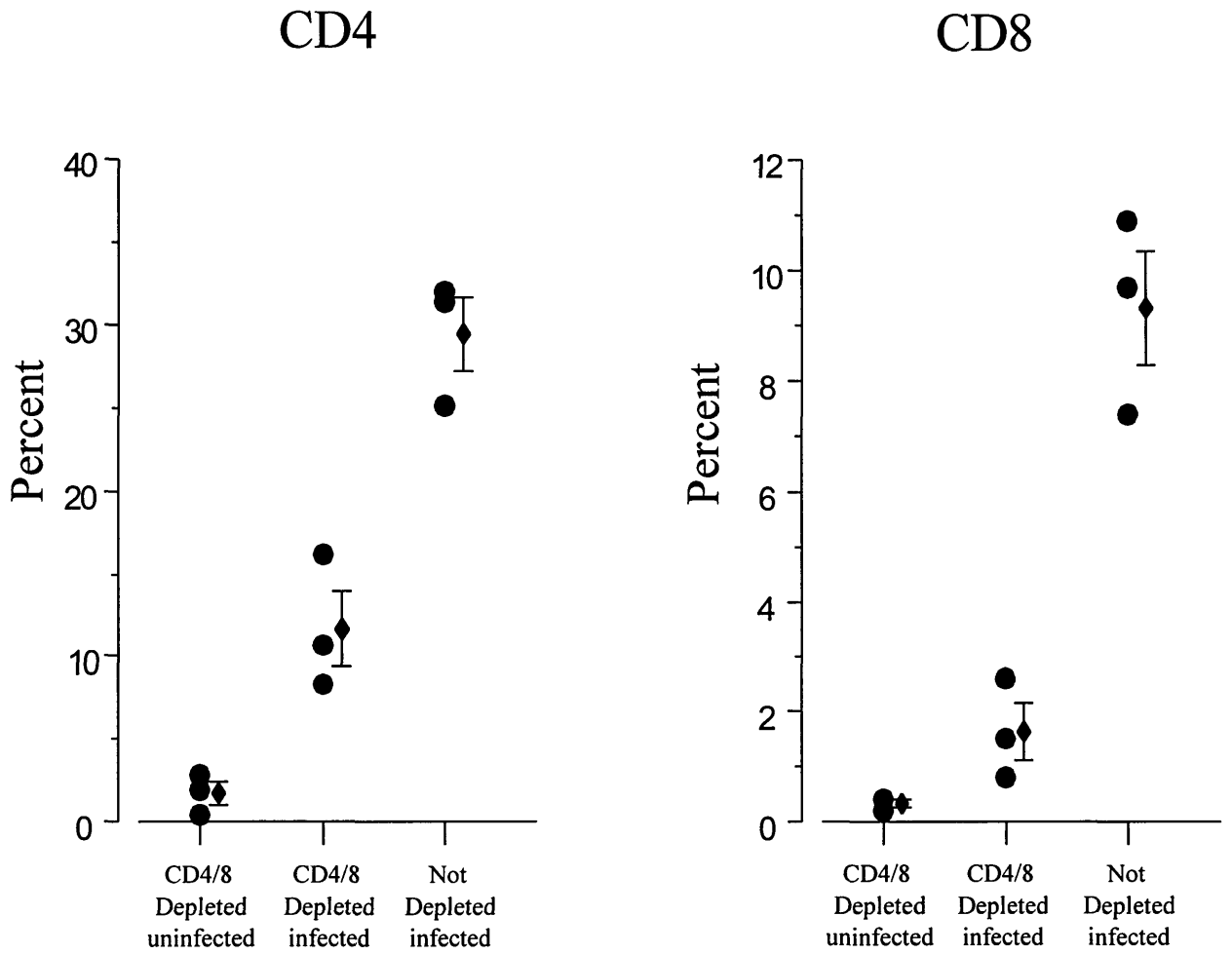


Figure 6.6 Percentages of CD4 positive and CD8 positive cells in spleen after depletion. 8-12 week old BALB/c mice were infected intranasally with RS virus or left uninfected (controls). 120 days after infection CD4 and CD8 lymphocytes were depleted using monoclonal antibodies (as in methods). Splenocytes stained with fluorochrome conjugatedantibodies (as in chapter 2) were analysed by flow cytometry. The percentage of CD4 and CD8 positive cells is shown above.

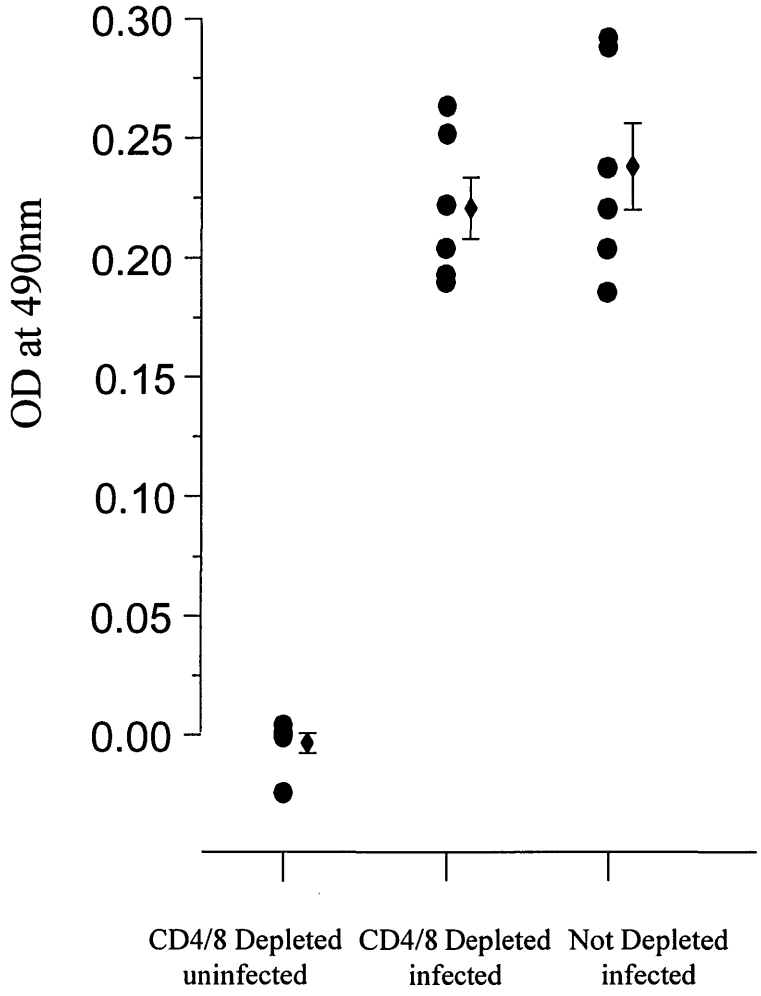


Figure 6.7 Serum antibody to RS virus after T cell depletion
8-12 week old BALB/c mice were infected intranasally with RS virus or left uninfected (controls). 120 days after infection CD4 and CD8 lymphocytes were depleted using monoclonal antibodies (as in methods). Specific serum antibody to RS virus was determined by ELISA.

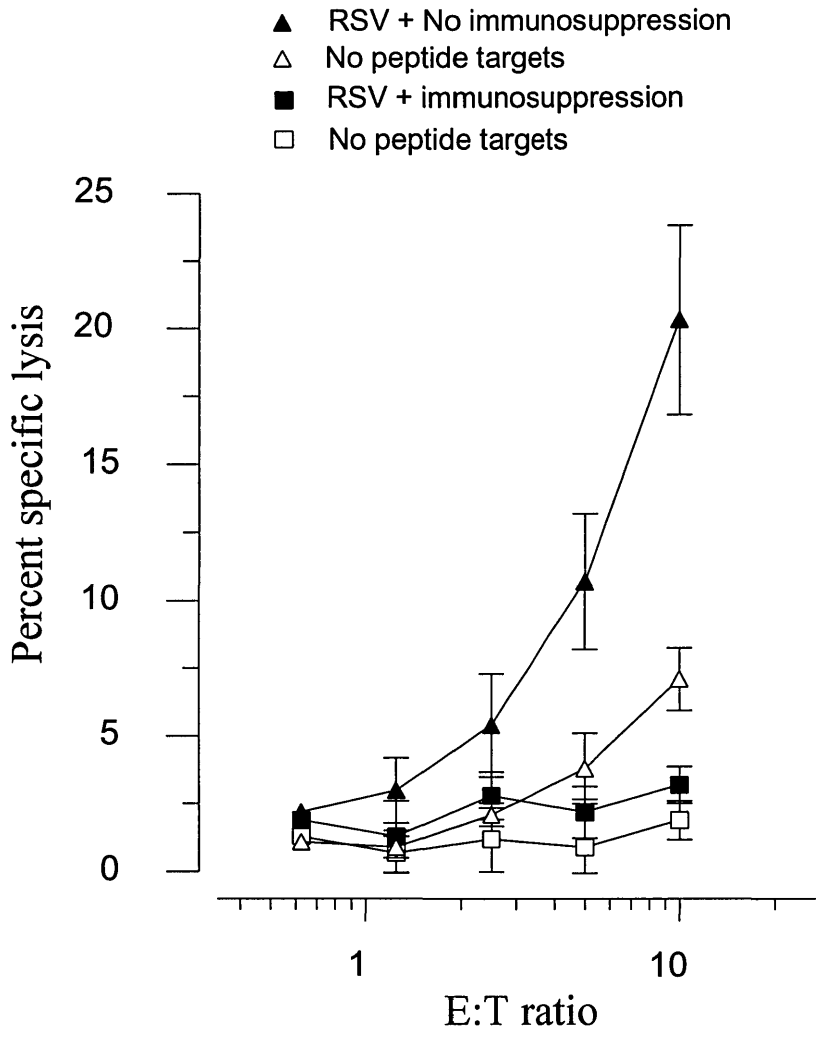


Figure 6.8 RS virus specific CTL memory after T cell depletion
8-12 week old BALB/c mice were infected intranasally with RS virus or left uninfected (controls). 120 days after infection CD4 and CD8 lymphocytes were depleted using monoclonal antibodies (as in methods). Splenocytes were restimulated in 5 day bulk (culture as in methods) using naive normal mouse splenocytes as stimulators. CTL lysis of P815 cells incubated with peptide SYIGSINNI was determined for infected mice. Yields of cells from bulk cultures from immunosuppressed mice were very, low limiting the effector to target ratios that could be tested.

Discussion:

Persistent viruses and immunity

Persistent viral infections are a cause of chronic disease in humans. It has recently been shown for example that adenovirus, a common respiratory pathogen, may persist in the lung and influence the development of chronic obstructive pulmonary disease in smokers (233). As discussed earlier, T cells are essential in controlling many viral infections. Diminished cellular immunity can be associated with prolonged disease or reactivation. For example in hepatitis B diminished CTL activity is associated with chronic infection (284) and immunosuppressed patients undergoing transplantation are susceptible to CMV (228,361). With HIV the decrease in CD4 lymphocyte counts later in infection, is associated with a decline in cell mediated immunity. As the CD4 counts fall the virus burden increases. Reactivation of herpes zoster or CMV which can become chronic or disseminated, sometimes heralds the onset of the acquired immunodeficiency syndrome in HIV infected individuals (reviewed in (159)). Children with defects in cell mediated immunity (137) shed higher amounts of virus for longer periods of time than other children. Mice that have had their T cells destroyed by irradiation (40) fail to clear RS virus and shedding is prolonged.

Control of acute and persistent virus infection by T cells

The mechanisms of RS virus clearance by the host are still being determined. Direct lysis of infected cells by cytotoxic lymphocytes is one important mechanism and was discussed in an earlier chapter. Cytokines produced by T cells also play an important role. Cytokines such as the interferons (IFN), interleukin-1 and tumour necrosis factor (TNF) act directly and indirectly to induce an antiviral state (reviewed in (211)). CTL can clear persistent RS virus

infection in irradiated mice (40) and specific CTL clear LCMV in persistent infection following neonatal inoculation (269,348). Tishon *et al* recently showed that specific CTL from mice deficient in IFN-γ were able to clear acute infection with LCMV but unable to control persistent infection (349). In both man and mice, IFN-γ induces the production of intracellular Mx proteins which are capable of inhibiting viral replication (283). Nitric oxide produced by macrophages through the action of IFN-γ may also inhibit viral replication (193).

Reactivation of viruses

A number of host and environmental factors influence viral reactivation. Immunosuppression using monoclonal antibodies was chosen in my experiments because it offered a selective elimination of CD4 and CD8 T cells without the toxicity risks of chemical agents, such as cyclophosphamide. Sub-lethal irradiation was also considered, but RS virus can be inactivated by ultraviolet irradiation and other forms of ionising irradiation could have a similar action. Greenlee *et al* (126), used cyclophosphamide to immunosuppress mice previously infected with papovavirus K. They cultured infectious virus 8 months after primary infection. Several mice in the treated group died during immunosuppression. In my experiments 3 mice died (one mouse from each group) but there was no evidence that the immunosuppression *per se* adversely affected mice compared with controls. I also considered using steroids as a means of immunosupression. Castrucci *et al* showed that latent parainfluenza virus infection unexpectedly re-activated from calves given dexamethasone. In this experiment dexamethasone immunosuppression was given to confirm that bovine herpesvirus 2 reactivation could occur (44) but was complicated not only by the emergence of parainfluenza but also bovine herpesvirus 1 reactivation.

In the experiment presented in this chapter, RS virus was found at low levels by virus culture. It is possible that immunosuppression using monoclonal antibodies to CD4 and CD8 T cells was not sufficient to allow higher levels of replication. Several other antiviral defences may have had some effect on virus growth *in vivo*. Antibody to RS virus was unaffected by depletion of CD4s and CD8s. Viral clearance may still have occurred through antibody binding and complement mediated cell lysis. Natural killer (NK) cells may also have inhibited viral growth. NK cells are cytotoxic lymphocytes that do not express CD3 or the T cell receptor for antigen (reviewed in (245)). They are considered to be a non-specific defence, recognising and killing virally infected cells and secreting IFN-γ. How NK cells recognise virally infected cells is still being determined but MHC class I expression may inhibit NK cell activation and lack of self antigens may be one factor in NK killing (42). $\gamma\delta$ cells, a subset of T cells, are frequently $CD4^-CD8^-$ (116) and would also remain after depletion of CD4s and CD8s. It has been suggested that cells that home to epithelial mucosal surfaces such as the gut and lung predominantly express the $\gamma\delta$ TCR. Openshaw showed that primary RS virus infection predominantly stimulates $\alpha\beta$ TCR lymphocyte that were almost exclusively single positive for either CD4 or CD8 (274). Only a very small number of lymphocytes in the BAL expressed $\gamma\delta$. However, Allan *et al* found that mice depleted of CD4s and CD8s given a primary challenge with influenza, had a greatly enriched efflux of $\gamma\delta$-TCR+, $CD4^-CD8^-$ lymphocytes into the BAL (7). These lymphocytes did not however compensate either in numbers or effect for the depleted subsets.

Summary

In this chapter it was shown that, 120 days after infection, immunosuppresion with T cell depleting antibodies allowed the recovery of RS virus from the lungs of mice by virus

culture.

Chapter 7 Studies of T-cell responses to non-viral antigens after viral respiratory infection.

Hypothesis

It has become accepted however that allergic asthma, rhinitis and other atopic diseases are likely to be multifactorial involving both familial and environmental influences. An important factor in sensitisation to allergens is irritation of mucosal epithelial surfaces. The respiratory tract is an important entry site for environmental antigens. Sigurs *et al* (332) recently found that RS virus bronchiolitis during the first year of life was an important factor in the development of asthma. Children who had severe RS virus bronchiolitis had high levels of serum IgE to common food and inhaled allergens. RS virus bronchiolitis was the single most important risk factor for this allergy. Severe bronchiolitis in infancy is associated with later wheezing episodes. However the relationship between viruses and the pathogenesis of asthma has remained controversial. One important approach to understanding the aetiology of asthma and allergy is through the use of experimental models. Most animal models have relied on the use of adjuvants such as alum to generate allergen specific antibody. It is possible that virus infections causing inflammation in the respiratory tract such as RS virus or influenza alter the immune response to unrelated inhaled protein antigens. The following chapter investigates the effect of RS virus infection on the immune response to inhaled antigen (ovalbumin) in mice. If RS virus does affect bystander immune responses then this should be apparent by investigating antigen-specific IgE, T cell cytokine production and proliferation.

Chapter 7 Studies of T-cell responses to non-viral antigens after viral respiratory infection.

Introduction:

Hayfever and allergy

John Bostock, a physician in the early 19th century and later the vice president of the Royal Society noted that every year he developed a cold for two months starting in June. It was characterised by profuse nasal discharge, sneezing, itching of the eyes and difficulty breathing (91). He recognised that these features were distinct from the common cold and recorded his case history in 1819 (27). This was probably the first complete description of allergic hay fever. This condition appears to have been unusual at that time since it took Bostock 9 more years to collect a series of 28 cases (28). Immediate hypersensitivity has become the most widespread immunological disorder in humans and is an increasing problem. It affects 1 in 4 people in developed nations (147). The reason particular people develop allergy is still not known despite many years of research. Much work has concentrated on identifying candidate genes. It has become accepted however that allergic asthma, rhinitis and other atopic diseases are likely to be multifactorial involving both familial and environmental influences (72). An important factor in sensitisation to allergens is irritation of mucosal epithelial surfaces. In particular, chemical irritation by inhaled pollutants (244) including vehicle exhausts may be associated with bronchial hyperresponsiveness. Diesel particles can act as an adjuvant in the sensitisation of mice to intraperitoneal Japanese cedar pollen (256). One hypothesis is that the increase in allergy is associated with rising air pollution (83,91).

Allergic asthma

The cause of allergic asthma is not known, but much is known about its manifestations; it is characterised by airways inflammation even in mild disease. Specifically this inflammation includes airway wall infiltration by T_H2 lymphocytes, eosinophils and mast cells (29,31,309). The net effect is the production of T_H2 cytokines such as IL-4 in response to certain antigens and switching to Ig isotypes such as IgE. IgE binds to the high affinity IgE receptor, $Fc_\epsilon R1$, on mast cells and primes them for activation by further antigen (119,120). The expression of $Fc_\epsilon R1$is pronounced on eosinophils, basophils and monocytes in atopic individuals (234). When activated these cells also secrete cytokines including: histamine, platelet activating factor and leukotrienes. Mast cells and eosinophils also release IL-4 and IL-5 further favouring a T_H2 state.

RS virus bronchiolitis and allergy

The respiratory tract is an important entry site for environmental antigens. Sigurs *et al* (332) recently found that RS virus bronchiolitis during the first year of life was an important factor in the development of asthma. Interestingly, in children who had severe RS virus bronchiolitis 3 years earlier there were high levels of serum IgE to common food and inhaled allergens, and by skin-prick testing sensitisation to allergens. The paper concluded that RS virus bronchiolitis was the single most important risk factor and that a family history of atopy or asthma further increased the risk.

Severe bronchiolitis in infancy is associated with later wheezing episodes. Bronchial hyperresponsiveness even 10 years later has been shown (299). However the relationship between viruses and the pathogenesis of asthma has remained controversial. Some authors

feel that 'asthma' is used to denote a wide range of clinically distinct conditions and that more careful definition is necessary (372). Others have suggested that respiratory viruses may be related to wheezing and asthma because of specific effects. Elias and coauthors have shown a potent effect of RS virus, rhinovirus (RV) and parainfluenza virus (PIV) to cause the release of IL-11 from human lung stromal cells after infection (81). They went on to show raised levels of IL-11 in the nasal secretions and aspirates of children with upper respiratory tract infections. When IL-11 was introduced into the lungs of BALB/c mice patchy inflammation, a mononuclear cell infiltrate and non-specific airways hyperresponsiveness was found.

With sensitive detection techniques such as PCR it has been possible to determine the importance of viruses in asthma, such as: Rhinoviruses, RS virus and Parainfluenza (189,191,282). Most would now agree that viruses are implicated in the majority of acute wheezing episodes (189,190). The mechanisms proposed for precipitating acute wheeze include: local epithelial damage, increased sensitivity of the cholinergic system (365) release of inflammatory mediators, the activation of the cellular immune response, the release of cytokines and the production of specific IgE.

One important approach to understanding the aetiology of asthma and allergy is through the use of experimental models. Animal models have been used to dissect the acute anaphylactic and the delayed-type hypersensitivity responses (298,318). Most animal models have relied on the use of adjuvants such as alum to generate allergen specific antibody. The use of allergens in the context of adjuvants complicates the models. It lays them open to the criticism that they do not reflect natural sensitisation since the responses cannot be generated

in the absence of adjuvant. Nevertheless, using adjuvants, models exist for the generation of allergic responses including anaphylactic shock with protein antigens (255). Using adjuvants it has been suggested that sensitisation via the respiratory tract may be enhanced in the context of acute viral infection (99,164,317). To my knowledge no study has elicited enhanced responses in the absence of adjuvant.

Animal models

Using ovalbumin nebulisation, sensitisation of guinea pigs has been demonstrated without the use of adjuvant. Sensitised guinea pigs exposed to ovalbumin challenge have immediate, late and delayed bronchoconstriction with eosinophil accumulation (173). Mice may also be sensitised by ovalbumin nebulisation (304). This model allows the evaluation of the effect of acute virus infections in the lung on sensitisation to allergens. I chose to investigate the effect of RS virus and influenza virus infection on the sensitisation of mice to ovalbumin in the absence of adjuvant. By developing a capture ELISA the ovalbumin specific IgE, IgG1 and IgG2a responses were determined. Splenocytes from sensitised mice were stimulated in culture with ovalbumin and the cytokine production determined using intracellular cytokine staining. The generation of acute anaphylactic shock was also used as a measure of sensitisation.

Results:

a) Weight loss during acute viral respiratory infection.

Design: 8 to 12 week old BALB/c mice were lightly anaesthetised with ether and infected i.n. with 5×10^6 pfu A2 RS virus (86µl), 86µl of influenza virus or 86µl of PBS (mock infection). X31 strain influenza virus was a kind gift from Dr Alan Douglas from the National Institute of Medical Research, Mill Hill; (4096 HAU per 50µl and it was diluted 1:100 in PBS before infection). After intranasal infection the mice were exposed daily for 20 minutes to nebulised 1% ovalbumin in PBS, for ten consecutive days. Control mice had nebulisation with PBS alone. The nebulisation occurred either from days 4 to 13 after infection, days 11 to 20 after infection or days 18 to 27 after infection. Mice were weighed regularly as an indication of the severity of illness.

Results: Mice exposed to ovalbumin nebulisation lost and regained the same amount of weight as controls (PBS alone). Weight loss followed the same time course in mice exposed to ovalbumin as those given PBS alone (figure7.1). In different experiments there was variation in the percentage of weight lost in RS virus infected mice. The weight loss was greater or less than the percentage seen after influenza infection. However, the timing of weight loss and recovery was consistent: influenza challenged mice lost weight from day 2, earlier than RS virus infected mice which lost weight after day 4 post infection. Peak weight loss occurred at day 6 or 7 after infection followed by steady recovery (figure 7.2).

b) Ovalbumin sensitisation during acute respiratory virus infection is associated with acute anaphylactic shock on intradermal challenge with ovalbumin.

Design: Mice were infected with influenza virus, RS virus or mock infected and then challenged with ovalbumin or PBS nebulisation (as above). A sterile solution of 500mg/ml ovalbumin in PBS was prepared. On day 18 after infection (5 days after last ovalbumin challenge) a $1cm^2$ patch was shaved on the abdomen of the mice and an intradermal injection of 20µl of ovalbumin solution given (total dose 10mg). For mice exposed to ovalbumin nebulisation from day 11 to 20 or day 18 to 27, intradermal challenge was 5 days after the last nebulisation. Mice primed by intraperitoneal injection of 200µl alum precipitated ovalbumin either once (ten days earlier) or twice (two months earlier and ten days earlier) were used as controls.

Result: Intradermal injection of ovalbumin caused acute collapse in mice given ovalbumin nebulisation during acute respiratory virus infection. The mice became immobile and appeared unwell. In some cases the nose became cyanosed rather than the normal pink appearance and the respiratory effort seemed increased. The onset of these changes was 15 to 20 minutes after intradermal injection of the ovalbumin. Mice allowed to recover from this anaphylactic collapse appeared normal 2 hours later. Acute collapse was only seen in mice given ovalbumin nebulisation after virus infection and not in mock infected animals. It did not occur in virus infected animals given PBS nebulisation instead of ovalbumin. Acute collapse was not seen in mice challenged with ovalbumin on days 11 to 20 or days 18 to 27 after virus infection. Anaphylactic collapse was seen in every animal tested, when primed by virus infection and ovalbumin nebulisation from days 4 to 13. Control animals given intraperitoneal alum precipitated ovalbumin (alum/ova) either once or twice (as above) also became immobile and unwell on intradermal ovalbumin challenge given 10 days after the last alum/ova dose.

c) Virus infection enhances T_H 2 cytokine production by splenocytes from mice exposed to ovalbumin.

Design: Mice were exposed to infection with influenza virus, RS virus or mock infected (as above) and exposed to ovalbumin nebulisation from day 4 to 13 after infection. 18 days after virus infection the mice were given a lethal intraperitoneal injection of pentobarbitone and exsanguinated. The spleens were removed aseptically and a single cell suspension made. 3×10^6 cells were set up in tissue culture (1.5ml total volume) for 72 hours with different concentrations of ovalbumin. Intracellular cytokine staining was performed for IL-4 and IFN-γ and cell surface staining for CD4 or CD8. Culture supernatants were tested for IL-2 or IL-4 using the CTLL cell proliferation assay in the presence or absence of anti IL-4 (11B11) or anti-IL-2 (S4B6).

Results: Intracellular cytokine staining of cells demonstrated higher IL-4 production in splenocytes from mice infected with influenza and RS virus than mock infected (figure7.3). Interestingly a higher proportion of CD8 cells were positive for IL-4 than CD4 cells. The influenza virus infected mice had more IL-4 producing cells than RS virus infected mice where the difference from mock infected mice was less marked. When the percentage positive was compared with the dose of ovalbumin in the culture the differences become clearer (figure 7.4 and 7.5). A dose response effect on cytokine production is seen in CD8 positive cells but not in CD4 positive cells: at higher concentrations of ovalbumin more CD8 cells are positive for both IL-4 and IFN-γ. In all cultures the production of IL-4 by CD8 positive cells was higher than IFN-γ. However, only in influenza infected mice did CD4 positive cells produce more IL-4 than IFN-γ. CTLL bioassay of culture supernatants failed to detect significant production of IL-4 in any cultures. IL-2 production was detected in

spleen cell cultures from RS virus infected mice only and was not specific for mice exposed to nebulised ovalbumin because it occurred in cultures from mice exposed to PBS alone (figure 7.6). The level of IL-2 production was consistently high in RS virus infected PBS challenged mouse splenocytes in different experiments. However, the level of IL-2 production seen in RS virus challenged and ovalbumin exposed mouse splenocytes varied between experiments from the level shown in figure 7.6 to less than half of this level.

d) Exposure to ovalbumin during acute respiratory virus infection leads to specific IgG1 but not IgE to ovalbumin.

Design: Mice were challenged i.n. by viral infection or mock infection followed by exposure to 1% ovalbumin or PBS by nebulisation (as above). Serum was tested by capture ELISA for specific IgG1, IgE and IgG2a to ovalbumin. Total IgE in serum was determined by ELISA and compared with a known standard (figure 7.7) (as described in methods).

Result: IgG1 to ovalbumin was detected in mice sensitised by ovalbumin nebulisation, only if they also had an acute viral respiratory infection (figure 7.8). Mice given alum precipitated ovalbumin intraperitoneally (IP) 10 days earlier were used as a positive control. Mice exposed to ovalbumin nebulisation after mock infection had no detectable IgG1 to ovalbumin. No significant IgG2a against ovalbumin was detectable except for control mice given alum precipitated ovalbumin intraperitoneally 10 days earlier (IP). No specific IgE to ovalbumin was detected in any group except controls (intraperitoneal alum precipitated ovalbumin) (figure 7.9). Total IgE levels were also not raised in any group except the controls (intraperitoneal alum precipitated ovalbumin).

Specific IgG1 to ovalbumin was only seen when ovalbumin nebulisation was performed during the period of acute weight loss (days 4 to 13). Mice given RS virus or influenza and then exposed to nebulised ovalbumin during the period of weight recovery (days 11 to 20) or after weight was regained (days 18 to 27) had no detectable IgG1 to ovalbumin (figure 7.10). Influenza infection was associated with higher levels of IgG1 to ovalbumin than RS virus infection. This did not appear to reflect the severity of illness, for the data shown mice lost more weight after RS virus infection than influenza (figure 7.2 Experiment 2).

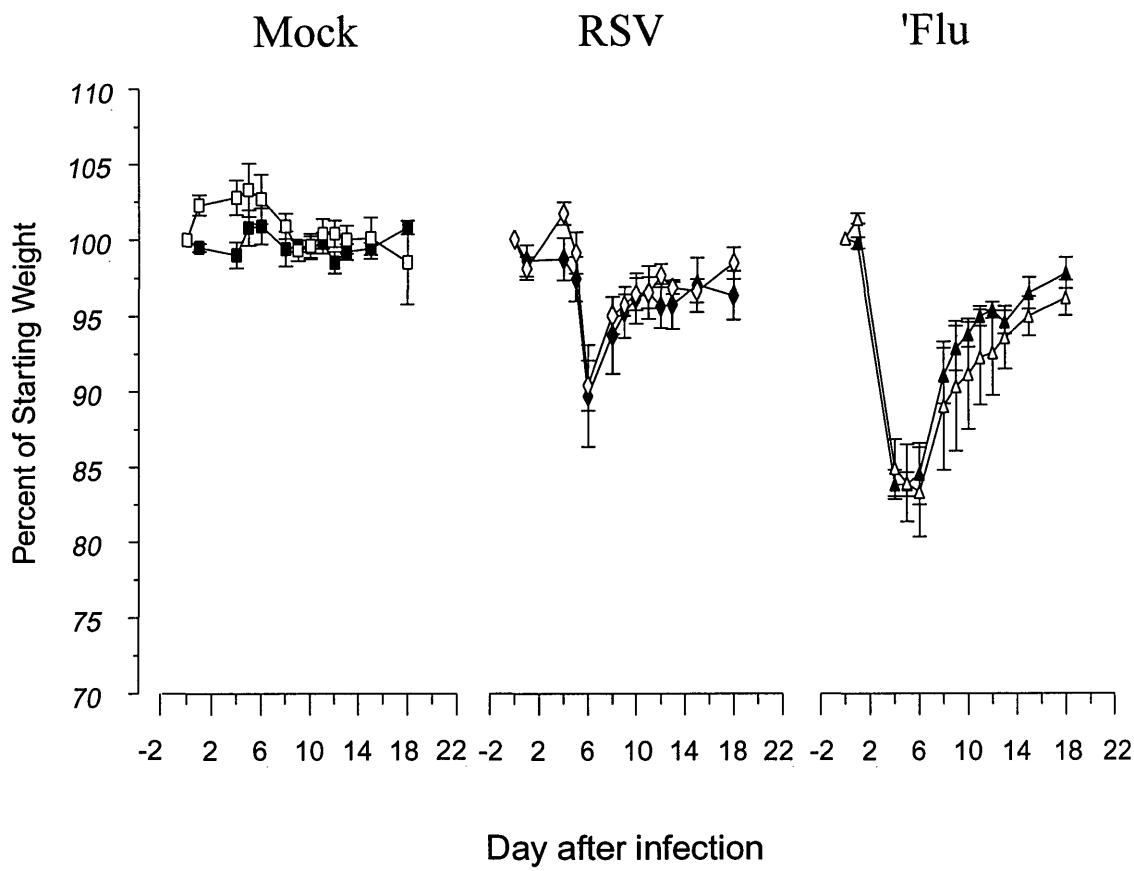


Figure 7.1 Weight loss after infection with and without exposure to ovalbumin. 8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu). After infection the mice were exposed to a daily 20 minute exposure to either 1% ovalbumin in PBS (filled symbols) or PBS alone (open symbols), from day 4 for 10 days. Weight loss, expressed above as percent of starting weight with mean and SEM, is given for each group.

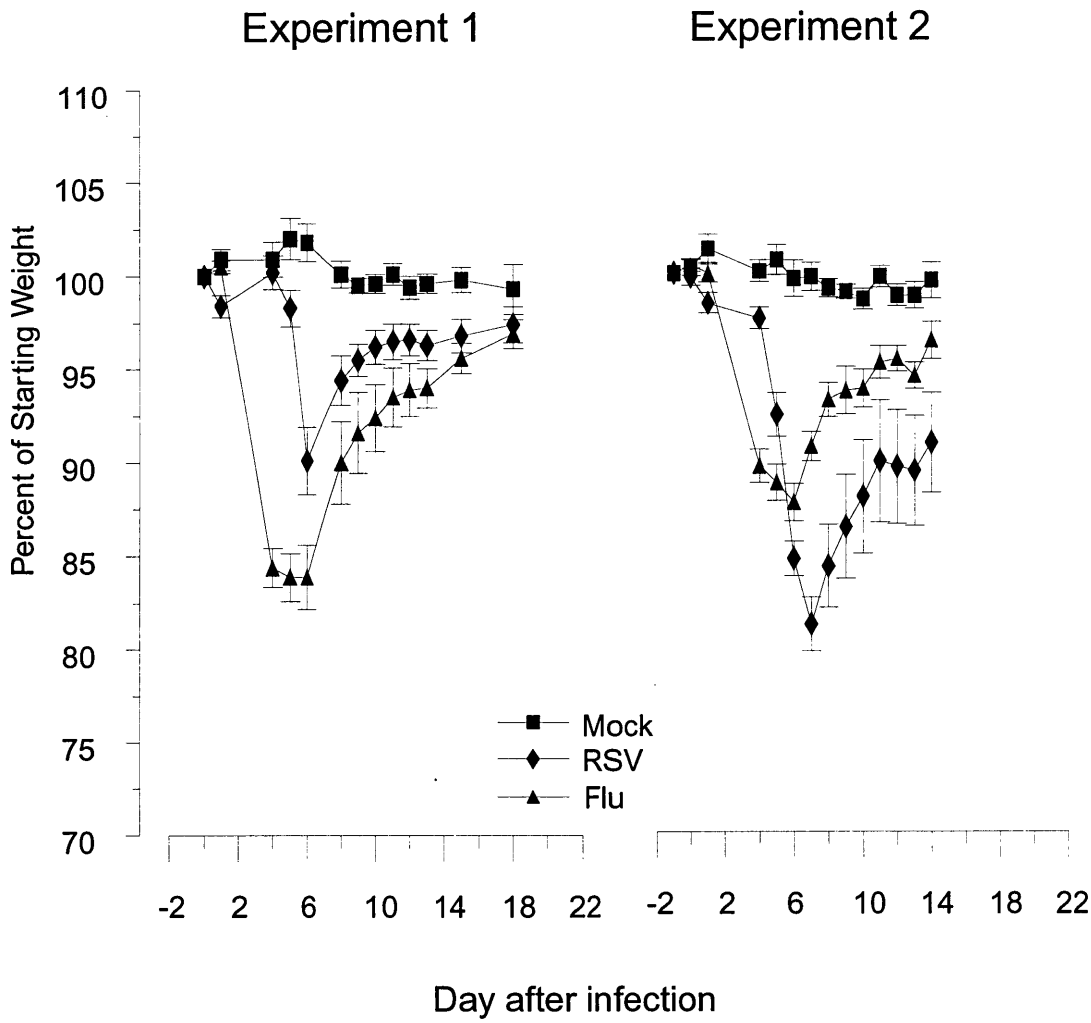


Figure 7.2 Weight loss of BALB/c mice during infection with influenza virus or RS virus. 8-12 week old BALB/c mice were challenged i.n. with PBS (mock infection), RS virus or influenza virus ('flu). Weight loss, expressed above as percent of starting weight with mean and SEM, is given for each group. Two experiments are shown.

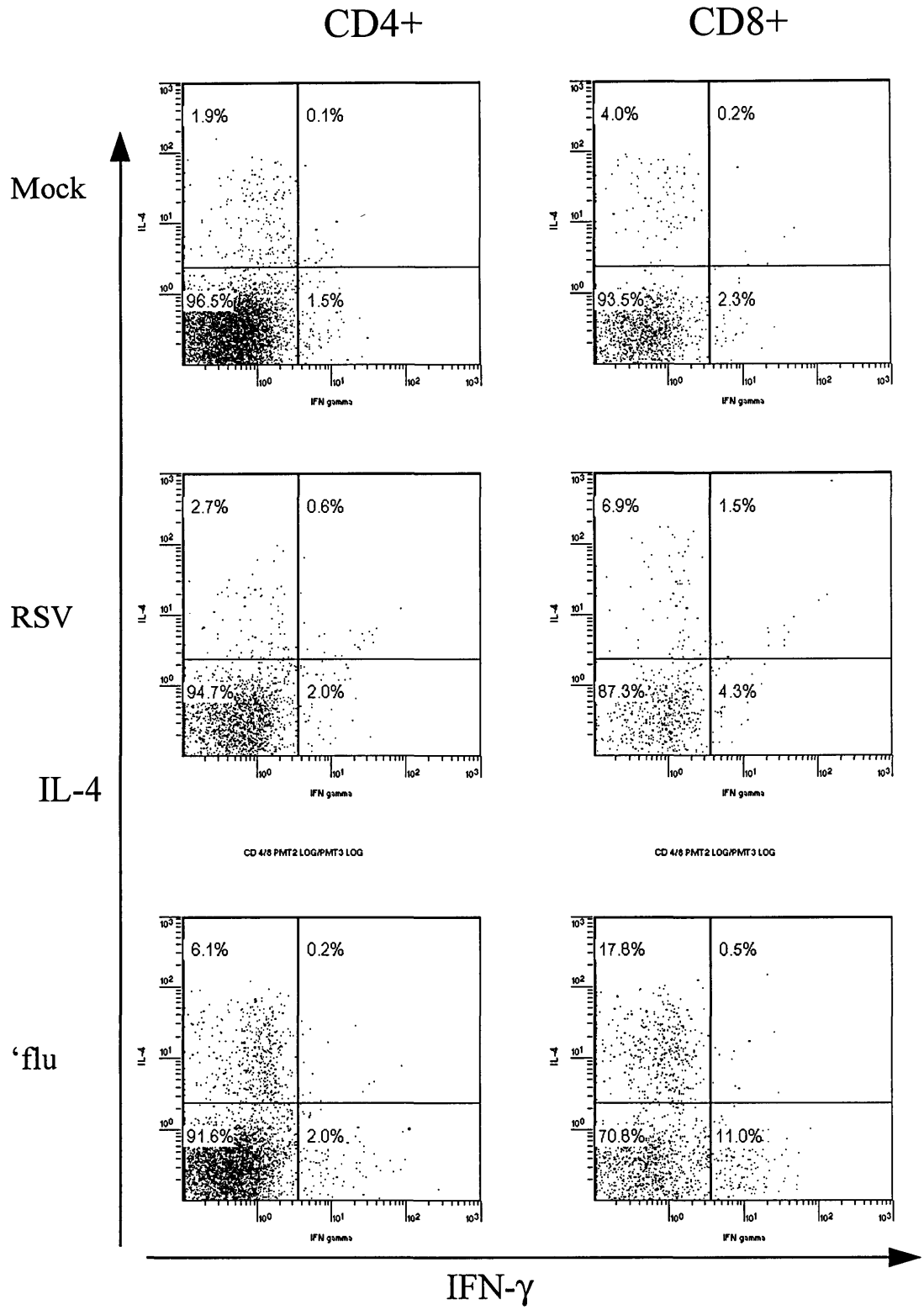


Figure 7.3 Flow cytometric analysis of splenocyte cytokine production.
8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu) andexposed daily for 20 minutes to either 1% ovalbumin in PBS or PBS alone for 10 days. Splenocytes were cultured in the presence of 500μg/ml ovalbumin for 72 hours. Intracellular IFN-γ and IL-4 in CD4+ (left panel) and CD8+ (right panel) cells in the lymphocyte gate was determined.

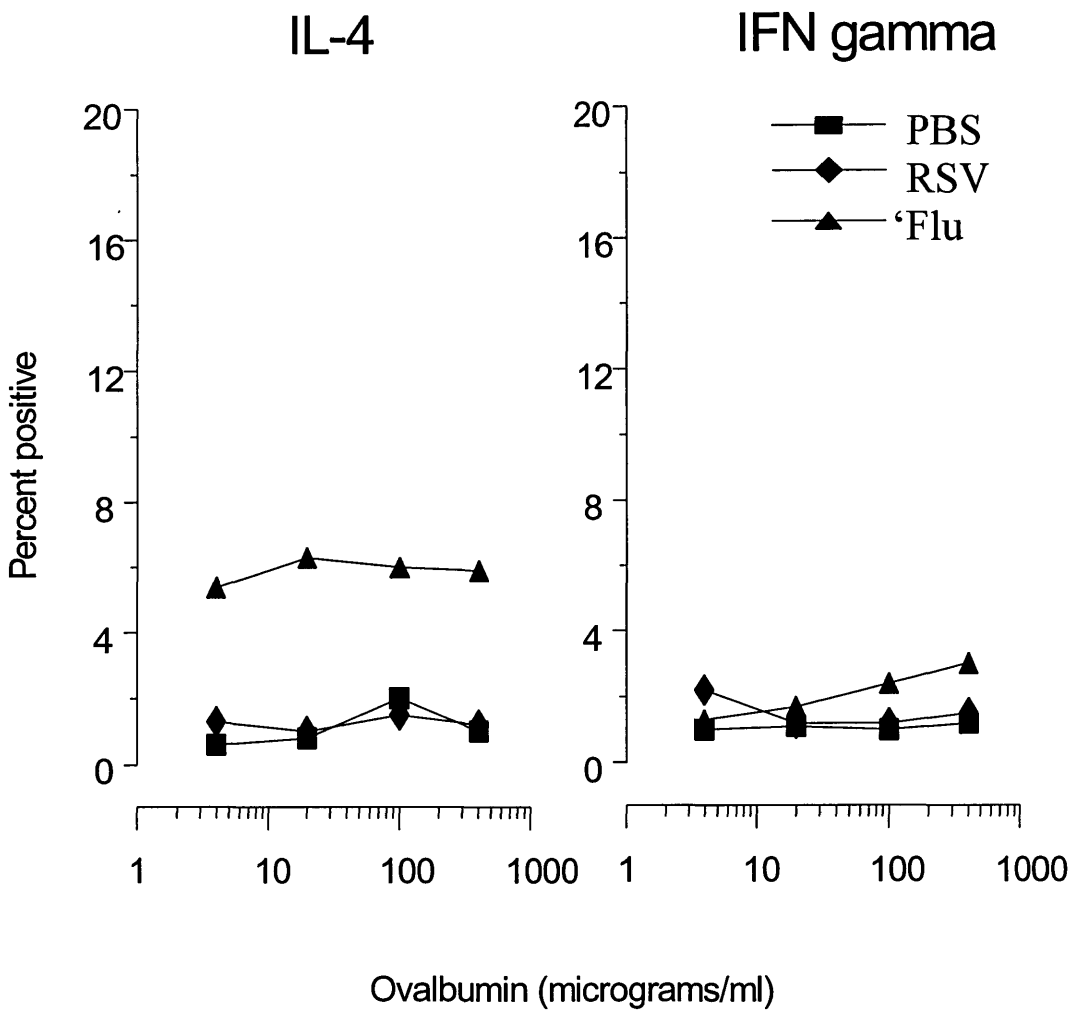


Figure 7.4 Flow cytometric analysis of $CD4^+$ splenocyte cytokine production. 8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu) and exposed daily for 20 minutes to either 1% ovalbumin in PBS or PBS alone for 10 days. Splenocytes were cultured in the presence of different doses of ovalbumin from 4μg/ml to 500μg/ml for 72 hours. Intracellular IFN-γ (right panel) and IL-4 (left panel) in cells in the lymphocyte gate were determined.

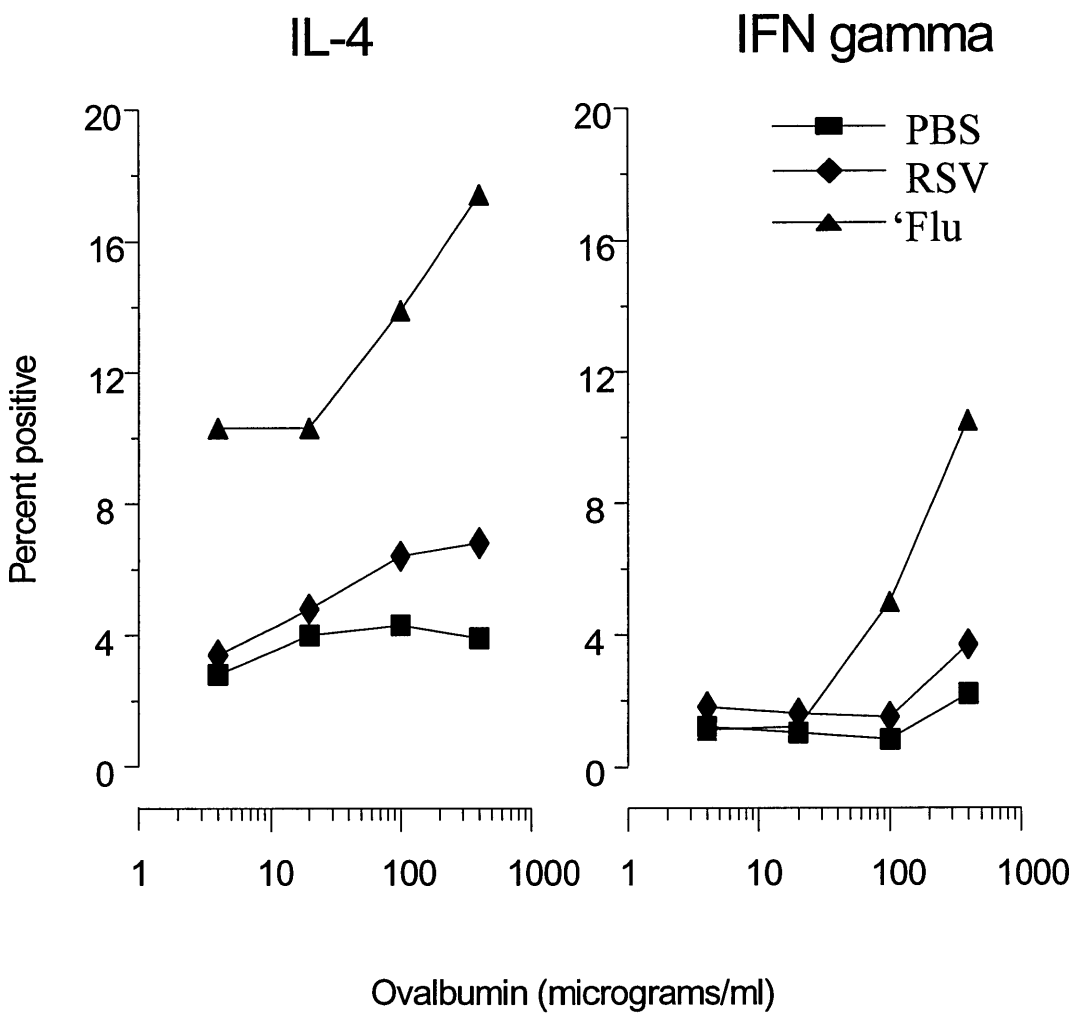


Figure 7.5 Flow cytometric analysis of $CD8^+$ splenocyte cytokine production. 8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu) and exposed daily for 20 minutes to either 1% ovalbumin in PBS or PBS alone for 10 days. Splenocytes were cultured in the presence of different doses of ovalbumin from 4μg/ml to 500μg/ml for 72 hours. Intracellular IFN-γ (right panel) and IL-4 (left panel) in cells in the lymphocyte gate were determined.

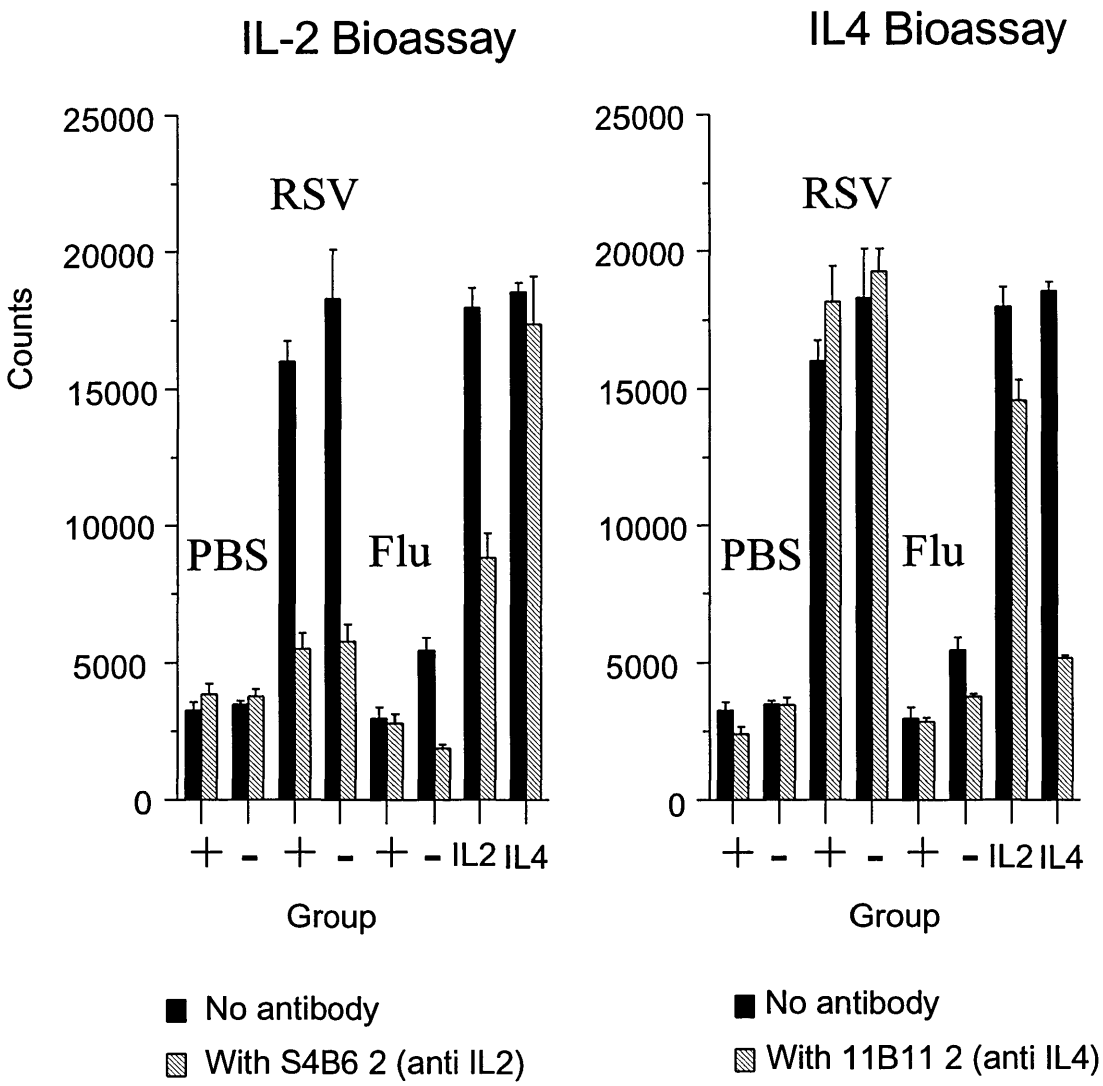


Figure 7.6 CTLL Bioassay of splenocyte cytokine production.
8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu) and exposed daily for 20 minutes to either 1% ovalbumin in PBS (+) or PBS alone (-) for 10 days. Splenocytes were cultured in the presence of ovalbumin (500μg/ml) for 72 hours. Supernatants were tested for proliferation of CTLL cells in the presence and absence of blocking antibody to IL-4 or IL-2. Inhibition of proliferation was compared with recombinant IL-2 or IL-4 at 1.5U/ml as positive controls.

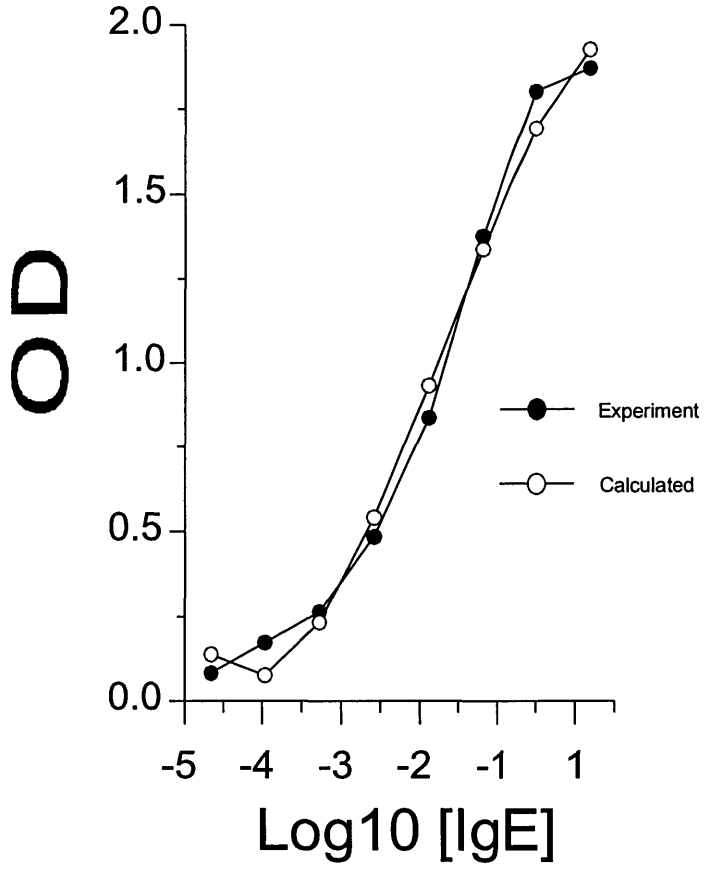


Figure 7.7 Standard curve for determining total IgE in serum
A total IgE ELISA was performed using a known standard. A polynomial expression was derived using "Minitab for Windows". IgE in test specimens was calculated according to the polynomial expression:

Log_{10} [IgE] = 8.75(OD) -7.17$(OD)^2$ + 2.19$(OD)^3$

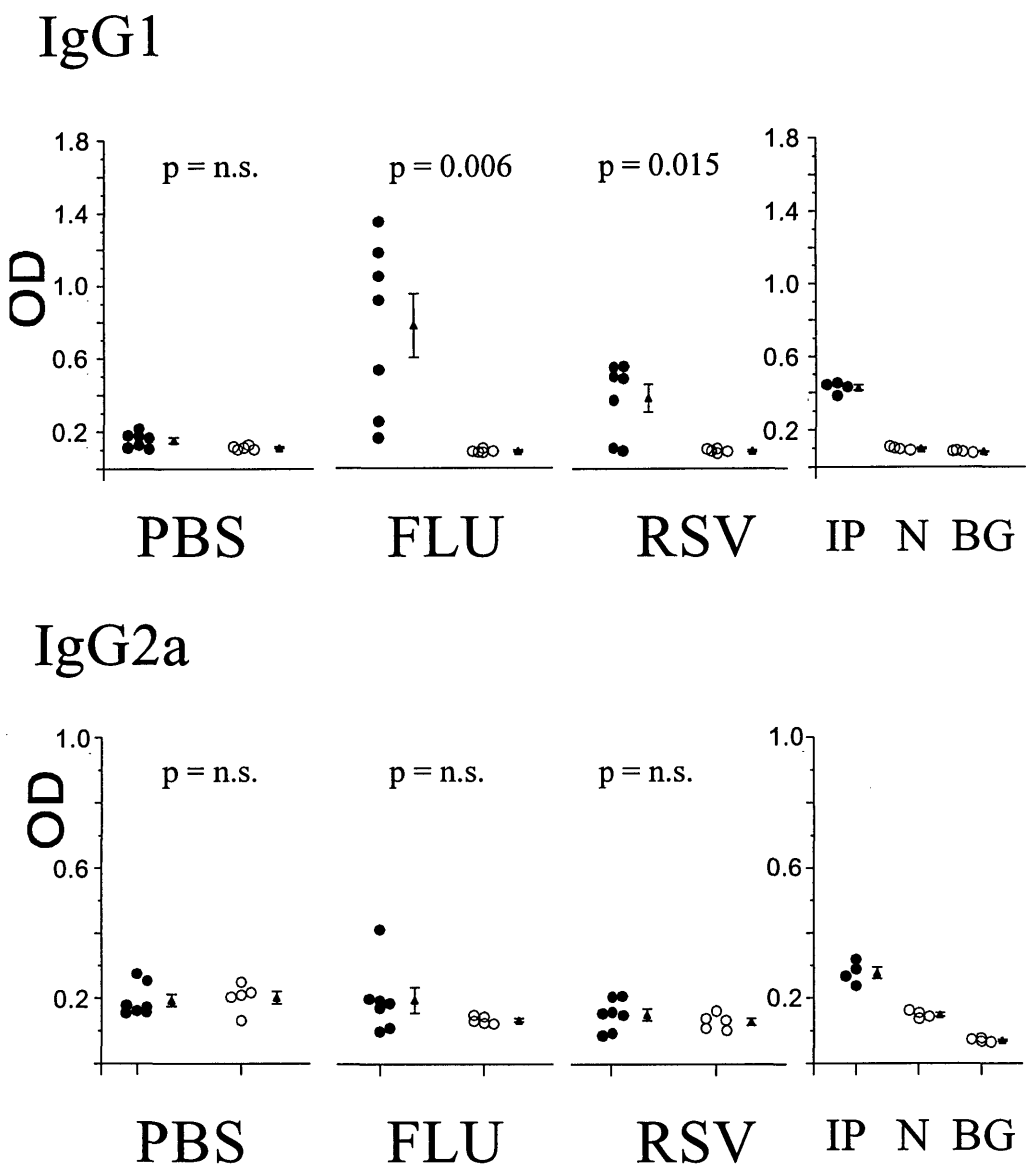
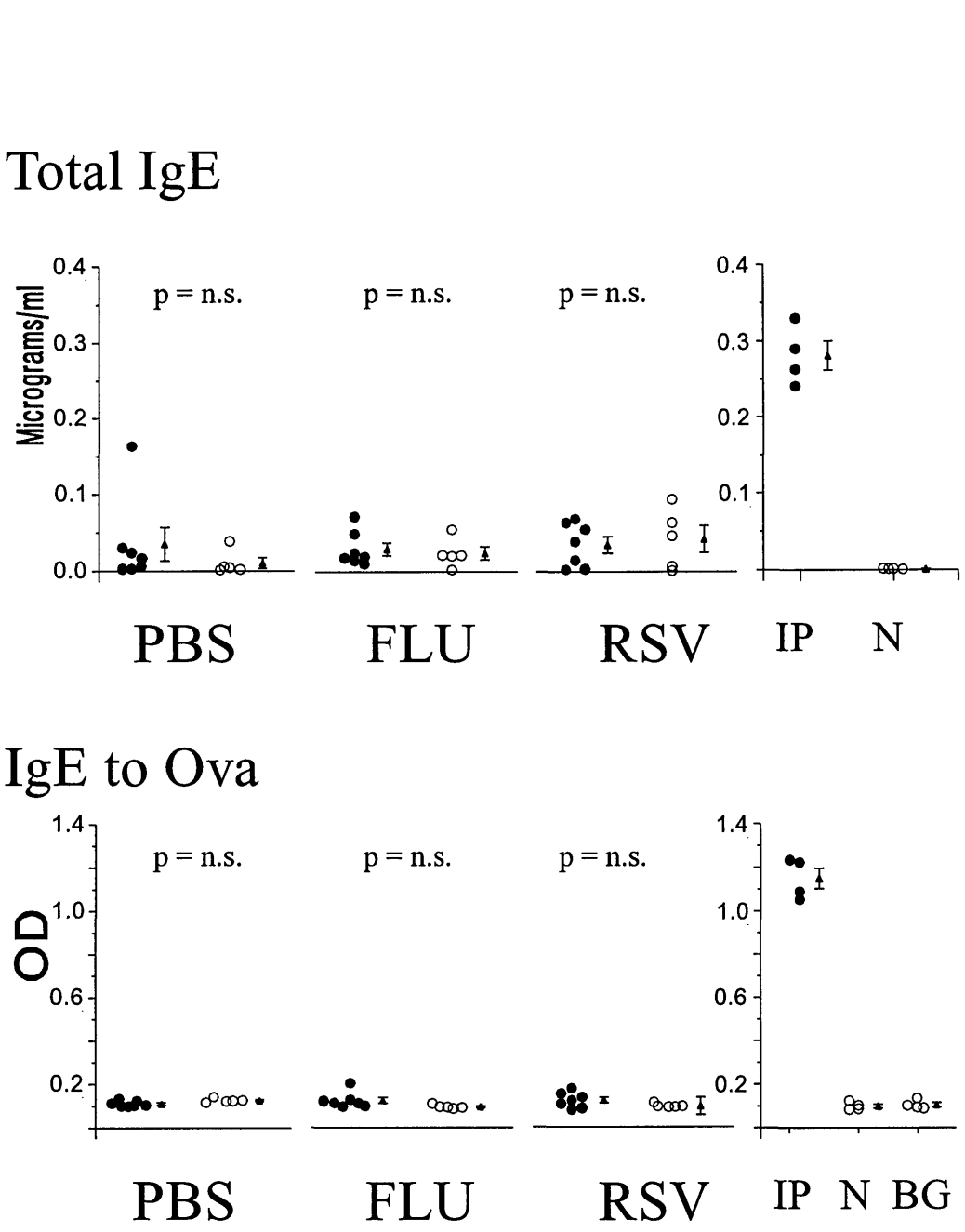


Figure 7.8 Specific serum IgG1 and IgG2a to ovalbumin.
8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu). After infection the mice were exposed to a daily 20 minute exposure to either 1% ovalbumin in PBS (filled symbols) or PBS alone (open symbols), from day 4 for 10 days. Serum was obtained 18 days after infection. Ovalbumin specific antibody was determined for different isotypes using a capture ELISA and digoxigenin conjugated ovalbumin (as described in materials and methods). Significance determined at 0.95 by Mann-Whitney test using "Minitab for Windows" (the groups are of unequal numbers). Each symbol represents an individual mouse except the right panel which is pooled sera from 5 mice given i.p. alum/ova (IP) or nil (N). BG is the test background with no serum added. Mean and SEM are shown



- Sensitised with nebulised ovalbumin
- ○ Sham sensitised with nebulised PBS

Figure 7.9 Total serum IgE and specific IgE to ovalbumin.
8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu). After infection the mice were exposed to a daily 20 minute exposure to either 1% ovalbumin in PBS (filled symbols) or PBS alone (open symbols), from day 4 for 10 days. Serum was obtained 18 days after infection. Ovalbumin specific antibody was determined for different isotypes using a capture ELISA and digoxigenin conjugated ovalbumin (as described in materials and methods). Significance determined at 0.95 by Mann-Whitney test using "Minitab for Windows" . Each symbol represents an individual mouse except the right panel which is pooled sera from 5 mice given i.p. alum/ova (IP) or nil (N). BG is the test background with no serum added. Mean and SEM are shown.

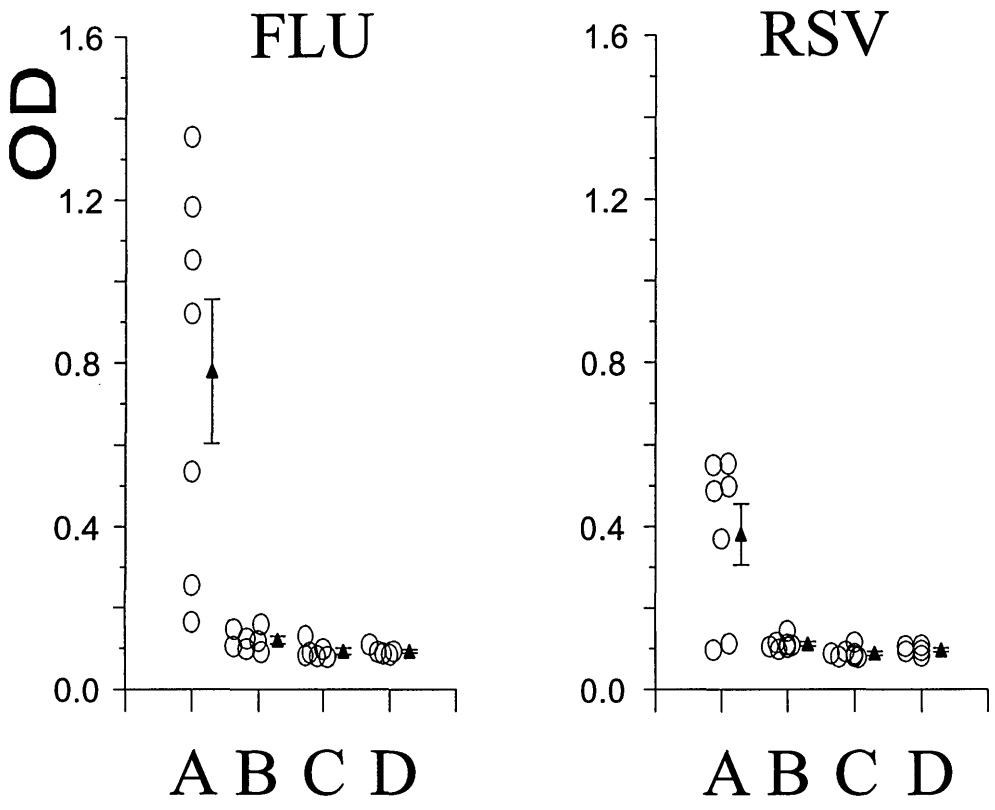


Figure 7.10 Specific IgG1 to ovalbumin according to timing of sensitisation.
8-12 week old BALB/c mice were challenged i.n. with PBS, RS virus or influenza virus ('flu). After infection the mice were exposed to a daily 20 minute exposure to 1% ovalbumin in PBS from day 4 for 10 days (A), from day 11 for 10 days (B), from day 18 for 10 days (C) or no exposure (D). Serum was obtained 5 days after the last exposure to nebulisation. Ovalbumin specific antibody was determined for different isotypes using a capture ELISA and digoxigenin conjugated ovalbumin (as described in materials and methods). Mean and standard error of the mean are also shown.

Discussion:

Anaphylaxis in Mice

Acute anaphylactic shock in mice is accompanied by marked hypoactivity that can be reversed with opiate antagonists (10). It can be mediated through IgE or IgG1, both of which bind to mast cells in the mouse. In some strains (CTS, DS, C57BL/6) anaphylaxis is far less common in younger mice (1.5 to 2.5 months). In other strains, such as BALB/c, DBA/2 and C3H anaphylaxis can be produced at all ages (144). The main mediator for anaphylactic shock in mice is platelet activating factor released from mast cells (11). IgG1 is estimated to be more effective than IgE in producing systemic sensitisation in some strains (143).

The observation of acute anaphylactic shock in my experiments was dramatic and completely unexpected. Unlike guinea pigs (221,232) anaphylactic shock in mice is less easy to produce. As far as I am aware it has only been shown with the use of adjuvants or passive transfer of serum from mice sensitised using adjuvants. Cutaneous responses to allergens have been shown after nebulisation of ovalbumin by Saloga *et al* (304) in the absence of adjuvant. Wheals were seen in my experiments at the site of intradermal injection in mice exposed to nebulised ovalbumin whether or not they had acute virus infections suggesting that some sensitisation may have occurred. However not all the mice primed with nebulised ovalbumin without virus had wheals on intradermal testing and the responses appeared smaller. This observation was made by Dr Lack whilst advising during the first set of experiments and was not made in a blinded manner. Future experiments will be able to confirm this finding. Because of the technical difficulty of detecting the very low levels of

antigen specific IgE in mice passive cutaneous anaphylaxis has been used to indicate and measure IgE. However both IgG1 and IgE are capable of eliciting cutaneous responses to cutaneous antigen when injected i.v. In mice deficient of mast cells, active cutaneous anaphylaxis is absent despite circulating IgE and IgG1 whereas passive cutaneous anaphylaxis can be generated with allogeneic hyperimmune serum (16). Therefore different mechanisms may exist for inducing passive cutaneous anaphylaxis. Evidence of active cutaneous sensitisation may reflect more closely what is occurring in allergic diseases such as allergic eczema .

Mast Cells in the Respiratory Tract

Mast cells are known to have a role in the pathogenesis of allergic asthma and are present throughout the respiratory tract. At mucosal surfaces, such as in the respiratory tract, mast cell activation is relatively restricted to cross linking of specific surface immunoglobulins (usually IgE) by inhaled antigen leading to degranulation and mediator release (162). Mast cells release histamine, leukotrienes, which cause bronchoconstriction, and TNF, IL-3, IL-4, IL-5 and IL-10 (118,251,365). Heterogeneity within mucosal mast cells may exist; evidence for this comes from differences in protease expression (162,305). Recent data from studies of mast cell knockout mice suggest that mast cells may have an important role in protection from bacterial infection (78,226) and may orchestrate or initiate responses through the rapid recruitment of circulating leucocytes by TNF-α (109).

IgE and RS virus

Welliver reported that the production and persistence of IgE in nasal epithelium after RS virus infection in infants was associated with subsequent wheezing. He noted, however, that

IgE was not usually raised in serum (368). Measles, also a paramyxovirus, causes respiratory epithelium inflammation and has also been implicated in elevating IgE levels. In a study in children with acute measles serum IgE levels were shown to be raised; it was suggested that this was another manifestation of altered immunoregulatory function during measles infection (127). A recent epidemiological study however has suggested that measles infection may protect against the development of atopy (329). In my experiments there was no elevation of total IgE levels. There was no evidence either of specific IgE to ovalbumin. Previous authors have suggested that respiratory virus infection may have a role in augmenting allergy type responses. Sakamoto *et al* and Holt *et al* exposed mice to alum precipitated ovalbumin (alum/ova) during influenza infection (164,317) and Leibovitz *et al* (214) during RS virus infection and suggested an augmentation of the allergic response to ovalbumin seen with alum/ova alone. In each case no direct measurement of IgE or IgG1 was made but passive cutaneous anaphylaxis (PCA) was used as an indirect measurement. Freihorst *et al* also used ovalbumin sensitisation during RS virus infection with and without alum (99). IgE was again assessed by PCA and no IgE was found without the use of adjuvant. The absence of detectable IgE in these experiments without adjuvant could have been due to the timing or dose of ovalbumin administration or the sensitivity of the method of detection.

Sensitisation of Mice to Ovalbumin

Recently a group in Denver has published data indicating that mice can be sensitised to ovalbumin and other protein antigens via the respiratory route (304). By this method of sensitisation mice develop allergy responses including airway hyperresponsiveness, allergen

specific IgE and cutaneous hypersensitivity (318). When IgE positive B cells were transferred from sensitised mice into naïve mice the recipients produced serum IgE (but not IgG1) and cutaneous responses to ovalbumin (210). When IgE negative ovalbumin specific B cells were transferred into naïve recipients they produced serum IgG1 and IgE to ovalbumin and cutaneous responses. In these experiments a direct ELISA was used and IgE was difficult to detect; large numbers of mice were often needed to show statistical differences (Dr G. Lack - personal communication). Because the ODs were usually very low, the level of IgE was expressed as units by comparison with a previously made serum standard which was assigned to be 1000 units. In my experiments no specific IgE to ovalbumin was found. It is possible that IgE was produced but for a number of reasons I could not detect it. The timing of the experiment may be important. In the work from Denver it was shown that IgE responses waned after the antigen exposure was removed and serum was tested shortly after antigen exposure ended. In my experiments the mice were bled 5 days after the last ovalbumin exposure by which time IgE may have fallen below the threshold for detection. IgE has a far shorter half-life (T½) than IgG1 in the blood; Haba *et al* have shown that IgE is cleared rapidly from mice with an initial T½ of 1 to 2 hours. They found only 0.2% remained after 48 hours. IgG1 is cleared less rapidly it has an initial T½ of 11 to 12 hours which then slows to a constant T½ of 9 to 11 days. Radiolabelled IgE injected into mice with pre-existing high levels of IgE from an IgE secreting tumour was cleared at the same rate suggesting that this clearance may not be due to adherence of IgE to saturable sites. Further study of the time course of ovalbumin specific Ig production in my model may help to determine whether IgE is produced. It would also be of value to determine whether later exposure to ovalbumin is associated with re-emergence of IgE and IgG1 which might suggest that a single priming episode during acute virus infection leads

to permanent sensitisation.

Mucosa Associated Lymphoid Tissue

The respiratory tract has a large surface area and as a mucosal surface shares a number of features in common with the gastrointestinal tract. Similarities in structure and lymphocyte homing between lymphoid aggregates in the lung and Peyer's patches in the gut lead to the concept of common mucosal associated lymphoid tissues (MALT) (163). However in most species bronchus associated lymphoid tissue (BALT) develops entirely postnatally and in man is not usually constitutively expressed (279) except in cases of inflammation or antigenic stimulation (68). Smoking is associated with significantly increased amounts of BALT (307). Higher levels of BALT have also been shown in a large study of infants under the age of 10 months (84). Common pathological abnormalities may exist in the gastrointestinal epithelium and the lung in atopic asthmatics. Wallaert *et al* recently showed increased expression of IL-3, IL-5 and GM-CSF in the intestinal mucosa of patients with asthma and atopy compared with controls (363). Furthermore a role in protection of the respiratory tract against bacteria (77)has been suggested for T cells after priming in the gastrointestinal tract.

Tolerance, immunosuppression or balance

Mucosal sites such as the gastrointestinal tract and lung are continuously exposed to soluble antigens and have a role in preventing harmful immune responses to nonpathogenic substances that do reach the body interior (65)as well as vigilance against pathogens. Therefore mucosal surfaces have been exploited as potential routes for the induction of

tolerance. Mucosally induced tolerance is an active process requiring an intact lymphoid immune system (106,107). Tolerance can be transferred both by serum and cells from tolerised animals and may involve the presentation of antigen by small resting B cells to naïve T cells (87,102). MALT especially BALT may have an important role in generating allergic responses. It has been suggested that tolerance may be produced through the expression of "immunosuppressive" cytokines such as IL-4, IL-10 and TGF-β (65,142). Also, rodent models of experimental autoimmune encephalomyelitis (EAE) have shown that oral feeding with antigen can be associated with TGF-β and IL-4 by $CD8^+$T cells which protects against EAE (197). It is striking that the cytokine that are involved in the generation of tolerance and the amelioration of disease are also involved in allergic responses. In the context of an inflamed mucosal surface these "immunosuppressive" cytokines may contribute to the development of allergy.

T_H2 $CD8^+$ Cells

Mouse airways smooth muscle hyperresponsiveness was shown after nebulised ovalbumin exposure by Saloga *et al* (304). Lack *et al* subsequently showed that this hyperresponsiveness was not transferred by passive transfer of IgE positive or IgG1 positive B cells unless the mice were again challenged twice with ovalbumin (210). Hamelmann *et al* (140) demonstrated that this airway smooth muscle hyperresponsiveness required the presence of $CD8^+$ cells. In mice depleted of $CD8^+$ cells IgE and cutaneous reactions to ovalbumin still occur but the production of IL-5, eosinophil accumulation and altered airways responsiveness do not. Reconstitution with $CD8^+$ cells from naïve animals during sensitisation restores these responses. In my experiments clear T_H2 cytokine production (IL-4) was shown in both $CD4^+$ and $CD8^+$ splenocytes cultured with ovalbumin. However, it

was only in $CD8^+$lymphocytes that high proportions of cells clearly had more IL-4 than IFN-γ. Other authors have recently indicated that an important functional role of $CD8^+$ cells in T_H2 responses may exist and T_H2 $CD8^+$ cells have recently been isolated from humans (224). A potential role of $CD8^+$ cells producing T_H2 cytokines in IgE production by rats has been shown by Sedgwick and Holt (328). Seder *et al* showed that naïve $CD8^+$ cells cultured in the presence of IL-4 on anti-CD3 coated plates produced IL-4 on restimulation (327). The same group used a transgenic mouse model to show that virus specific $CD8^+$ cells could induce airway eosinophilia by a switch to IL-5 production (61). A T_H2 environment was generated by first giving intraperitoneal alum precipitated ovalbumin and challenging with intranasal ovalbumin. $CD8^+$ cells extracted from the lung were cultured on anti-CD3-coated plates with peptide and IL-4. IL-5 production was seen in peptide specific $CD8^+$ cells shown to have the transgenic TCR. The authors suggest that a similar mechanism may exist for the link between wheezing in asthma and acute virus infection. However the model used relied on the use of adjuvants to create a T_H2 environment and in my hands virus infection *per se* was associated with an effect equivalent to or greater than adjuvant. Therefore I suggest that virus infection may allow the generation of $CD8^+$cells producing T_H2 cytokines in response to antigen stimulation that allow subsequent wheezing on exposure to allergens or viral infections. This hypothesis should be testable using my model.

Summary

The data presented in this chapter indicate the possible effects respiratory viruses may have in the development of allergy to protein antigens. Primary sensitisation to an allergen would be unlikely through an intact mucosal epithelium. The respiratory mucosal surface has, after all, developed with the immune system over millions of years to function in an environment

with pollens, grasses and other antigens. However, inflammation of the respiratory mucosal epithelial surface may allow the entry of allergens, or the exposure of mast cells, B cells and T cells to allergens in a cytokine rich environment and lead to sensitisation. Respiratory virus infections are common and cause severe airways irritation and inflammation. They are especially common in childhood. RS virus outranks all other respiratory infections as a cause of severe respiratory illness in young children during a time when their relatively immature immune system is exposed to a wide range of antigens for the first time. Apart from the increase in hayfever and asthma, a great deal of concern exists about the increasing incidence of severe anaphylactic reactions to food antigens such as peanuts, extracts of which are found in a wide range of foods. Some authors are now recommending that children should not be exposed to peanuts or peanut extracts during the first years of life (319). The peanut antigen triggering anaphylaxis is easily identified. However, the acute phase of a mucosal viral infection would long since have disappeared and such a potent cause of inflammation could have been responsible for allowing sensitisation in the first place. Through this model it will be possible to examine further the way respiratory virus infections could induce this type of severe allergic response.

Chapter 8 Discussion

Since RS virus was identified in the 1950s our understanding of the pathogenesis of many diseases has undergone a profound change. Powerful new tools in virology and immunology, like PCR, have made it possible to answer important questions. In this chapter I will review the key findings from this thesis and discuss how they enhance our understanding of the infectious cycle of RS virus and its role in disease.

Viraemia in children during primary infection

I have shown, using nested RT-PCR, that a cell associated viraemia occurs in children during primary RS virus infection. No virus was found in the cerebrospinal fluid or in serum. Viraemia may be a route by which the virus reaches other organs such as the central nervous system, the liver or bone (213,296). As discussed earlier, apnoeas that occur in young infants with RS virus infection may be due to central infection. Although viral RNA was not found in the CSF in my experiments, RS virus could be deeper in the brain within cells. Further studies to determine this may be possible using RT-PCR or *in situ* PCR.

Immunosuppressive effects of paramyxoviruses

Domurat *et al* found that adult peripheral blood monocytes are infectable *in vitro* (73) and that RS virus antigen can sometimes be found on circulating mononuclear cells. Others have shown that RS virus induces the secretion of cytokines from human monocytes such as IFNα and that this secretion may decrease the lymphoproliferative response to RS virus (203,289). This may be one of the factors that allows reinfection with RS virus. Measles virus, which is closely related to RS virus, also has profound effects on the immune system. Measles infection of PBMCs in vitro causes inhibition of phytohaemagglutinin (PHA) induced

proliferative responses (321). Sometimes intercurrent measles infection is associated with a transient remission of immunological disorders such as idiopathic thromocytopaenia (217). Infection of immunologically active cells like monocytes or lymphocytes may confer an advantage to RS virus that allows it to avoid rapid clearance and enhances the chance of transmission and persistence.

Implications for antiviral therapy

Small particle administration of ribavirin by inhalation is effective in children who catch RS virus and have defined risk factors, such as immunodeficiency or chronic lung disease (1). *In vitro* ribavirin is a potent broad spectrum antiviral with its most pronounced activity against ortho- and paramyxoviruses (66). Given intravenously, it is life-saving in Lassa fever with few side-effects except for reversible mild anaemia and sometimes rigors (94). Nebulised drug delivery during severe RS virus bronchiolitis is made more difficult by airways plugging and ventilation perfusion mismatch. Intravenous ribavirin administration in severe bronchiolitis may make logical sense in view of the viraemia shown in chapter 3. It may be an adjunct to or even alternative to nebulisation. This route of administration would also decrease the potential risk to staff (297).

Persistence of RS virus in the lung

There is no known animal reservoir for RS virus. One question has been where RS virus resides during the summer months when clinical isolates are extremely unusual. In chapter 4 it was shown that RS virus persists in lung tissue in the mouse model. The mechanisms of persistence were not determined but it is clear that serum antibody and cytotoxic responses are present. Selection for mutations in the major epitope for CTL recognition does not seem

to occur. Another mechanism presumably exists to allow persistence and the mouse model will allow further experiments to determine it.

In chapter 6 I showed how immunosuppression could allow the isolation of RS virus from the lungs of mice that had recovered from infection. Whether RS virus can be reactivated in the immunocompetent host to produce spread to other animals also needs to be determined. If reactivation occurs in man it may explain the sudden re-emergence of RS virus in yearly epidemics. The long term effects of bronchiolitis include recurrent wheezing in children even ten years later and persistence of the virus may go some way to explaining this effect. RS virus replication inducing a continuous low level inflammation may alter the immune response to other antigens, including respiratory viral infections, leading to more severe disease. In young children with smaller airway diameters, this may be the cause of wheezing episodes, and would explain why children appear to grow out of virus associated wheeze at 3 to 6 years of age (367,372)as airway diameters increase with growth. From that time responses associated with allergy might then appear more important, as will be discussed below.

Associations with allergy

In chapter 7 it was shown that exposure to ovalbumin at the same time as influenza virus or RS virus infection caused sensitisation to ovalbumin. Sensitisation was associated with acute anaphylaxis on intradermal exposure to ovalbumin, a dramatic and unexpected finding. It was shown that this sensitisation was associated with high levels of ovalbumin specific IgG1. In mice sensitised to ovalbumin during acute respiratory virus infection a higher proportion of splenocytes produced IL-4 in response to ovalbumin than in uninfected

controls. As far as I am aware this is the first time enhanced sensitisation to an allergen has been shown in the context of an acute respiratory virus infection without the use of adjuvants. It clearly indicates that respiratory viruses can act as cofactors for the generation of allergic responses to protein antigens. Although influenza virus appeared to have a more potent effect on both specific IgG1 and IL-4 levels, it should be remembered that far more infants are infected with RS virus in early years than with influenza. Therefore, in numerical terms, RS virus may represent more of a potential threat in promoting allergic responses.

The role of respiratory viruses in predisposing to asthma is still controversial. Recently it has been suggested that virus exposure in early life has at most a minor role in the aetiology of wheezing and may in fact be protective against both allergies and asthma (229,230). Martinez has argued that acute respiratory viruses produce T_H1 responses predominantly and may inhibit the development of T_H2 clones that may be associated with allergy (229). Kudlacz *et al* recently showed that sensitisation to ovalbumin during parainfluenza virus infection diminished lung histamine release by guinea pigs on rechallenge with ovalbumin (205). However these findings and interpretation may not conflict with my data. Firstly, although acute RS virus infection is associated with a vigorous T_H1 antiviral response, individual RS virus proteins may be associated with T_H2 responses, that although difficult to identify during acute infection, may be very important. Secondly, the timing of antigen exposure may be very important, as was seen in my experiments. Levels of environmental antigens, such as house dust mite and pollens, are likely to be at a constant level throughout the acute phase of virus infections when infants have a primary infection. In the work of Kudlacz *et al*, for example, ovalbumin exposure was limited to 2 days separated by a week and commenced on day 7 or day 19 after infection and when parainfluenza antigen was no

longer detectable in the lung. This may have had a very different effect on the ovalbumin response.

What needs to be done next?

a) In the mouse

We do not know the mechanisms by which RS virus persists in the lung. First, it will be important to determine which cell types it persists within. Initial *in situ* hybridisation experiments (not discussed in this thesis) suggest this technique may not be sensitive enough and it may be that this question has to be answered using the more sensitive method of *in situ* PCR. Mechanisms by which the virus avoids elimination by the immune system need to be determined, the effect of the virus on class I MHC expression in infected cells, for example. As discussed in chapter 1, the proteins of RS virus may have important immunological effects. Information about the possible role of G protein in stimulating T_H2 cells has already come from Dr Openshaw's group (272). Collins *et al* suggest that the non-structural proteins of RS virus should also be investigated to see whether they have an interaction with the immune system. NS2 has a short half life and may be secreted suggesting a potential role in interacting with the host (reviewed in (58)).

In chapter 6 it was shown that when mice infected 120 days earlier were immunosuppressed using depleting antibodies, RS virus could be recovered from their lungs. It remains to be shown whether reactivation could be produced without profound immunosuppression. RS virus epidemics may begin because of reactivation in some individuals. This could be due to specific triggers such as local irritation or inflammation. It is possible that atmospheric conditions could have an important role in this, bearing in mind that epidemics occur

specifically during the winter months. There may be a role for stress and chemical pollutants (such as exhaust fumes and smoking) on virus reactivation. RS virus persistence may alter the response to other pathogens such as rhinovirus or pseudomonas. There is some evidence that colonisation and acute infections with pseudomonas in children with cystic fibrosis may coincide temporally with the RS virus season (183,270,285). Experiments can be designed to examine the effect of RS virus infection in dual infections with other pathogens.

In chapter 7 a model of allergic sensitisation associated with RS virus and influenza infection was described. A great number of questions are raised by these novel findings. It will be important to determine whether specific T cells are involved in generating this sensitisation. Depletion of CD4 cells, CD8 cells and B cells may indicate the importance of these subsets. It may be interesting to use mice deficient of mast cells to examine the role these cells may have. Furthermore the role of specific cytokines including IL-4 and IL-5 may be important. It needs to be determined how long this sensitisation lasts and whether tolerance to ovalbumin can be generated after they have been sensitised. It may be that other viruses such as parainfluenza and rhinovirus may also be able to promote allergic responses. Site specific responses could also be examined, for example the response in the upper respiratory tract and local draining lymph nodes. These questions can be addressed in this model.

b) In man

Recently it was suggested that persistent adenovirus infection may be associated with chronic obstructive pulmonary disease in adult smokers (233). A role of RS virus in chronic wheezing and asthma has been discussed for many years. RT-PCR could be used to determine whether RS virus is in lung biopsy and postmortem specimens in children and

infants that have recovered from primary infections. It may be possible to develop an *in situ* PCR to try to determine which cells become infected and whether the brain is infected. Of particular interest are infants who are victims of sudden infant death syndrome, in whom a possible role for viruses has been suspected (12,96,356) possibly through immunopathology (310). Further sorting experiments may be able to discover which peripheral blood cells carry RS virus. However, this may be difficult because of the large amount of blood that may be required and the speed needed to sort blood cells into highly purified populations. Following the results demonstrating sensitisation of mice to ovalbumin, the T cell cytokine responses to common allergens during and after RS or influenza virus infection may provide useful information about whether these viruses are involved in sensitisation in children. It may that reinfections also promote sensitisation and that reinfections of adults during periods of high pollen counts may account for later onsets of hayfever and allergic rhinitis. Prospective case control studies examining IgE to allergens before and after RS virus infection may test this link.

In Conclusion

In this thesis I have shown that RS virus can cause a cell associated viraemia in primary infection. Circulating peripheral blood mononuclear cells can contain RS virus and may be a route for dissemination to other organs. RS virus persists in the lung for more than 100 days after primary infection of mice. The whole virus is present but the cells that are infected have not yet been determined. Both RS and influenza virus may augment the response to inhaled ovalbumin and cause profound sensitisation. In mice, this sensitisation produces acute anaphylactic shock following cutaneous challenge with ovalbumin and occurs without the use of adjuvant. Using this model it is possible to explore some of the roles of

RS virus in causing chronic wheezing, asthma and allergy

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