Mapping of B-cell Epitopes on the Fusion Protein of Human Respiratory Syncytial Virus

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ABSTRACT

HRSV is the leading cause of lower respiratory tract (LTR) infection in the new-born and during early childhood, accounting for approximately 90% of the reported cases of bronchiolitis in infancy. The WHO estimates that of the 12.2 million annual deaths in children under five years of age, a third is due to acute infections of the LRT. RSV may also be the cause of up to 2.4 % community-acquired LRT infections in adults of less than 60 years of age and in elderly people.

The fusion (F) and attachment (G) proteins are the major protective antigens in RSV. Almost all the available F-specific monoclonal antibodies efficiently neutralize RSV in vitro and monoclonal antibodies have been successfully used for *in vivo* treatment of human RSV infection. Since the F-protein is genetically more stable than the G-protein it represents a better candidate for vaccine development. Immunity to RSV includes serum antibody, secretory antibody, and cytotoxic T-cell responses. Secretory antibodies are mainly effective in the upper respiratory tract, while serum antibodies are the key for resolution of LRT infection. However, immunity to RSV is incomplete and re-infections occur throughout life. The work described in this thesis was designed to:

A: Identify both linear and conformational B-cell epitopes of the fusion protein of human RSV as a part of a programme of work to develop an epitope based vaccine against RSV. 55 overlapping peptides covering the whole of the fusion protein of RSV were synthesized by solid phase synthesis using the RAMPs system these peptides were used to screen sera from RSV-infected individuals (9 adults and 49 infants) for reactivity with linear epitopes using an Enzyme Linked Immunosorbent Assay (ELISA). In addition, a human monoclonal antibody (RF2) that recognizes a conformational epitope on the fusion protein of HRSV was used to screen a

combinatorial peptide library to identify mimotopes of this conformational epitope. The linear epitopes and the mimotopes identified in this way were used *in vivo* experiments to assess their ability to induce anti-peptide antibodies that can cross-react with RSV.

B. In an attempt to throw light on the question of how RSV can re-infect the same individual, serial serum samples were obtained from infants prior to exposure to RSV at 6 months of age, following primary exposure at 9-12 months and following second exposure at 12-18 months. The reactivity of these sera with the 55 overlapping peptides from the F protein was assessed by ELISA to determine if there were differences in the pattern of peptide recognition at different stages of exposure to RSV.

DEDICATION

TO MY FAMILY

TO

- The soul of my grandfather (Muhammad Al-Sanie) which was the most precious thing I lost during my PhD he encouraged me and believed in me, unfortunately he did not see this day.

- My loving supporting mother, the most wonderful mother in the world.

- My father who I lived all my life to gain his respect and meet his expectations. He made me who I am, and I hope one day he can be proud of me and trust my decisions in life.

- My grandmother, constant lovely prayers, and food supplies.

- My beloved sisters and brother.

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List of abbreviations

ADCC	Antibody-dependent cell cytotoxicity
BBG_2Na	The sequence 130-230 of the RSV G protein expressed in *Escherichia .coli* (G2Na) and bound to the C terminus of an albumin-binding region of the streptococcal G protein (BB)
Boc^t	tertiary butoxycarbonyl
BSA	Bovine serum albumin
CFA	complete Freund's adjuvant
CTL	cytotoxic T lymphocytes
DCs	dendritic cells
DIC	diisopropylcarbodiimide
DMAP	2-dimethylaminopyridine
DMF	N, N-dimethylformamide
DMSO	Dimethyl sulphoxide
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid disodium
ELISA	Enzyme linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FI-RSV	
Fmoc	flourenyl methoxy carbonyl
Fmoc-gly	
GM-CSF	Granulocyte macrophage colony-stimulating factor
GXM	*Cryptococcus neoformans* capsular polysaccharide glucuronoxylomannan
HOBt	Hydroxy-benztriazole
HPLC	High performance liquid chromatography
HRSV	Human Respiratory Syncytial Virus
HSV-1	Herpes simplex virus type 1
ICAM-1	intracellular Adhesion Molecules- 1
IFA	in_incomplete Freund's adjuvant
IL-1	Interleukin- 1
IL-2	Interleukin- 2
IL-4	Interleukin- 4

IL-10	Interleukin- 10
IL-12	Interleukin- 12
ISCOMs	ImmunoStimulating Complexes
k_a	Association- rate
k_d	Dissociation -rate
K_D	Dissociation- constants
KLH	Keyhole limpet hemocyanin
LD	Lyme disease
LRI	lower respiratory tract infection
LRT	lower respiratory tract
mAb	Monoclonal antibody
Mab 19	Monoclonal antibody 19
Mab 2E9	Monoclonal antibody 2F9
Mabs	Monoclonal antibodies
MEM	Minimal Essential Medium
MHC	Major histocompatibility complex
mRNAs	Messenger ribonucleic acids
Mtr	4, methoxy, 2,3,6, trimethyl
MV	measles virus
NDV	Newcastle disease virus
NDV-F	Newcastle disease virus-Fusion protein
NP	nucleoprotein
NS1	Non-Structural protein 1
NS2	Non-Structural protein 2
Obu^t	tertiary butyl ester
Opfp	pentafluorophenol ester
PIV	parainfluenza virus
PS	Polysaccharide
RF2	Human monoclonal antibody
RSV	Respiratory Syncytial Virus
RSV Ag	Respiratory Syncytial Virus Antigen
RU	Resonance Units
SCID	Severe combined immuno-deficient mice
SLE	Systemic Lupus Erythematosus
SPR	Surface Plasmon Resonces

ssDNA	**Singlestranded deoxyribonucleic acid**
Stat-11	**Signal Transducers and Activators of Transcription-11**
Stat-4	**Signal Transducers and Activators of Transcription- 4**
$TCID_{50}$	**Tissue Culture Infectivity Dose that kills 50% of infected cells**
TCR	**T-cell receptor**
TFA	**Trifluoroacetic acid**
TLR4	**Toll-like receptor 4**
TM domain	**transfer domain**
TNF	**Tumor Necrosis Factor**
TNF-α	**Tumor Necrosis Factor- gama**
TRT	**Trityl for histidine**
ts	**temperature-sensitive**
URT	**Upper respiratory Tract**
URTI	**Upper Respiratory Tract Infection**
WHO	**World health Organization**
WT infection	**Wild type infection**

TABLE OF CONTENTS

LIST OF FIGURES

Numerous Originals in Colour

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Human Respiratory Syncytial virus

Human Respiratory Syncytial Virus (RSV) is the single most important cause of lower respiratory tract infection (LRI) in newborn infants and immuno-suppressed individuals worldwide (WHO 2002).

The virus was first isolated in 1956 as the cause of chimpanzee coryza (symptoms that are similar to the common cold) and subsequently it was repeatedly isolated in Baltimore from a group of children with pneumonia (Morris, Blunt et al. 1956; Chanock, Roizman et al. 1957; Simoes 1999). It accounted for virtually up to 98% of reported cases of LRI in the USA, and total medical costs for all RSV-related hospitalisations for children <5 years old were estimated at $US652 million in the year 2000 (Paramore, Ciuryla et al. 2004). In a survey carried out in Maryland -USA between 1997 to 2000, an estimated 390 RSV-related deaths occurred in 1999 only (Leader S 2003). In the UK around 20000 infants are hospitalized result of an RSV infection annually (Handforth, Friedland et al. 2000). The WHO estimates that there are repectively 160000 annual deaths in children less than 5 years of age caused by RSV, and up to 64 million worldwide infections (WHO 2002). This has made the WHO to assign the virus for rapid vaccine development.

Although traditionally it is considered a pediatric pathogen, RSV infection can lead to a life-threatening disease in immuno-compromised individuals and in the elderly (Webster 1999). Although the severity of disease declines with repeated infection, previous infection does not prevent disease and any immunity developed therefore appears to be incomplete (Hall, Walsh et al. 1991).

RSV is a member of the genus *Pneumovirus* of the family *Paramyxoviridae*; which are enveloped non-segmented negative-stranded RNA viruses. Negative-stranded RNA viruses encode and contain their own RNA transcriptase but mRNAs are only synthesized once the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires the continuous synthesis of viral proteins (Fields and Howley 2001) (Figure.1.1). The RSV genome is a single strand of RNA composed of approximately 15,000 nucleotides that are transcribed into 10 major sub-genomic mRNAs that encodes 11 major viral proteins.

Cross-neutralization studies have shown that RSV isolates can be classified into two groups, RSVA&RSVB of roughly equivalent virulence (Anderson, Hierholzer et al. 1985; Mufson, rvell et al. 1985).

The development of an effective vaccine against RSV could reduce RSV-related mortality and although the importance of RSV has been known for more than 30 years and the virus targets a wide range of high-risk groups, there is still no approved vaccine nor a highly effective antiviral therapy against this pathogen (Polack and Karron 2004).

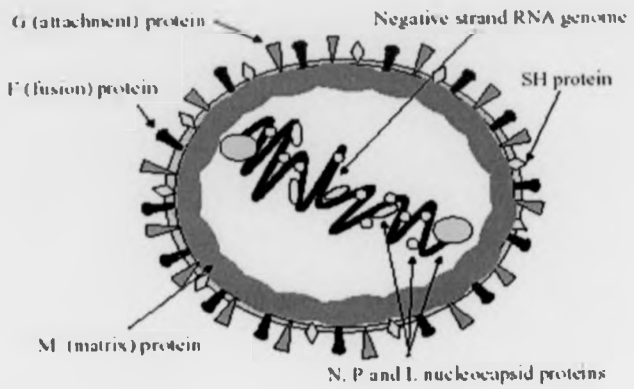

Figure 1.1

The structure of respiratory syncytial virus.

The RSV virion is about 200 nm in size and consists of a nucleocapsid within a lipid envelope. The nucleocapsid is a symmetrical helix with a helical diameter of 12-15 nm. The lipid bilayer is derived from the host plasma membrane and contains virally encoded transmembrane surface glycoproteins which are 11-20 nm in size and closely spaced at intervals of 6-10 nm. (from Hacking and Hull 2002).

1.2 The Virus

RSV virion consists of the nucleocapsid enclosed within a lipid envelope that is derived from the plasma membrane of the target cell. The virions are irregular in shape, range in diameter from 15-300 nm and can be seen by electron microscopy or when negatively stained. They can be visualized in the tissue culture as filamentous forms which are 60 to 100 nm in diameter and up to 400 nm in length. Enclosed in the envelope are three viral trans-membrane surface glycoproteins: the attachment protein G, the fusion protein F, and the small hydrophobic protein SH (Fields and Howley 2001) (Figure. 1.1).

The RSV genome is a single-stranded negative sense RNA of 15,200 nucleotides that is transcribed into 10 sub-genomic mRNAs. Transcription is initiated from the 3' end with only a fraction of the polymerase moving on to the next gene (Figure 1.2).

RSV encodes 11 proteins: (Figure.1.3).

- Four proteins are associated with the genomic RNA to form the viral nucleocapsid (N, P, and L) and M2. The RSV M2 gene contains two partially overlapping open reading frames (ORFs), designated M2-1 and M2-2, which give rise to two proteins, M2-1 (194 amino acids) and M2-2 (90 amino acids), M2 ORF-2 is a second distinct protein transcribed from the M2 gene, which has a defined function in transcription regulation. The mechanism for translating the M2-2 ORF is not clear, but it may involve a ribosomal stop-restart. The M2-1 protein is an essential transcriptional elongation factor and in its absence, the polymerase does not transcribe beyond the NS1 and NS2 genes. The M2-1 gene also increases RNAP processivity across the gene junctions, attenuating transcription termination. The M2-2 gene is not essential for RSV growth, as it can be deleted from a recombinant RSV. However, the

M2-2 deficient virus grows slowly in tissue culture and there is an increase in transcription and decrease in RNA replication, suggesting that the M2-2 protein is involved in regulating transcription and RNA replication.

- The N protein (391 amino acids) binds tightly along the entire length of genomic RNA to form an RNAse-resistant nucleocapsid.
- L is the major polymerase subunit- it contains six discrete segments, are highly conserved and represent the functional domains of the protein (polymerase motifs).
- The P protein is about 507 to 603 amino acids long and is thought to associate with free N and L to maintain them in soluble forms and might also participate as a cofactor in RNA synthesis (Fields and Howley 2001).The three proteins N, P and L form a complex that is essentially adequate to direct RNA replication.
- Three major proteins of RSV are the trans-membrane surface proteins G, F, and SH. The surface fusion (F) protein and the attachment (G) glyco-protein are the two major immunogenic proteins. Both proteins induce the production of RSV neutralizing antibody responses and they are therefore important targets for vaccine development (Fields and Howley 2001).
- One protein is a non-glycosylated virion matrix protein (M), which is about 341 to 375 amino acid long.
- Finally, there are two non-structural proteins that accumulate in infected cells but are present in only trace amounts in mature virions the NS1 and NS2 proteins. The RSV NS1 (139) amino acids) and NS2 (124 amino acids) are considered to be non-structural proteins. Neither protein is thought to be essential for virus growth in cultured cells or in chimpanzees, as the genes can

be deleted from a recombinant RSV, but the growth rate was reduced substantially both in vitro and in vivo.

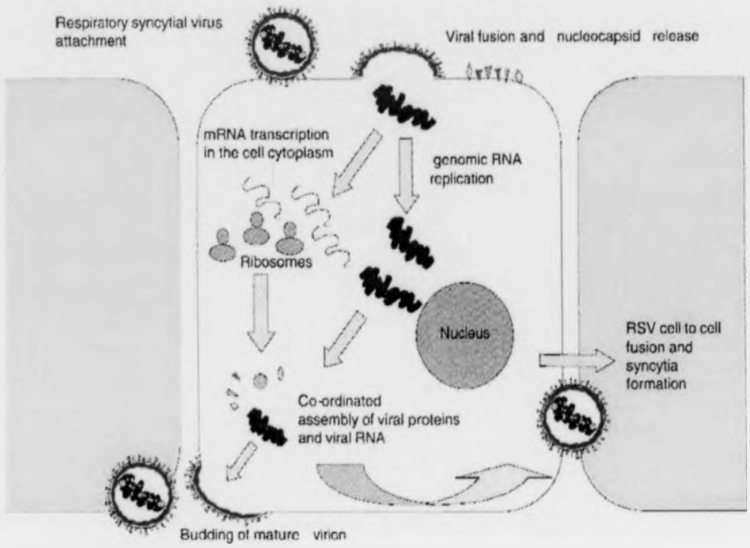

Figure 1.2

The replicative cycle of RSV.

RSV attachment occurs via the G protein. Fusion is mediated by the F protein after which the viral envelope is incorporated into the cell membrane and the nucleocapsid is released into the cytoplasm. RSV mRNA accumulates for about 15 h after infection and then remains at constant levels thereby allowing for RNA replication and subsequent viral assembly. The M2-2 gene governs this transition from transcription to production of genomic RNA. The M protein and membrane-destined G protein meet in the Golgi and can interact through a six amino acid motif at the cytoplasmic end of the G protein. It is likely that the F and G proteins interact with each other via their cytoplasmic domains. Meanwhile the N, P, L and M2-1 proteins form inclusions in the cytoplasm and it is then thought that M2-1 interacts with the M protein. Through these series of interactions the M proteins can be seen to co-ordinate the assembly of the envelope proteins F and G with the nucleocapsid proteins N P and M2-1. Budding appears to be the reverse of penetration and occurs in vitro on the apical cell surface (from Hacking and Hull 2002).

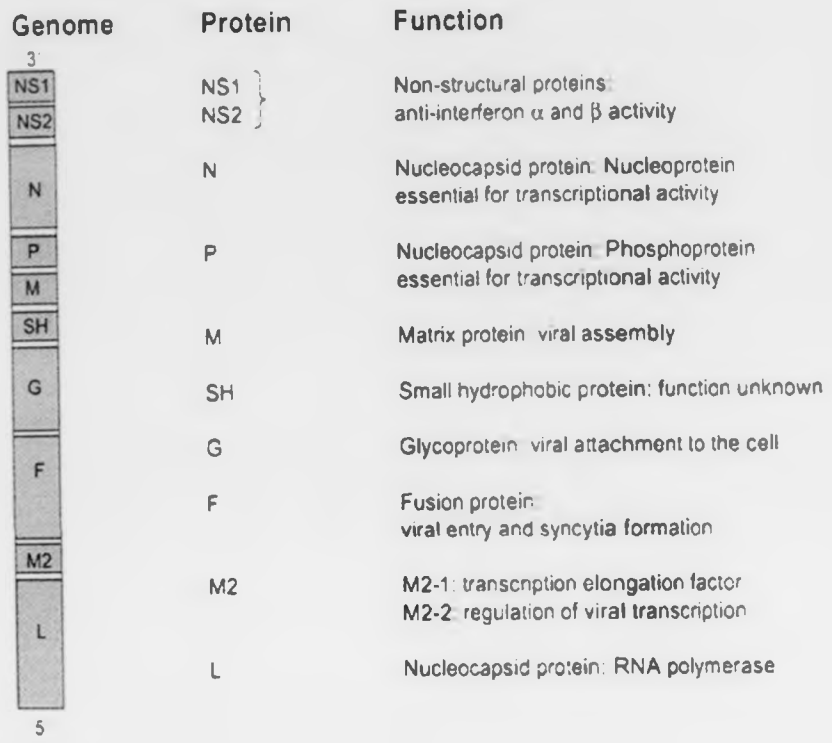

Figure 1.3

Proteins encoded by RSV (from Hacking and Hull 2002)

1.3 Fusion Protein

The F protein is responsible for virus penetration through fusion between viral and host cell plasma membrane. It was first identified when anti F-specific monoclonal antibodies (Mabs) inhibited syncytium formation in tissue culture (Walsh and Hruska 1983). While the F protein is able to mediate fusion in recombinant viruses lacking G and SH, efficient fusion occurs only when all the three proteins are expressed (Karron, Wright et al. 1997). The F protein of RSV shares structural and functional features with the F-proteins of *Paramyxoviruses* for example: the size of the protein (the *Paramyxovirus* F genes encode 540 to 580 amino acids residues), the location of the hydrophobic domains, the cleavage peptide and the cysteine residues (mostly clustered in a 150 amino acid long segment of the F1 subunit) (Walsh and Hruska 1983).

The fusion process starts by the fusion peptides being inserted into the target membranes, later in the course of the infection the F proteins at the membrane of an infected cell fuse with the nearby cells forming syncytia (giant cell formation), and viral spread (Fields and Howley 2001).

F is a type I glycoprotein, which is synthesized as a precursor of 574 aa, the precursor (F_0) needs to proteolytically cleaved to form the disulphide linked-biologically active protein (F_1- F_2) (Vey, Schafer et al. 1994). This cleavage of F_0 is believed to take place in the Golgi or trans Golgi network (Collins and Mottet 1991; Bolt, Pedersen et al. 2000), where it is glycosylated and results in the release of the biologically active protein consisting of the disulphide-linked chains F_1 and F_2: the F_2 domain (amino acid 1 to 130), the cleavage peptide (amino acids 131 to 136), and the F1 domain (amino acids 137 to 574) (Fields and Howley 2001). Recent data show that this cleavage process of F_0 is a unique feature of RSV. The furin-like protease cleaves the

molecule at two sites (furin consensus sequences) RAR/KR109 (FCS-2) and KKRKRR136 (FCS-1), which release the region between the two cleavage sites as a separate peptide that consist of 27 amino acids (pep27) (Calder, Gonzalez-Reyes et al. 2000; Zimmer G 2001). Data from Calder et al., 2000 suggest that this third peptide is released from the processed molecule and its function is unknown.

Structural studies on the F protein

The F2 is the most divergent fragment of the F molecule. It has the bulk of the carbohydrate content and therefor; it is a potential site for N glycosylation in the F protein. A region of 28 hydrophilic amino acids that is unique to HRSV is located at the N-terminal end of the F2 (Lopez, Bustos et al. 1998).

The model of the secondary structure of the protein shows:

- Three hydrophobic regions; the first is the signal peptide, located at the N terminus of the F2 chain. The second is the fusion peptide at the N terminus of the F1 chain, and the third is the transmembrane region, located near the C terminus of F1 (Lopez, Bustos et al. 1998).
- Two heptad repeat sequences; two sequences, HRA and HRB are located next to the fusion peptide.
- A third heptad repeat region (residues 53-100) is within the sequence of the F2 chain of RSV-F (Lambert, Barney et al. 1996).
- A cluster of cysteines region is located between the two heptad repeat in the middle of the F1 chain (Lopez, Bustos et al. 1998).

Calder et al.2000 recently suggested two morphologic shapes that the protein takes- the full-length F formed rosettes that had two types of protein rods, one cone-shaped and the other lollipop shaped. The secreted form of the protein formed individual

cone-shaped rods. These authors suggeste that the morphologic shapes might play role in membrane fusion (Calder, Gonzalez-Reyes et al. 2000).

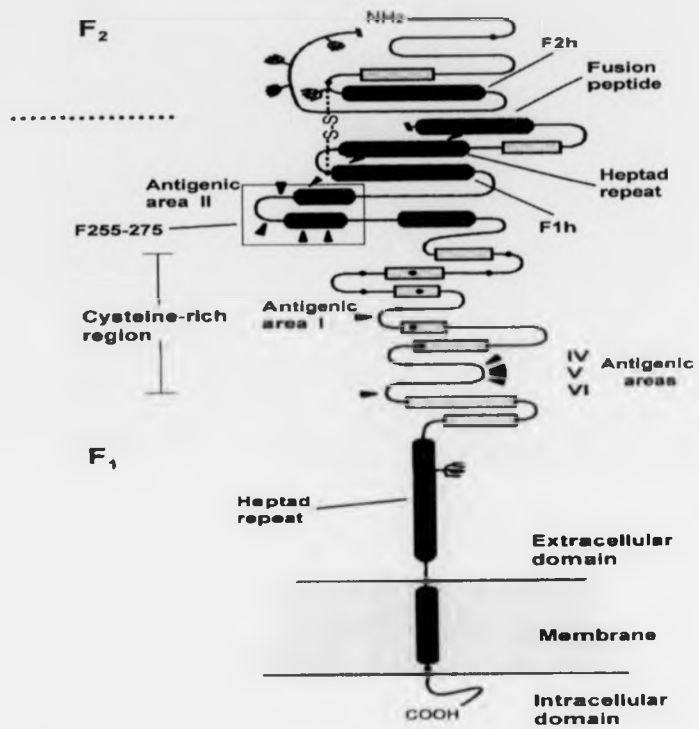

Figure 1.4

Predicted secondary-structure of the F protein.

This figure demonstrates the location of secondary –structure motifs of the F protein and shows cysteine residues (•), potential N-glycosylation sites (ψ), site of proteolytic processing (↓) (2), amino acid changes in escape mutants (▼) hydrophobic regions (—) and S-S, disulphide bridge. The predicted secondary-structure elements of the F protein are: α-helices (cylinders), β-sheets (rectangles) and loops (turns).The segment between residues 255 and 275 (F255-275) are predicted to fold in α-helix-loop-helix structure, is boxed. F1h and F2h are amphipathic α-helices in the F1 and F2 chains, respectively (Lopez, Bustos et al. 1998).

1.4 Epidemiology

RSV has seasonal epidemics which occur annually. Peak rates of infection occur in the winter months in areas of temperate climate while in tropical or hot climates the outbreaks are expected during the rainy summer season (Glezen and Denny 1973).

RSV is highly infectious and it spreads via personal contact or through exposure to contaminated surfaces, for example bedding and sheets. The virus is inactivated in an aerosol so there is a very limited chance of transmission through aerosol droplets (Collins and Pollard 2002). Infected respiratory secretions can transmit RSV, not by small droplets but by contamination of environmental surfaces such as hands or bedding (Simoes 1999; Webster 1999). By the age of one, 50 % of all children will have been infected with RSV and by 24 months, 95% of all children will be sero-positive for RSV; half of these children will have been exposed to the virus twice (Kim, Arrobio et al. 1973).

High-risk groups include infants with a history of premature birth; those with bronchopulmonary dysphasia, congenital heart disease, cystic fibrosis, immuno-suppressed patients, and those with underlying disorders of cellular immunity. Furthermore, individuals living in institutions and elderly people are at particular risk. During epidemics, spread within hospitals and day nurseries often takes place, facilitated by close personal contact and the ability of those infected to shed virus for up to 3 weeks after the acute phase (Simoes 1999).

RSV A and B viruses co-circulate during epidemics, with group A being predominant (Hall, Walsh et al. 1990). The impact of antigenic diversity on RSV epidemiology is not completely understood, but it may partly be explained by the susceptibility to re-infection through life and the yearly variation in the severity of epidemics within communities. Epidemiological studies have highlighted several factors that could be involved in increasing the risk of infections. In developing

countries, risk factors have not been defined; although overcrowding, indoor pollution and malnutrition may play a part in the development of more severe disease. In a recent study carried out in the Gambia, a higher incidence of lower respiratory infection (LRI) was found in boys than in girls and case incidence of infection was greater in small or rural villages than in urban or large villages, possibly due to poverty (Weber 2002).A very interesting observation was made in this study-the possibility of cross- protection between RSV and the measles virus. After 3 years of regular outbreaks of RSV, a low number of cases were then observed associated with a measles epidemic that followed several years of low numbers of measles cases. This observation was supported by the results from a study in the US that suggested that an increase in RSV cases was correlated with the use of measles vaccination to control measles infection (Weber 2002).

In developed countries, factors that can increase the risk of infection include: family history of atopy, having a sibling in school, exposure to passive cigarette smoking, and attendance at a day care centre or a child minder (Simoes 1999).

1.5 Management of RSV

Although RSV was discovered 25 years ago and bronchiolitis caused by RSV remains the most common disease in infants and childhood against this virus infection, up to the present time there is neither a vaccine nor effective treatment (Kimpen 2001).

An early study showed that infants under 2 months of age were relatively spared from infection which was confirmed by the results of a later study showing the inverse correlation between the severity of RSV and pneumonia, and the level of neutralizing maternal antibodies (Bruhn and Yeager 1977). In another study by Ward et al.1983, sera from 100 new- born babies and maternal antenatal sera were tested. The infants of mothers who had higher titres of neutralizing antibodies remained protected from infection. In contrast, infants of mothers who had lower levels of neutralizing maternal antibodies caught RSV infection before 6 months of age (Ward, Lambden et al. 1983).

Data from humans and animal models highlighting a protective role for antibodies in RSV infection initiated research that focused on passive immunization of high risk groups. Thus research focused on the preparation and use of immuno-globulin obtained from individuals with high anti-RSV antibody titres. Three trials were carried using RSV-IG (Groothuis, Simoes et al. 1993). It was concluded from these trials that during the RSV season, the monthly administration of 750mg/kg body weight RSVIG was safe. RSVIG administration was tolerated by infants and was effective in reducing the incidence and severity of RSV LRI in high risk infants.

On the basis of these trials, the product was approved for use in the USA in 1996, and later, the American Academy of Pediatrics (AAP) produced guidelines for the use of the product. Potential disadvantages associated with the use of RSIG include the risk of blood-born disease contamination, unreliability of the supply, and the possibility of interference with childhood vaccination (Rodriguez, Gruber et al. 1997).

All these factors resulted in efforts to develop humanized monoclonal antibodies against RSV that could be used in therapy. Palivizumab (MEDI-493, Medimmune Inc) was produced. This is an IgG-1 humanized monoclonal antibody against the fusion protein of RSV. The antibody was tested in premature infants with or without chronic lung disease and its use resulted in the reduction RSV-related hospitalization by 55 % (IMPACT RSV Study Group 1998), which was comparable to data from using RSVIG. Based on this study, AAP approved and updated the guidelines for the use of RSVIG in 1998 (Rodriguez, Gruber et al. 1997) (IMPACT RSV Study Group 1998).

Antiviral therapy

Antiviral therapy against RSV includes the use of ribavirin and corticosteroids. Ribavirin first showed promising in-vitro antiviral activity but was stopped because of its financial costs. Subsequently, the use of ribavirin was reviewed between 1975-1996 and it was concluded that there was not enough data to support the benefits of using Ribavirin in infants with bronchiolitis (AAP 1996). Still, the AAP advised the use of Ribavirin in infants with underlying diseases and severe cases of bronchiolitis, as well as children receiving ventilation. The use of corticosteroids was mainly considered because of the similarity of bronchiolitis and asthma syndromes. Since the 1960s, six trails have been carried out to test the use of corticosteroids (Kimpen 2001). Data from these six studies suggest significant improvement in the clinical symptoms and the duration of symptoms in RSV bronchiolitis. Corticosteroids were also assessed for the prevention of wheezing after an RSV episode and the data were conflicting. Data so far suggest that only severe cases in which mechanical ventilation was required could benefit from the use of corticosteroids, but still guidelines are needed (Hemming, Prince et al. 1995; Kimpen 2001).

1.6 Immunity to RSV

Immunity to RSV is mediated first via humoral and later via cellular immune mechanisms. Immunity thus includes serum antibody (induced following infection or maternally-derived in young infants), secretory antibody and major histo-compatibility complex class I-restricted cytotoxic T lymphocytes (CTL) (Dudas and Karron 1998). Exposure to RSV gives only partial immunity and it is incomplete, short lived, and re-infection occurs throughout life, as demonstrated by epidemiological studies and challenge studies in healthy young adults (Crowcroft, Cutts et al. 1999; Simoes 1999).

1.6.1 Innate immunity

RSV mainly infects epithelial cells in the nasal airways and induces the enhancement of the expression of intercellular adhesion molecule-1 (ICAM-1) on epithelial cells (Patel, Kunimoto et al. 1995). Therefore the respiratory tract is the major site of inflammation. Respiratory epithelial cells produce opsonins and collectins as well as several cytokines and chemokines such as RANTES and IL-18 (Noah, Ivins et al. 2002) that attract neutrophils, CD4 helper cells and eosinophils (Figure1.5). Studies on the lungs of the children who died from RSV infection following immunization with formalin inactivated vaccine (FI) - showed strong lymphocytic infiltration around small airways, cell debris in the airway lumen and a large number of neutrophils in the lungs (Prince, Curtis et al. 2001).Using a murine macrophage-like cell line (P388D1) RSV showed viral persistence, enhanced phagocytosis and an increase in the expression of Fc-γ receptors and production of IL-1 β and IL-6. These results indicate that respiratory macrophages play a role in the innate response to RSV infection (Guerrero-Plata, Ortega et al. 2001). Bartz et al, (Bartz, Buning-Pfaue et al. 2002) investigated the consequence of the early interactions between RSV and macrophages or dendritic cells (DCs), using *in vitro* cultured human cord blood-derived DCs and

macrophages. Both DCs and macrophages were infected with RSV and macrophages appeared to be more effective than DCs in taking up the virus. Analysis of the cell surface marker expression showed that both DCs and macrophages infected with RSV had high levels of co-stimulatory and adhesion molecules following infection, and a significant increase of MHC class II expression was also observed following infection. The up-regulation of these surface markers in infected cells highlights the need for antigen presenting cells to mature following RSV infection, and to present antigen to T lymphocytes. The cytokine profile was also analyzed in supernatants of RSV-infected macrophages and DCs. IL-6 was produced by macrophages as well as by DCs while IL-12p75 was produced by dendritic cells but not by macrophages. Results from this study are in agreement with previously published work (Bartz, Buning-Pfaue et al. 2002). Monocytes from RSV infected infants exhibit increased production of IL-10 by macrophages, associated with RSV infection but not by influenza or para-influenza virus. IL-11 and PGE2 were also produced in large quantities by macrophages as well as by DCs. Although IL-11 has been detected before in nasal aspirates from RSV infected infants and in biopsies from adult asthmatics (Bartz, Buning-Pfaue et al. 2002), its production following RSV infection of DCs was a novel finding in this study. Overall, enhanced IL-10 production by macrophages and enhanced IL-11 and PGE2 production by DCs *in vivo* could explain the dominant Th2-type response seen in ongoing RSV bronchiolitis and might also contribute to the delayed development of a protective RSV specific immune response (Bartz, Buning-Pfaue et al. 2002).

Several RSV proteins have been shown to modify or influence the innate immune response. For example, RSV F induced IL-6 production by CD14 and Toll-like receptor 4 (TLR4) expressing monocytes. When purified RSV F (purified by either immunoaffinity or by lectin chromatography) was incubated with either human (PBMC) or mouse peritoneal macrophages, a noticeable increase in the production of

IL-6, IL-8, and TNF-α and IL-1β was observed. Furthermore, IL-6 production was blocked by mAb to CD14 (Kurt-Jones, Popova et al. 2000). Macrophages from CD14 or TLR4 knockout mice could not produce IL-6 (Kurt-Jones, Popova et al. 2000). This study suggested the role of TLR4 as a receptor for innate immune recognition of RSV F protein since TLR4 deficient mice had higher levels of RSV in their lungs and were either unable to clear the virus or the virus was present a few days longer than in the control mice.This suggests that the inflammatory response to the infection is mediated by TLR4 and CD14 expression or signaling. In a further investigation by the same group, it was shown that in the absence of TLR4, there was a reduction in IL-12 expression. In the lungs of RSV infected mice ($TLR4^{null}$) only a limited number of NK and $CD14^{+}$ cells had trafficked to the site of infection and had a significantly reduced cytotoxic activity resulting in their decreased ability to clear the infection (Kurt-Jones, Popova et al. 2000; Haynes, Moore et al. 2001).

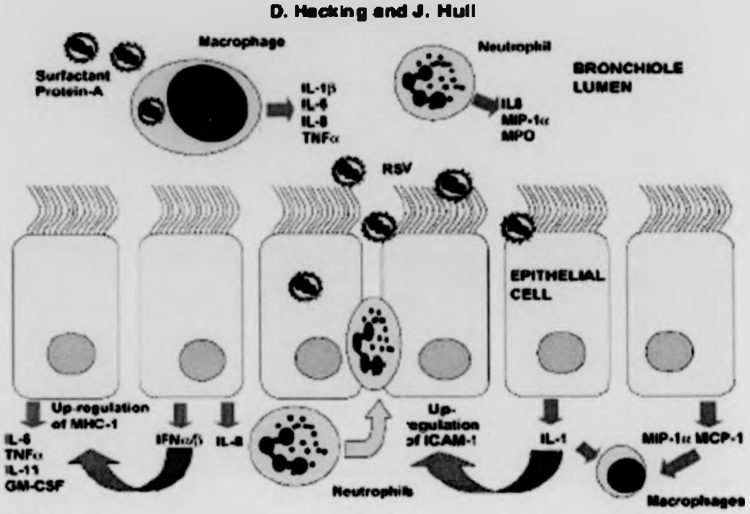

Figure 1.5

RSV infection of the respiratory epithelium.

RSV is bound by surfactant protein A and engulfed by macrophages which leads to IL-1β, IL-8, IL-6 and TNF-α release. Infection of epithelial cells stimulates release of a number of cytokines including the neutrophil and macrophage chemoattractants IL-8 and MCP-1. IL-1 secretion leads to the up-regulation of ICAM-1, a receptor that is vital for neutrophil adherence and activation. IFN α /β secretion causes the up-regulation of the major histocompatibility complex I (MHC-1) expression. (From Hacking and Hull 2002).

1.6.2. Cellular immunity

$CD4^+$ Lymphocytes

$CD4^+$T-cells normally play an important role in immunity against viruses by providing help to B-cells in the process of antibody production and by the synthesis of important anti-viral and inflammatory cytokines such as IFN-α and TNF-α . However, their role in immunity to RSV has not been fully studied. The first evidence for the role of $CD4^+$T-cells and the cell-mediated immune response to RSV was observed following lung autopsies from the children who received the FI-RSV vaccine. In the lungs of the children who died, a strong eosinophilic infiltrate was seen along with another strong cellular infiltrate composed of mainly lymphocytes. The virus was also isolated at up to 10^4 $TCID_{50}$ per gram tissue from the lungs (Kim, Canchola et al. 1969).

Pathology was also enhanced in BALB/c mice following RSV challenge of mice previously immunized with a recombinant vaccinia (vv) expressing the G protein (Openshaw, Clarke et al. 1992). Challenge of mice immunized with vv expressing F or M2 also produced pulmonary inflammation associated with a memory T-cell response but without the development of lung eosinophilia (Openshaw, Clarke et al. 1992). It has also been demonstrated that $CD4^+$cells specific for the G protein can mediate pathology and exacerbation of the disease (Varga and Braciale 2002). Furthermore, when G-specific Th2 $CD4^+$T-cell lines were passively transferred to naïve mice, the same pattern of enhanced disease that was observed in FI-RSV immunized children was seen in the challenged, immunized animals. These and other data suggest that the development of lung pathology and eosinophilia is driven by $CD4^+$ Th2 cytokine- producing T cells (IL-4, IL-5, and IL-13) that are directed towards the G-protein of RSV(Hussell, Khan et al. 1997). The role of G protein-specific $CD4^+$cells has been further evaluated (Srikiatkhachorn, Chang et al. 1999;

Varga, Wissinger et al. 2000; Tripp, Hou et al. 2001; Varga, Wang et al. 2001), and this work showed that the Th-2 biased response to the G-protein is directed towards an immuno-dominant epitope on the protein represented by amino acids (aa183-195) that elicits both Th1-and Th2 $CD4^+$ effector cells. The study showed that $CD4^+$IFN-γ -secreting cells are predominant in the G-protein specific $CD4^+$-memory cell population in mice immunized with G and challenged with RSV. However, it is not clear why, among a population of cells directed to one epitope in the presence of Th1 $CD4^+$ cells, the rest of the $CD4^+$cells develop into Th2 cytokine –secreting cells.

$CD8^+$ Cytotoxic T-lymphocytes (CTL)

As mentioned above, the first evidence for the involvement of the cellular immune response to RSV was observed in the FI-RSV vaccination trail in 1960. CTLs are now thought to be the major effectors for the resolution of established primary infections with respiratory viruses. The first indication for the role of $CD8^+$ CTL in RSV infection was seen in patients with congenital heart immune deficiency in whom the virus continued to be shed for longer than in controls and highlights the need of an intact cell-mediated immune response for viral clearance (Fishaut, Tubergen et al. 1980).

The dominant role of CTLs in the resolution of infection is suggested most strongly by the incidence of extended RSV shedding in immuno-suppressed rodents and in immuno-deficient human patients lacking T-cell mediated immunity (Dudas and Karron 1998; Domachowske and Rosenberg 1999). Other supporting evidence for the primary role of $CD8^+$ T cells in the resolution of RSV infection includes:

1. The early detection of CTLs in infants with primary RSV infection in whom CTL responses are detectable within the first 10 days of the infection. CTL activity in PBMCs from infants with acute RSV infection peaks early and can usually be detected within 1 week after infection (Chiba, Higashidate et al. 1989).

2. The restriction of virus replication following the passive transfer of virus-specific CTL clones into RSV-infected immuno-deficient mice and the temporal relationship between peak CTL activity and virus clearance in mice (Pemberton, Cannon et al. 1987; Anderson, Norden et al. 1990; Dudas and Karron 1998).

3. Although $CD8^+$T-cell epitopes on the G protein have not been identified so far, $CD8^+$ epitopes expressed on the N, and M protein have been identified. Alwan et al showed that $CD8^+$ cells directed towards an epitope on the M2 protein reduced the eosinophilia in mice that had received passively-transferred Th2–G specific $CD4^+$T cell (Alwan, Kozlowska et al. 1994). In addition, insertion of a $CD8^+$/M2 T-cell epitope into the G protein used to prime mice prevented the induction of pulmonary eosinophilia (Srikiatkhachorn and Braciale 1997).

4. The CTL epitope from the M2 of RSV has been shown to induce virus-specific CTL responses in mice (Hsu, Obeid et al. 1998). Chimeric peptides in which the CTL epitope from the RSV M2 protein (amino acids 81-95) was covalently linked to a fusion peptide from the F protein of measles virus (F1-aa 113-131) were used to immunize mice intra-nasally three times with 7 days intervals without an adjuvant. Mice were then challenged with RSV at 1, 3, or 6 weeks after the last immunization. Immunization with the chimeric peptides induced both a peptide and RSV-specific CTL responses. The CTL response was correlated with a significant reduction in the viral load in the immunized mice but it was short lived and declined within 6 weeks of the last dose (Hsu, Obeid et al. 1998).

5. The first CTL epitope to be described from the fusion protein of RSV was defined by Jiang et al.2002. The epitope was a 15-mer peptide from the F protein (F92-106). BALB/c mice were immunized with this peptide, and both peptide -and RSV-specific CTL responses were induced. When the immunized animals were challenged intranasally with RSV, a reduction in viral load was observed in the lungs

of the peptide-immunized mice compared to those in the control group. Furthermore, naïve mice passively receiving purified $CD8^+$ cells from peptide immunized animals showed a reduction in the viral load following challenge with RSV, again indicating the importance of $CD8^+$ cells in the response to the virus (Jiang, Borthwick et al. 2002).

6. Deletion of F protein- specific $CD8^+$ T cells from F-sensitized mice resulted in the induction of pulmonary eosinophilia in the F immunized mice following RSV challenge. Further work showed the importance of IFN-γ production by the $CD8^+$ T cells for the prevention of pulmonary eosinophilia produced by F-specific CD4 T-cells (Hussell, Baldwin et al. 1997).

7. More Recent work by Braciale et al. 2002 investigating the role of $CD8^+$ cells during an RSV infection, showed an important role of M2-specific $CD8^+$ cells in the RSV infected lungs of Balb /c mice in terms of their effector-cell activity and their ability to produce cytokines. This activity was restricted to lung lymphocytes and was not seen in splenocytes from these animals (Varga and Braciale 2002).

All these data and the observed failure to produce a $CD8^+$ response to the G protein in the FI-RSV vaccinated children give a potential explanation for the development of the serious immuno-pathology seen in the lungs of the children who died. It seems likely that CTLs have a crucial role in the resolution of infection in addition maintaining some kind of balance between Th1 and Th2- type responses.

In a publication of work on bovine RSV infection, Viuff et al, (2002) showed that the virus was cleared by neutrophils in the lumen by apoptosis of the infected epithelial cells and subsequent phagocytosis of these cells. Both $CD4^+$ and $CD8^+$ cell numbers increased during the acute phase but most interestingly, no $CD4^+$ nor $CD8^+$ cells could be detected between the infected epithelial cells of the bronchi and T cells were only seen when apoptosis was no longer demonstrable (Viuff, Tjornehoj et al.

2002). This set of data and those from Braciale et al. 2002 imply that there is a role for intact CTLs in the resolution of the infection and that, other factors such as TCR signalling or an RSV gene product may underlie the inhibition of $CD8^+$ effector cell activity. The inhibition of the $CD8^+$ cell activity was seen only in the lungs and was not seen in the spleen, a secondary lymphoid organ. This means that RSV has a selective capability of inhibiting $CD8^+$ cell activity in the primary infection and subsequently memory development, and this may lead to re-infection of the host.

These data are recently supported by results from a study carried on hospitalized children with RSV bronchiolitis and rotavirus infected infants. IFN-γ, and IL-4 producing γδ cells were measured in the peripheral blood of these infants. The number of IFN-γ producing γδ cells was reduced during the RSV infection compared to an increase in IL-4 producing γδ cells, while the IFN-γ producing γδ cells from rotavirus infected infants were not affected. Furthermore, during the convalescent phase, levels of IFN-γ producing γδ cells returned to normal. Further work carried out by the same group showed that when cytokine production by γδ T- cells was measured in school children with different acute respiratory infections (excluding RSV) there was no inhibition of the production of IFN-γ producing γδ T-cells (Aoyagi, Shimojo et al. 2003).

These studies clearly demonstrate the suppressive effect of RSV infection on the production of γδ cells and may thus explain the relatively Th2-biased response during primary RSV infection (Aoyagi, Shimojo et al. 2003). Growing evidence for a role for CTLs in cell injury or immunopathology has been obtained in several studies. For example, Scott et al showed that CTL responses in infants included in their study could be detected in 78% of infants under 6 months of age while CTL responses were only detected in 46% of the children greater than 6 months of age (Scott, Kaul et al.

1978). The greater CTL activity found in the age group predominantly affected by the virus indicates a role for CTL in pathogenesis (Scott, Kaul et al. 1978).

The role of IFN-γ

IFN-γ is a type II interferon (IFN) produced by T and NK cells that has direct anti-viral activity and provides help for the generation and activation of CTLs. It also stimulates antigen presentation through up-regulating the expression of MHC Class I and II. IFN-γ also activates NK cells and has a role in antibody isotype switching (IgM to IgG2a) and thus it is considered as one of the crucial cytokines in protective immune response to viruses.

The role of IFN-γ in RSV pathogenesis is not very clear. Many studies have shown that the Th-2 driven immune response and the eosinophilic lung damage are associated with exposure to either FI-RSV or G protein (Hussell, Baldwin et al. 1997) showed that the development of lung eosinophilia was diminished by IFN-γ production by CTLs and enhanced by treatment with anti-IFN-γ or anti $CD8^+$ antibodies. Similar data were obtained by (Srikiatkhachorn and Braciale 1997) who showed the development of lung pathology in IFN-γ deficient animals. In a further study, it was shown that in the absence of IFN-αß and IFN-γ signaling, or Stat-11, mice developed an enhanced eosinophilic lung response and a strong Th-2 cytokine profile (Durbin, Johnson et al. 2002). The pro-inflammatory response to RSV infection in these animals suggests that IFN-αß which is produced in response to viral infection along with IFN-γ (produced by activated NK or T cells), not only limits virus replication but also has a role in moderating or controlling a biased Th2- type response (Durbin, Johnson et al. 2002). Hsu et al (1998) showed that spleen cells from mice immunized with a plasmid encoding a single CTL epitope from the M2 protein of RSV (pSecTag-M2) re-stimulated with the synthetic peptide that represent a CTL

epitope M2:82-90 *in vitro* produced significant amounts of IFN-γ. Also the reduction in viral load in the lungs of plasmid-immunized mice was abolished by *in vitro* treatment with anti-IFN-γ antibody on the day before challenge. The results indicate that the clearance of RSV from the lungs by plasmid DNA induced immunity involved the production of IFN-γ (Hsu, Obeid et al. 1998; Hsu, Chargelegue et al. 1999).

The role of IL-12

IL-12 is an important cytokine in the response to virus infection and in the development of Th1-type responses. It normally drives viral clearance and host recovery from viral infection. In RSV, the development of a Th1-type response was associated with termination of infection and with mild RSV infection. The role of IL-12 has been examined in C57BL/6 mice, which develop a milder airway infection with RSV than do BALB/c mice. C57BL/6 mice showed a fourfold increase in IL-12 levels post-RSV infection with very little inflammation. However, when these animals were treated with anti-IL-12 antibody, an increase in airway hyperactivity and the production of eosinophilia was observed. Since IL-12 activation is dependent on Stat-4 signaling, a similar experiment was carried out on Stat-4 deficient mice: there was an exacerbation in the inflammation seen in the airways which supports the need for balancing of Th1-type cytokines in controlling the disease (Tekkanat, Maassab et al. 2001). The same group showed that IL-13 is produced in high levels in BALB/c and DBA/2 mice during the course of the RSV infection. Neutralization of IL-13 eliminates airway hyperreactivity, decreased mucus production, and increases IL-12 production. This suggested that IL-13 production is inversely associated with IL-12 production and highlights the importance of IL-12/IL-13 balance during an RSV infection.

1.6.3. Humoral immunity

Antibodies play an important role in limiting the infectivity of respiratory viruses. However, the mechanisms which contribute to resolution of or protection against viral infection and diseases caused by these viruses are only partly understood. There are four principal ways in which antibodies contribute to immunity to viruses: a) neutralization, b) prevention of endocytosis of the virus c) inhibition of fusion, and d) inhibition of the un-coating of virus inside the endosome. In addition, there are the other general anti-microbial functions of antibodies including; i) promoting phagocytosis or opsonisation; ii) activation of the complement system; iii) induction of inflammatory responses and attracting phagocytes and further serum antibodies to the site of the infection;and iv) antibody-dependent cell cytotoxicity (ADCC) via IgG attached to the surface of the target cell (Crowe 1996). Several observations from the natural infection with RSV highlight the role of antibodies in resolving RSV infection and protection from re-infection. These include:

1. RSV can still be neutralized at the cell surface even following attachment to the target cell, by antibodies during the first 60 minutes of infection (Osiowy and Anderson 1995). Other studies also suggeste that some anti-F neutralizing antibodies decrease infectivity by inhibition of fusion at the plasma membrane (Beeler and van Wyke Coelingh 1989).
2. Local antibody response is induced by RSV (Both IgG, IgA, and IgM antibodies can be detected in the nasal washes during acute infection), this response is associated with viral clearance (McIntosh, Masters et al. 1978).
3. In B cell defective mice or when B cells are depleted, virus clearance following RSV infection is delayed and the animals show increased morbidity (Graham, Bunton et al. 1991).

4. The relative protection from RSV infection in infants during the first weeks or months of life was associated with the level of passively transferred maternal antibody , while severe pneumonia was shown to be associated with low levels of neutralizing antibodies (Glezen, Paredes et al. 1981; Englund 1994).

5. Following primary infection in young infants, the rate of re-infection with RSV (LRI) at the time of re-infection is correlated inversely with the level of serum neutralizing antibody against RSV (Glezen, Taber et al. 1986).

6. Data from passive-transfer studies in rodents have demonstrated the protective effect of polyclonal immune sera and neutralizing RSV monoclonal antibodies (MAbs) in the LRT. The serum RSV-neutralizing-antibody titre following passive transfer is correlated with the level of protection against virus replication in the LRT. While in contrast, the presence of serum neutralizing RSV antibodies usually has little effect on virus replication in the upper respiratory tract (URT) (Walsh, Schlesinger et al. 1984; Prince, Horswood et al. 1985; Prince, Hemming et al. 1987; Groothuis, Simoes et al. 1993; Sami, Piazza et al. 1995).

7. RSV neutralizing antibody also protects young infants as shown in a randomized study, where the titre of RSV neutralizing antibody achieved in infants who received a dose of RSVIG in that was comparable to that previously demonstrated to protect the lungs of cotton rats against RSV infection (Groothuis, Simoes et al. 1993; The PREVENT Study Group 1997).

8. Following primary infection with RSV, serum antibody levels to the virus decline several months after the infection to reach undetectable levels; this is normally also associated with primary infection occurring at a very young age. (Welliver, Kaul et al. 1980). However, these antibodies do provide a degree of

protection against a second infection (Kreil, Burger et al. 1997). Serum antibodies are generally long-lived after a second or third infection (Domachowske and Rosenberg 1999) (Crowe 1999).

9. Secretory neutralizing antibody also plays a role in protection against RSV as shown in studies in adults and the presence of secretory neutralizing antibody but not serum antibody is correlated with protection of the upper respiratory tract against RSV (Mills, Van Kirk et al. 1971). The development of RSV-specific immunoglobulin A antibodies (IgA) in nasal secretions in infants is associated with virus clearance (McIntosh, Masters et al. 1978).

Specific Maternal antibody in the immunity against RSV

Epidemiological studies have shown protection against RSV in babies born to mothers with high levels of neutralizing RSV antibody. A correlation between IgG anti-fusion or F protein antibodies and its contribution to immunity from disease also has been demonstrated. Several studies have shown that pre-existing antibody could protect infants from RSV since babies born to mothers with high levels of IgG antibody to RSV were protected against infection during the first few months of life. It has been demonstrated that in 575 randomly selected cord blood samples, protection against RSV infection in early days is correlated with the level of maternal antibody. Furthermore, the level of antibody at birth directly correlated with the age at the time of infection. Kasel et al. studied passively-transferred maternal antibody and naturally-induced serum antibody to specific viral proteins in 34 children followed from birth up to 3 years of age using immunoglobulin class-specific ELISA techniques. Levels of IgG antibody to the F protein were correlated with immunity and children with less severe RSV disease had significantly higher anti-F antibody titres prior to infection. Other studies demonstrated that infants younger than 6 months do not produce

antibody to RSV polypeptides following natural infection, which could explain the lack of specific RSV antibody and may account for high re-infection rates in young children (Englund 1994).

1.7 Development of a vaccine against RSV

There are serious difficulties to be faced in the development of an RSV vaccine including:

a) the need for the vaccine to be effective in seronegative children before six months of age, and in very young infants with maternally-derived RSV specific antibodies;

b) the vaccine needs to take into account the existence of two antigenically diverse RSV groups, A and B;

c) the vaccine should be designed to elicit neutralizing antibodies, since such antibodies have been shown to play a major role in resistance to RSV disease in humans, as well as in protection from infection in experimental animals; and

d) it is likely that it will be necessary that the vaccine be designed to induce CTL, the problem lies in the uncler role that CTls play in man. It is likely that more than one type of vaccine will be needed in various human populations.

In the last 20 years, and since the FI-RSV trial in 1960s, several approaches have been used in the search of an effective RSV vaccine. These include the generation of a genetically engineered (cDNA derived) RSV vaccine, synthetic peptides and viral protein subunits and live virus vaccines. Of these, only inactivated, subunit, and live attenuated RSV vaccines have been evaluated in human clinical trials to date. (These include the subunit vaccines PFP-l, PFP-2, BBG_2Na and cold-passaged /temperature-sensitive mutants (Simoes 1999; Kneyber and Kimpen 2002).

1.7.1. Formalin inactivated vaccine

In the 1960s, the potential of a formalin-inactivated vaccine (FI-RSV) to protect against RSV disease was assessed. An alum-precipitated RSV vaccine (Lot 100) was developed by proliferation of the Bernett strain of RSV in human embryonic kidney cells and passage in vervet monkey kidney cells and finally, inactivation of the virus by formalin. The vaccine was tested in children between the ages of 2 months to 7 years.

Thirty-one children were immunized with 2 or 3 separate doses of FIRSV and 40 were given placebo. Unfortunately, the vaccine failed to protect against naturally-acquired RSV infection, and the use of this vaccine resulted in the development of an abnormal immune response to natural infection. As a result, 69% of the vaccine recipients developed pneumonia following subsequent infection with RSV and two of the children died (Kapikian, Mitchell et al. 1969; Kim, Canchola et al. 1969).

Sera from vaccinated children contained high titres of anti-F antibodies but these had low virus neutralization activity. Low levels of antibodies to F and G protein were observed following WT infection as compared to the placebo control group. Lymphocytes from vaccinated children showed far stronger proliferative responses to RSV Ag than those seen in lymphocytes from children naturally infected with WT-RSV. Subsequent analysis of the sera from vaccinated children showed that anti-F antibodies had low fusion inhibitory activity, which may be a result of changes in the F protein function resulting from the treatment with formalin (Prince, Curtis et al. 2001).

In the lungs of the children who died, a strong eosinophilic infiltrate was seen along with other strong cellular infiltrates composed of mainly lymphocytes, and the virus was also isolated at up to $10^4 TCID_{50}$ per gram tissue from the lungs. One of the

children who died had *Escherichia coli* in the trachea, lungs, blood and spleen; the other had *Klebsiella* in the trachea, lungs, and nose (Kim, Canchola et al. 1969).

At the time of this study it was proposed that the relatively low level of neutralizing antibodies induced to both F and G protein may have delayed the clearance of the virus or facilitated the enhancement of disease along with the increased lymphocyte proliferative responses. The study was brief in its description of the histopathology and the details of the autopsies, and considered " peri-bronchiolar monocytic infiltration " with increasing eosinophils as the mark disease enhancement (Kim, Canchola et al. 1969). This study lead to the acceptance of the view that eosinophils are the marker of the disease as suggested later by others (Prince, Curtis et al. 2001). In order to understand the mechanisms by which the vaccine contributed to disease enhancement and in order to develop a new safer vaccine, the availability of a good animal model that reflects the pathology seen in the lungs of the dead children is necessary.

A study that followed the trail pioneered by (Prince, Jenson et al. 1986) used the FI-RSV lot 100 to vaccinate cotton rats. Lungs from the vaccinated animals showed strong infiltration of lymphocytes, macrophages and neutrophils and the sera contained low levels of neutralizing antibodies. It was suggested that a protective epitope must have been changed as a result of the formalin treatment and that another non-protective epitope induced high titres of antibodies that could have formed immune complexes. Other studies suggested peri-bronchiolitis as the marker of the disease caused by the vaccine (Openshaw, Culley et al. 2001; Prince, Curtis et al. 2001). Again, a major criticism of this study was the limitation of the lot 100 stock plus the fact that it was 20 years old, and the limited histopathology induced by its use.

Recently, a batch of the vaccine 100 was re-produced by Wyeth-Lederle following the old protocol and to control for interference by any non-viral components, a mock infected (MFI-RSV) vaccine was produced along with the vaccine. Lungs of cotton rats immunized with either the old or the new batch of the vaccine had enhanced lesions. Although the vaccine helped decrease viral replication, when animals were primed with the vaccine and exposed to WT infection, they developed marked lesions in the lungs, which were characteristic of those seen in animals with primary or secondary infections.

Comparative histological studies of lungs from the dead children with the animals vaccinated with either (FI-RSV or MI-FIRSV) revealed that peri-bronchiolitis consisting of lymphocytes was a general finding while alveolitis consisting of neutrophils was associated mainly with the use FI-RSV vaccine (Figure 1.6 a, and b). The study involved 500 cotton rats and the authors considered alveolitis to be the main characteristic of enhanced disease caused by the virus. As a result of these studies, a case was made for the use of cotton rats as a reference model for the study of FI-RSV pathology along with other factors such as cytokines and chemokines.

The BALB/c mouse model has also been adopted for the study of immunity to human RSV and the development of immunopathology to RSV. Priming mice with FI-RSV resulted in a typical Th-2-type response with production of IL-4 and 5, strong eosinophilia and cytopathological changes in the lungs. In mice deficient in $CD4^+$T cells or in mice following administration of blocking antibodies against 1L-4 and IL-10, these responses were not observed (Connors, Kulkarni et al. 1992).

Pathology similar to that seen in the lungs of children who died in the FI-RSV vaccine trial was also seen when BALB/c mice were primed with either FI-RSV or vaccinia recombinant expressing the G-protein of RSV (vvG), followed by challenge with RSV. When immunized with vv expressing F or M2 proteins (vvF, vvM2) mice

still showed the same inflammatory profile but without lung eosinophilia. The inflammation rather showed a typical memory T-cell response (Openshaw, Clarke et al. 1992). Earlier work by Alwan showed that transfer of G-specific Th2 $CD4^+$T-cell lines to naïve animals induced lung eosinophilia following intranasal RSV challenge (Alwan, Record et al. 1992). More recently, Johnson, et al. 2003 investigated the involvement of IL-13 along with IL-4 in RSV disease using mice immunized with vaccinia recombinants expressing the G –protein (vvGs) and in FI-RSV immunized mice (Johnson TR 2003). In the vvGs immunized mice, inhibition of both IL-4 and IL-13 production was necessary to induce eosinophilia and a type 2 response. On the other hand, either IL-13 or IL-4 alone needed to be blocked in FI-RSV immunized mice in order to reduce the eosinophilia and type 2 cytokine production. This study further highlights the contribution of cytokines and other local environmental factors for the enhancement of the RSV disease (Johnson TR 2003).

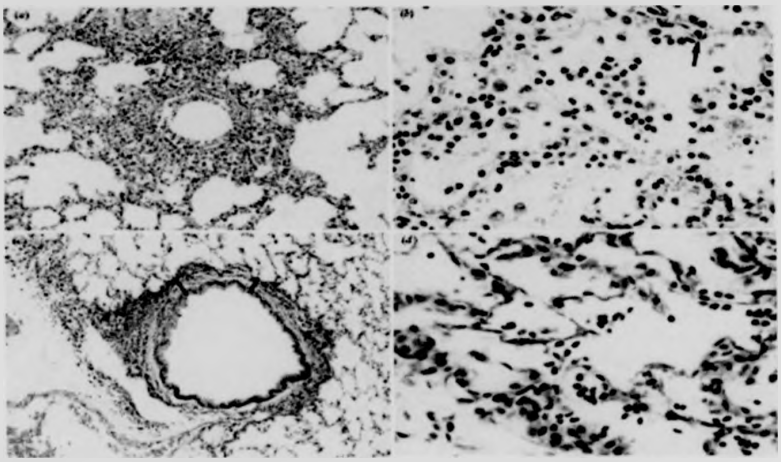

Figure 1.6

Photomicrographs of peribronchiolitis and alveolitis in Lot 100 autopsy (frames a and b) and FI-RSV immunized cotton rat autopsy (c and d).

H&E stain x100 (a and c, peribronchiolitis) and x400 (b and d, alveolitis). Neutrophilic alveolitis (presence of neutrophils in the alveoli), which is the key feature of vaccine-enhanced disease, is seen in both instances. Eosinophils, an infrequent finding in both Lot 100 autopsies (highlighted by arrow, frame b), were not seen in cotton rats (Price et al. 2001)

1.7.2 Live attenuated vaccines

Considerable effort has been expended on the development of a live attenuated RSV vaccine since at the present time, this seems to be the best approach to develop an effective vaccine as soon as possible.

This approach has the following advantages:

1. A live attenuated RSV vaccine will induce an immune response that resembles the wild-type RSV; and
2. The immune response provoked by a live attenuated vaccine will be sufficient to protect young infants (< 1 year) in the presence of maternal anti-RSV antibodies, since RSV infection can occur in the presence of maternal antibodies, as demonstrated by Crowe et al (Crowe, Firestone et al. 2001).

Several live attenuated viruses have been developed and tested over the last 20 years and the first attempt used an attenuated Bovine RSV but this was shown to be poorly immunogenic in chimpanzees (Crowe 1995). Following this, two attenuated viruses have been produced: a) a cold passage mutant *cp or (cpRSV)*; where the virus was attenuated by decreasing temperature during culturing. b) temperature-sensitive *ts* mutants where virus replication was dependent on a specific temperature (*RSV ts-1, RSV ts-2)*.These candidates failed in clinical trials because either the mutant was genetically unstable and replication of the vaccine became temperature independent (*RSV ts-2)*, or the mutant was weakly attenuated or over-attenuated. However children vaccinated with either *cpRSV, or RSV ts-1* had no enhanced disease-related effects (Crowe 1998).

Crowe et al. 1995 produced several live attenuated vaccine candidates, produced by a combination of chemical mutagenesis and cold passaging. All the candidates induced protective response against natural infection and retained their

genetic stability when tested in chimpanzees. To test the efficacy of the vaccine in the presence of maternal antibodies, anti-RSV antibodies were administered intravenously to chimpanzees; all four mutants were genetically stable and induced neutralizing antibodies(Crowe, Bui et al. 1995).Two of the candidates *(cpts248/404, and cpts-530/5009)* were tested in infants and young children. *Cpts-248/404* induced a 4-fold increase in virus neutralizing antibody titre in 68% of sero-negative children aged 6 to 24 months; in 44% of sero-negative infants aged 3 to 5 months; and in 29% of sero-negative infants aged 1 to 2 months. *Cpts-248/404* induced URTI in children younger than 6 months of age and a strong response to the G protein in younger infants. Both strains remained immunogenic and phenotypically stable. However, the *cpts* 248/955 was shown to be genetically un-stable and the vaccine induced symptoms in very young infants and the children shed the virus (Crowe, Bui et al. 1995; Wright, Gruber et al. 2002).

Pringle et al.1988 had further investigated the possibility of developing a *ts* vaccine using chemical mutagenesis and produced 4 mutants (*ts19A, ts1A, ts1B, and ts19B).* All four mutants remained genetically stable but all induced upper respiratory tract symptoms in healthy adults which made the four candidates unsuitable for testing in infants. Another mutation was produced from *ts1B (ts1C).* This last candidate was also tested in healthy adults and did not cause any associated illness, but it still needs to be tested in infants (Mckay 1988 , pringle 1993).

1.7.3 Subunit vaccine

Purified F or G proteins of RSV have been shown to induce protective immunity in the animal model of RSV. This protective immunity was limited to the lower airways in mice immunized with RSVF (Walsh, Brandriss et al. 1985). When purified F protein was used to immunize cotton rats, higher levels of anti-F antibodies were induced but the vaccine enhanced histopathological damage in the lung following challenge with wild-type RSV (Murphy, Sotnikov et al. 1990). Furthermore, when this vaccine was adminstrated to sero-negative chimpanzees, low titres of neutralizing antibodies were induced (Crowe 1995).

Another subunit vaccine has been developed for use with the adjuvant *Quillaja saponaria* (QS-21) which may improve the immunogenicity of the subunit proteins. This vaccine was tested in mice and induced highe levels of virus neutralizing antibodies and cytotoxic T lymphocytes (Hancock, Speelman et al. 1995). Similar data were obtained by (Bastien and Trudel et al. 1999) who immunized mice with synthetic peptides representing the amino acid region 174 to 187 of the G protein from both human and bovine RSV together with cholera toxin as adjuvant. Both the peptides induced IgG responses in mice when immunized intranasally and higher titres were achieved when the peptides were combined with the adjuvant. Immunized mice were protected from RSV infection after challenge but no secretory IgA was detected (Bastien, Trudel et al. 1999). Another subunit vaccine candidate is the BBG2Na peptide vaccine. This vaccine consists of an epitope from the G protein (130-230) expressed in *Escherichia coli* (G2Na) and bound to the C terminus of an albumin-binding region of the streptococcal G protein (BB). BBG2Na induced high neutralizing antibody titres against RSV and did not induce a Th-2 type response, nor pathology in the lungs (Power, Plotnicky-Gilquin et al. 1997). BBG2Na is currently being evaluated in Phase III clinical trials (Kneyber and Kimpen 2002).

Other candidates which were tested in several clinical trails are the purified F protein (PEP) subunit vaccines (PEP-1 and PEP-2). PFP-1 consists of~90 to 95 % purified F protein, whereas PEP-2 consists of ~98 % purified F protein. Tristram et al, tested the efficacy of PEP-1 in 26 children aged from 18 to 36 months (these children had previous RSV infections). The vaccine induced a 4-fold increase in neutralizing antibody titre in a majority of the immunized children, and all immunized children remained protected against subsequent natural RSV infection for 6 months (Tristram, Welliver et al. 1993). The efficacy of PEP-2 has also been evaluated in children with cystic fibrosis and in pregnant women and has induced a 4-fold increase in neutralizing antibody titre in both populations. In summary, both PEP-1 and PEP-2 induced high titres of anti-F and RSV neutralizing antibodies and the frequency of RSV infection was lower in PEP vaccinated groups during the time of the follow up. Although the PEP vaccines were immunogenic, they were tested either in sero-positive children (under one year of age with immune deficiencies) or in an elderly population. Thus, the efficacy of the vaccine is still to be evaluated in a healthy high-risk group suvh as young infants. Furthermore, the number of individuals in the study was too small to effectively evaluate the outcome of the use of the vaccine (Munoz FM 2003) (Kneyber and Kimpen 2002).

1.7.4 DNA vaccines

The use of naked DNA as a vaccine is a promising approach to immunization against human disease that avoids disease-enhancement associated with some vaccines available on the market. There are several advantages in using DNA vaccines including:

- the low economic cost of production, at a high level of purity and in large quantities;
- the vaccines are genetically and heat stable and can be genetically manipulated;
- the vaccines mimic natural viral infection which induces humoral as well as a cellular immune responses even in the presence of maternal antibody;
- the vaccines have a very low risk of generating pathogenic infection;
- the vaccines provide the possibility of immunizing against different pathogens with a single vaccine; and
- the vaccines can induce long-lived immune responses, including persistent and protective antibody levels, which has important implications for vaccination strategies (Henke 2002).

However, some potential problems with DNA vaccines include:

- possible random integration into the genome of the vaccine
- the potential of induction of immunological tolerance
- the induction of hyper-immunity
- the potential of induction of anti-DNA antibodies
- the potential induction of autoimmune responses , and
- the need to limit the persistence of antigen

In RSV, the observation that DNA immunization induced protective immunity in mice in the presence of maternal antibodies makes this form of immunization of particular

interest for use in neonates and for developing an RSV vaccine to target this high risk group. Immunization of 1-week-old BALB/c mice with DNA encoding RSV protein F resulted in the induction of strong CTL responses and protective immunity (Martinez, Li et al. 1999).

A plasmid encoding 248-amino acids of the G protein and not the full length (pND-G) was used to immunize Balb/c mice. The pND-G vaccine markedly inhibited the RSV-induced airway inflammation and decreased viral loads in the lungs of immunized mice. Both IFN-γ and IL-12 were induced by the vaccine and no airway eosinophilia was seen in the lungs of the immunized animals. Sera from vaccinated animals contained higher levels of anti-RSV antibodies than those induced following RSV infection (Miller, Cho et al. 2002).

In an attempt to develop an epitope based DNA vaccine, Hsu et al .1998 constructed plasmid DNA vectors encoding a single cytotoxic T lymphocyte (CTL) epitope from either the M2 protein of RSV or as a contol, from the nucleoprotein (NP) of measles virus (MV) with or without a signal sequence. Following intra-dermal immunization, plasmids in which the CTL epitopes were expressed with the signal sequence (pSecTag-M2/NP) were more effective at inducing peptide- and virus-specific CTL responses than plasmids expressing CTL epitopes without the signal sequence (pcDNA3-M2/NP). CBA mice immunized with pSecTag-NP showed greater peptide – and MV-specific CTL responses than did mice immunized with PcDNA3-NP. Spleens from mice immunized with pSecTag-M2 induced both peptide and virus-specific CTL responses, while mice immunized with pcDNA3-M2 had neither demonstrable peptide nor RSV-specific CTL activity. DNA immunization induced protection against MV-induced encephalitis and a significant reduction in viral load following RSV challenge. The reduction of viral load following RSV challenge was abrogated by prior injection with anti-IFN-γ antibodies again highlighting the

importance of IFN-γ in CTL responses These results underline the ability of epitope-based DNA immunization to induce protective immune responses to specific epitopes and point out the potential of this approach for the development of vaccines against infectious diseases (Hsu, Obeid et al. 1998).

A further study by the same group showed that the M2-RSV epitope expressing DNA plasmid formulated with chitosan and delivered intranasally to BALB/c mice induced both peptide- and virus-specific CTL responses that were comparable to those induced via intra-dermal immunization. Intranasal immunization with the free plasmid in the absence of chitosan failed to induce detectable CTL responses. A significant reduction in viral load following RSV challenge of chitosan /DNA immunized mice was observed in the lungs of immunized mice compared to that in the control group. The result highlights the potential of the intranasal route for the induction of local and systematic CTL responses (Iqbal, Lin et al. 2003).

1.7.5 Other RSV vaccine approaches

Other approaches to the development of a vaccine against RSV include:

- Vaccinia was used to express F or G protein and these constructs were immunogenic in rodents, and vaccinia expressing the M2 protein induced short lived protection in BALB/c mice.

However, recombinant adenovirus expressing RSV proteins failed to induce demonstrable protection against RSV (Kneyber and Kimpen 2002).

- Genetically engineered vaccines have been developed to provoke a longer-lived cell-mediated response as well as humoral immunity. A combination of both DNA and live viral vectors has being used/ In this case, the DNA vaccine is used to prime a T-cell response and the recombinant viral vaccine is used to boost the response. In addition, a combination in which one recombinant viral vector is used for priming and a second viral vector for boosting has also being tested (Henke 2002; Robinson 2003).These heterologous "prime-boost" immunizations induce greater immune responses than can be achieved by priming and boosting with the same vector. The first immunogen initiates a memory cell response and, the second immunogen expands the memory response. The two vaccines do not induce responses against each other and therefore do not interfere with each other's activity (Robinson 2003).

- ISCOMs have also been used for a vaccine against RSV. Trudel et al. were the first to show that subunit vaccines expressed in ISCOMs induced both neutralizing and CTL responses to RSV in BALB/c mice (Trudel, Nadon et al. 1992). Hu et al.1999, immunized Balb/c mice with ISCOMs containing RSV F-protein and G protein (with lower expression of the latter) both intra-nasally and subcutaneously. Virus neutralizing antibodies were detected in serum and

in all mucosal organ extracts irrespective of the immunization route used (Hu, Elvander et al. 1998).

1.7.6 Synthetic epitope-based vaccines

The antibody response to a protein antigen is predominantly focused to restricted parts of the molecule termed B-cell epitopes. These epitopes are mainly located on the surface of the molecule and thus it is essential that the antigen is in its native conformation for the interaction with antibodies to occur.

The complete surface of a protein molecule is now in general accepted as being antigenic and comprises many overlapping B-cell epitopes. B-cell epitopes are made up of short linear sequences of amino accids along the polypeptide chain (continuous or linear B-cell epitopes), or consist of amino acid residues brought together by the folding of the polypeptide chains when the protein is in its native conformation (discontinuous or conformational B-cell epitopes) (Figure1.7).However, some hidden residues in the protein structure seem to be directly involved in binding to the antibody. Such buried residues might be revealed following the binding of antibody to the surface exposed residues in the protein, and might induce side-chain adjustments in adjoining epitopes (Partidos and Steward 2002).

It is generally agreed that the majority of anti-protein antibodies recognize discontinuous or conformational B-cell epitopes, and only a small fraction have specificity for continuous B-cell epitopes. In the latter case, antibodies with specificity for continuous B-cell epitopes might be induced as a result of partial denaturation of the antigen during the immunization procedure.

The demonstration that peptides can induce antibodies of predetermined specificity that can interact with the native protein has prompted the examination of their potential as candidate vaccines. The cross-reactivity of the anti-peptide

antibodies with the native protein might be explained by their inherent flexibility in solution and immunization with unconstrained peptides normally induces a diverse set of antibody specificities that bind several different conformations similar to that of the native epitope (Partidos and Steward 2002).

The use of synthetic peptides has contributed significantly to the understanding of the nature of epitopes (Morris 1996). The synthesis of overlapping peptides covering the complete amino acid sequence of an antigen allows repeated screening with different monoclonal antibodies to assess their reactivity with specific linear sequences. Because of cross-reactions, this technique also identifies a range of sequences that are related to, but not 100% identical with the antigen sequence that induced the production of the specific monoclonal antibody.

A good deal of work has been focused on the problem of identifying the structural characteristics of linear B-cell epitopes and a number of empirical approaches have been used in attempts to predict such epitopes from the primary amino acid sequences of protein. These include the identification of regions of hydrophilicity, solvent accessibility, protrusion, atomic mobility and secondary structure (e.g. β–turns).

The value of these methods for potential vaccine use is limited as they only identify linear B-cell epitopes. As mentioned above, it is very likely that induce virus-neutralizing antibodies will often be conformational in nature and therefore, these epitopes cannot be predicted from the primary amino acid sequence.

The development of combinatorial peptide libraries where random peptide sequences serve as a pool of diverse molecular shapes has provided important means for analyzing the specificity of immune responses to an antigen (Muller, Plaue et al. 1990). Combinatorial peptide libraries can be constructed with bacterio-phages expressing random peptide sequences and also by solid-phase peptide synthesis of

random sequences on a solid support such as (resin beads) (Partidos and Steward 2002). In the latter instance, following interaction of a target molecule (antibody or other ligand) with the peptide-bead, and using a simplified detection system based on ELISA, resin beads carrying novel peptide sequences recognized by the antibody or ligand can be selected and the peptide sequence identified by micro-sequencing.

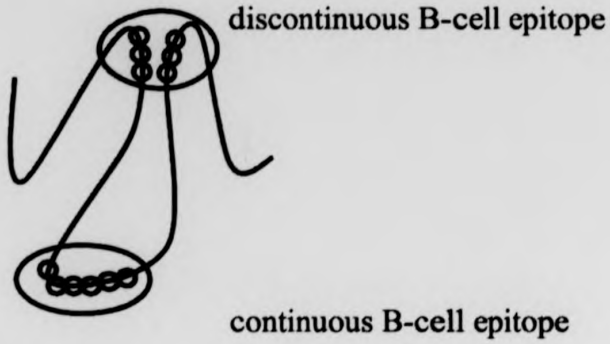

Figure 1.7

Types of B-cell epitopes.

In the case of the F-protein of RSV, several linear B-cell epitopes have been identified using synthetic peptides and conformational epitopes have been identified following the use of Mabs or polyclonal antisera to screen solid phase combinatorial peptide or bacteriophage libraries. These epitopes from the F-protein of RSV are listed in Table 1.1, and are matched with the corresponding linear overlapping peptides used in the present study.

The concept of a mimotope was first defined by Geysen in 1985 as: " a molecule able to bind to the antigen-binding site of an antibody molecule, not necessarily identical with the epitope inducing the antibody, but an acceptable mimic of the essential features of the epitope (Figure.1.8)(Partidos and Steward 2002). Although atomic identity might explain the same antigenicity displayed by an epitope and a mimotope, this molecular mimicry might result from the fact that the epitope and the mimotope bind to different subsites within the same paratope of the antibody. As a result, a mimotope may react with an antibody directed to the native epitope without actually binding to the same paratope as the native epitope. In this case, anti-mimotope antibodies are unlikely to cross-react with the native epitope (Figure.1.8). For a mimotope to be considered as a candidate for vaccine development, it should:

(i) mimic the antigenicity of the epitope by binding to the antibody that recognizes the native epitope, preferably with high affinity; and

(ii) mimic the immunogenicity of the epitope by inducing antibodies cross-reactive with the native epitope. In spite of the potential difficulties with this approach, it has proved successful in a number ofinfections.

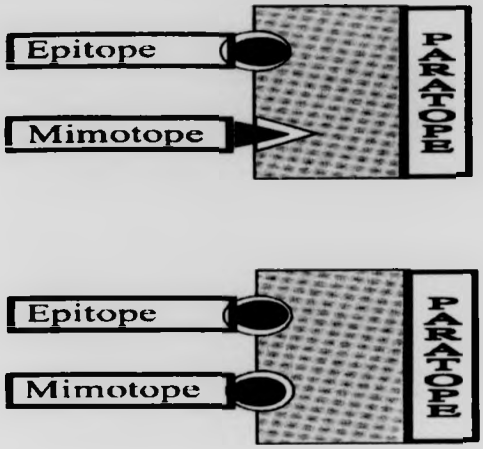

Figure 1.8

The difference between an epitope and a mimotope (Partidos 2002).

A) is the top diagram and b) is the bottom diagram

A) The epitope and the mimotope bind to different subsites within the paratope

- The mimotope is not a true mimic of the native epitope
- The mimotope is antigenic since is recognised by the antibody directed to the native epitope
- The anti-mimotope antibodies will not cross-react with the native epitope
- The mimotope is of no use for vaccine development

B) The epitope and the mimotope bind to the same subsite within the paratope

- The mimotope is a true mimic of the native epitope
- The mimotope is antigenic since it is recognised by the antibody directed to the native epitope
- The anti-mimotope antibodies will cross-react with the native epitope
- The mimotope can be a candidate for vaccine development

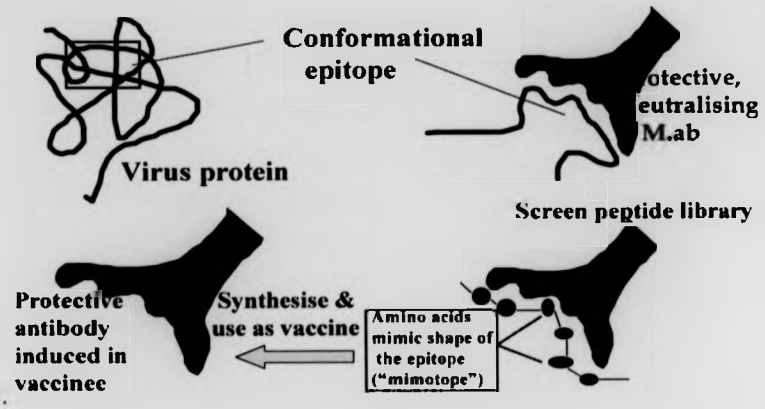

Figure 1.9

Concept of "mimotopes" of conformational B-cell epitopes.

A monoclonal antibody with specificity for a protective, neutralizing epitope on the viral protein is allowed to react with the combinatorial peptide library. Strong binding to a particular bacteriophage or resin bead carrying a specific peptide implies a "good fit" of the antibody binding site with that peptide sequence. This peptide is thus a mimic of the conformationally-determined epitope in the protein i.e. a "mimotope". The sequence of the mimotope can be determined and the peptide can be synthesized and used as a vaccine to induce an antibody response hopefully with specificity similar to that of the original protective monoclonal antibody.

Several mimotopes have been characterized and have been shown to produce antigen –specific neutralizing and protective antibodies when used as immunogens. For example, a protective neutralizing murine monoclonal anti-RSV antibody (mAb 19) was used to screen an 8 mer solid phase combinatorial peptide library. Five resin beads were selected and the peptide sequence obtained by micro sequencing. In an attempt to improve the binding of mAb 19 with the mimotopes, single amino acid substitutions at each position of two of the peptides (S1 and S2) were performed. Three sequences were derived from the S1 peptide (S1S, S1K, and S1P) that showed enhanced binding to the antibody. The three mimotopes were synthesized as multiple antigenic peptides on a polylysine backbone (MAPs) and were used co-immunize BALB/c mice together with a peptide representing a promiscuous T-helper epitope from measles virus fusion protein to induce T-cell help. This resulted in the induction of specific anti-peptide antibody. The anti peptide antibodies inhibited the binding of mAb19 to RSV antigen and neutralized the virus *in vitro* with similar titres to those found in sera from RSV-infected animals. Considerably higher titres of antibody were induced following immunization with a MAP construct with mimotope S1S to which the Th peptide was covalently synthesized. However, immunization with the mimotope S2-MAP complex induced antibodies that failed to neutralize RSV *in vitro*. Following RSV challenge of S1S-MAP-immunised mice, a 98·7% reduction in the titre of virus was observed and a markedly reduced cellular infiltration in the lungs of immunized mice compared to that in controls was observed. Although the covalent synthesis of the Th epitope to the S1S-MAP construct resulted in higher *in vitro* reactivity of the anti-peptide antibodies for RSV, immunization with this construct did not result in a detectable increase in the clearance of RSV *in vivo* (Chargelegue, Obeid et al. 1998).

It is likely that the ability to induce virus-specific cytotoxic T-lymphocyte responses will also be a necessary property of an effective vaccine against RSV. Immunization of BALB/c mice with a synthetic peptide or recombinant vaccinia virus expressing a single CTL epitope from the M2 protein of the virus (M2-9) induces virus-specific CTL and results in a reduction in virus titre following challenge with virus (Hsu, Chargelegue et al. 1998). The work of Chargelegue et al. cited above shows that immunization with a synthetic peptide vaccine induces virus-specific antibodies and is also protective. Taken together, these results indicate that responses directed to either the antibody-or cell-mediated arm of the immune response are effective in the absence of a response in the alternative arm of the response (Hsu, Chargelegue et al. 1999).

To determine whether peptide-induced humoral or cell-mediated responses would act synergistically to enhance virus clearance to an extent greater than the individual antibody- or cellular responses alone, peptides representing B-cell, Th-cell and CTL epitopes were injected together in a peptide cocktail. This resulted in a more effective clearance of RSV in the peptide cocktail-immunized, challenged mice than in mice immunized with peptides inducing B-cell or CTL responses alone. While the mechanisms underlying the synergistic effect on protection following immunization with the peptide cocktail were not investigated, it is possible that protection results from the production of appropriate cytokines or from other unknown co-operative mechanisms between humoral and cellular immune response.

In a study by Jiang, et al., 2002 a 15-mer peptide (aa92–aa106) from the F protein of RSV was shown to act as an MHC class I-restricted (H-2Kd) epitope for RSV-specific CD8+ CTL. This was the first report of murine CTL epitope in the F protein. When this peptide P8 was used to immunize BALB/c mice, peptide and RSV-specific CTL responses as well as peptide-specific proliferative responses were

induced. Following intranasal challenge with RSV, lungs from immunized mice showed a significant reduction in viral load compared to that seen in the control non-immunized group. Even though both peptide specific $CD4^+$ and $CD8^+$T cells were isolated from the lungs of immunized mice, a decrease in CTL activity was shown following treatment with anti-CD4 antibody which suggests that the CTL activity was controlled mainly by the $CD8^+$ population of cells. Furthermore, passive transfer of purified peptide specific $CD8^+$ lymphocytes into BALB/c *scid* mice prior to challenge with RSV also resulted in a reduction in the virus load in lungs of challenged mice (Jiang, Borthwick et al. 2002).

This last result and previous results from the same group indicate the potential of synthetic peptide epitopes for the induction of protective immune responses against RSV infection. However, this approach involves the identification of the critical epitopes able of inducing both the appropriate antibody (B cell epitopes) and cellular (T-cell) immune responses, and that is by no means a straightforward task. Furthermore, careful consideration needs to be given to the question of MHC restriction of CD4 and CD8 T-cell responses. Although there is evidence that there are promiscuous CD4 and CD8 T-cell epitopes, much work will need to be done for each vaccine to ensure adequate coverage of genetically diverse human populations.

Reference	Amino acid residue	Type of epitope	Linear-conformational	Corresponding peptide in this study
(Trudel, Nadon et al. 1987)	216-236/F1	Mab /neutralizing	C	P 20-21
(Bourgeois, Corvaisier et al. 1991)	200-225		L	P 18-25
(Trudel, Stott et al. 1991)	216-232 221-237 275-288	Mab	L	P 20-21 P 20-21-22 P 26-27
(Levely, Bannow et al. 1991)	F1(328-355)	Human Th neutralizing site		P 31-32-33
(Partidos, Stanley et al. 1991)	283-327	Mab /neutralizing	C	P 27-28-29
(Lopez, Andreu et al. 1993)	215-275 255-275	Mab /neutralizing	C	P 20™25 P 24-25
(Lounsbach, Bourgeois et al. 1993)	265-272 (12mer)	Mab	C	P 25
(Lopez, Bustos et al. 1996)	F255-275 Helix-loop	CD, and NMR spectroscopy	C	P 23-24-25
(Corvaisier, Guillemin et al. 1993)	205-225	T-cell		P-18-21
(Langedijk, Meloen et al. 1998)	N→F1(173-182)	Mab /Neutralizing site	L	
(Chargelegue, Obeid et al. 1998)	S1 (HWYISKPQ) And S2 (HWYDAEVL)	Mab / Neutralizing	C	none

Table 1.1

Linear B-cell epitopes identified from the F-protein of RSV.

1.8 Aims and objectives of the thesis:

- To identify linear human B-cell epitopes of the fusion protein of RSV using the reactivity of human sera with 55 overlapping peptides covering the whole of the fusion protein of RSV.
- To identify peptide mimics (mimotopes) of a conformational human B-cell epitope using a human monoclonal antibody (RF2) that recognizes a conformational epitope in the fusion protein.
- To test the reactivity of human sera with previously identified mimotopes: S1S, and SdS (2 mimotopes that mimic a conformational shape in the fusion protein).
- To attempt to identify any differences in the profile of peptide recognition by sera taken at different stages of exposure to the virus

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection and synthesis of peptides

Conventional (solid phase peptide synthesis) using Fmoc chemistry was used to synthesize 55 overlapping peptides of RSV A2 strain fusion protein covering the whole protein. Each peptide was 15 amino acids long and overlapped by 5 residues with the following peptide (except for peptide 55 which was 13 amino acid long).

The peptides were synthesized by Dr Shisong Jiang, Immunology unit, LSHTM.

The amino acid sequence of the fusion protein used for the synthesis of these peptides is shown in Figure (2.1).

2.1.1 Solid phase peptide synthesis

The concept of solid phase peptide synthesis is to retain chemical reactivity of the peptide seen in solution when the peptide is on a solid phase by adding a covalent attachment step that links the peptide to an insoluble polymeric support (resin). The resin must contain appropriate functional groups onto which the first amino acid can be anchored. The next step is the systematic elaboration of the growing peptide chain. Even when a residue has been incorporated effectively into the growing resin-bound polypeptide chain, it may still undergo irreversible structural modification or rearrangement during subsequent synthetic steps.

Temporary protecting groups are therefore used that can be removed with minimal level of side reactions that can affect the desired product e.g. the N^{α} 9-amino flourenyl methoxy carbonyl (Fmoc) group (Grant 2002). The 55 peptides were synthesized in two batches of 25. 0.3g resin was distributed equally into 25 cartridges of the RAMPS multiple peptide synthesis system (Dupont), washed with DMF and allowed to swell. Each peptide bond was built from the carboxyl to the amino-

terminus, adding one amino acid at a time. Fmoc protected amino acids (at 5-fold excess) previously converted to the hydroxyl-benzotriazol-activated esters by treatment with 1.5mmol HOBT and 1.2 mmol DIPICI/mmol of amino acid for 20 minutes in DMF/DCM were used to elongate the peptide chain. The coupling reactions were carried out in DMF, and the cartridges were rocked for 2 x 60 minutes. At the time of coupling the first residue, dimethyl-amino-pyridine was added to catalyse incorporation. The resin was then washed in DMF, Fmoc groups were removed with 20% piperidine in DMF for 20 minutes, and followed by 10 washes in DMF. After the final de-protection of the last amino acid, the peptide-resin was washed in DMF, shrunk in DCM and diethyl ether and dried. The coupling and de-protection reactions were monitored by the addition of 3, 4-dihydro-3-hydroxy-4-oxo-1, 2, 3-benzotriazine (10 mg/ml in DMF) which turns bright yellow in the presence of free amino groups. Side chain protection was provided by the following protecting groups: Mtr (4, methoxy, 2, 3, 6, trimethyl) for arginine, TRT (trityl) for histidine, Boc^t (tertiary butoxycarbonyl) for lysine and tryptophan for serine, threonine and tyrosine, Obu^t (tertiary butyl ester) for aspartic acid and glutamic acid, except for, (Asn and Qln), where the Opfp (pentafluorophenol ester) was used .

MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT

GWYTSVITIE LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST

PPTNNRARRE LPRFMNYTLN NAKKTNVTLS KKRKRRFLGF LLGVGSAIAS

GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS LSNGVSVLTS KVLDLKNYID

KQLLPIVNKQ SCSISNIETV IEFQQKNNRL LEITREFSVN AGVTTPVSTY

MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIM SIIKEEVLAYV

VQLPLYGVI DTPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS

FFPQAETCKV QSNRVFCDTM NSLTLPSEIN LCNVDIFNPK YDCKIMTSKT

DVSSSVITSL GAIVSCYGKT KCTASNKNRG IIKTFSNGCD YVSNKGMDTV

SVGNTLYYVN KQEGKSLYVK GEPIINFYDP LVFPSDEFDA SISQVNEKIN

QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIVILLS LIAVGLLLYC

KARSTPVTLS KDQLSGINNI AFSN

55 (13mers)

Figure 2.1

Amino acid sequence of the F protein of RSV (Collins *et al.* 1984).

The numbered and underlined sequences represent the overlapping peptides used in this study.

2.1.2 Peptide-resin cleavage

For peptide cleavage and side chain deprotection, peptides were rocked in 95% TFA and 5% scavengers for approximately 3 hours at room temperature. Longer cleavage times were allowed for sequences that contained any arginine residues due to the lower acid lability of the mtr group. Water was used as a general scavenger, but for peptides containing methionine, 1, 2 ethanedithiol was used and for arginine, phenol was used. If the peptide contained both methionine and arginine, a combination of both phenol and ethanedithiol was used.

The resin was then filtered, washed with the cleaving solution and dried to an oily residue in a rotary evaporator. The peptides were precipitated by the addition of cold diethyl ether (50-60 ml). Finally, after a couple of washes and trituration with diethyl ether, the peptides were then desiccated and the white flaky powder was collected and stored-20°C.

2.1.3 Peptide Characterization

The peptides were characterized by:

(i) High performance liquid chromatography (HPLC) to exclude any contamination with deletion products and to ensure the presence of a single peak of the major product.

(ii) Mass spectrometry to determine the molecular weight of the synthetic peptides (analysis performance by Eurogentec UK Ltd).

2.2 Human monoclonal antibody (RF2)

2.2.1 RF2 Production

A human monoclonal antibody (RF2) specific for a conformational epitope on human RSV was kindly provided by Dr.P.Brams, IDEC Pharmaceuticals, San Diego California. The antibody was isolated from a spontaneous abdominal tumour in a severe combined immunodeficient mouse (SCID) reconstituted with human spleen cells from one human donor with idiopathic thrombocytopenic purpura. The reconstituted mouse was immunized with the F protein of RSV. The tumour was shown to have developed spontaneously as *EBV*-transformed human B cells. The tumour cells expanded rapidly in culture, were stable more than 18 months and secreted high levels of specific human anti-RSV monoclonal antibody. Antibodies were purified from supernatants by adsorption to a protein A Sepharose 4B column and Protein A-bound human monoclonal IgG was eluted with citrate buffer, pH 3.5 RF2 had human γ_1 heavy chain and κ light chain and was shown to recognize a conformational epitope, since it required both 48K and 23K disulphide-linked fragments of the F protein for binding by immuno-blotting (Chamat, Walsh et al. 1999).

2.2.2 Synthesis of the solid-phase combinatorial peptide library

The 8 mer peptide library was synthesized on Novasyn TG resin, (a polystyrene-poly oxythylene resin functionalized with amino groups) so that each resin bead bears a different 8 amino-acid peptide. The resin allows the de-protection of the peptide without its cleavage from the resin. There are 2.8×10^6 beads /g of resin with a capacity of 0.24 mol/g. Nineteen reaction vessels containing the resin were used for the synthesis by Fmoc chemistry with all natural amino acids except cysteine. After each synthesis step the resin in each vessel was pooled, thoroughly mixed and re-dispensed

into the 19 vessels for the next synthesis step (Hsu, Crowe et al. 1995). The library was synthesized by C.Stanley, and M. Long in the Immunology unit, LSHTM.

2.2.3 Bead selection

0.05g of resin was blocked with blocking buffer (10% BSA 1% Tween 20 in PBS) for 2 hours at room temperature with continuous shaking, centrifuged and excess buffer removed. The resin was divided into ten equal parts in microfuge tubes. The human monoclonal antibody to the RSV F protein (RF2) was diluted with blocking buffer into half $\log_{10}$ dilutions at 10 mg/ml, and 200µl of each dilution was added to the resin. This was left overnight at 4°C with continuous shaking. It was then washed five times with washing buffer (PBS with 0.05% Tween 20). Goat anti-human IgG peroxidase was added and left for 2 hours at 37°C. The substrate, consisting of 10mg of 3, 3', 4, 4' diaminobenzidine-HCl in 5ml methanol, 30mg 4-chloro-1-naphthol in 5ml methanol, and 30mg 4-chloro-1-napthol in 5 ml methanol was added to 40 ml PBS with 10 µl of hydrogen peroxide added just before use. 200 µl of substrate was added to each tube and the color was allowed to develop for 5 minutes, after which the reaction was stopped with 100 µl of 2 M of sulphuric acid. The resin was placed in a 24-well microtitre plate (NUNC) and dark brown stained beads were selected with a capillary tube. The antibody was stripped from the peptide on the beads by incubating in TFA for 1 hour. Excess TFA was washed away with distilled water. Dr Herve Drobecq (Pasteur Institute of Lille) generously determined the sequence of the 8-mer peptide attached to the beads by micro-sequencing.

2.2.4 SPOTs Synthesis

Combinatorial peptide synthesis is a new development in work with peptides which allows the synthesis and screening of several peptides in parallel. SPOTs synthesis is one example of this procedure. That has the advantage of being relatively inexpensive and requiring only simple apparatus. It is straightforward to perform and can be

carried out on the open bench, except for the de-protection steps. It is also relatively quick to perform and provides a rapid system for analyzing antibody responses and for epitope mapping. The disadvantage of the SPOTs synthesis is that the peptide length is usually limited to around 10 residues. The solid phase support used in the spot technique is a cellulose membrane that can be bought commercially or prepared in-house, and materials for the whole assay can be purchased commercially. The first step of the synthesis involves derivatizing the hydroxyl functions on commercially available filter paper or introducing a modifying group upon which the peptide can be synthesized. The commercial membrane normally has 96 spots, but when the membrane is synthesized manually, the number of individual peptides on the membrane can be controlled.

2.2.4.1 Synthesis of peptides by the SPOTs technique

The first stage of synthesis is to prepare the paper by introducing a modifying group onto which the peptide can be built. The method uses conventional reagents: diisopropylcarbodiimide (DIC), hydroxy-benztriazole (HOBt), FMOC-gly with addition of a catalyst (2-dimethylaminopyridine (DMAP)) to couple glycine to sugar hydroxyls by an ester linkage (Suchankova, Ritter et al. 1990):

$$\text{Fmoc-NH-CH}_2\text{-CO}_2\text{H} + \text{HO-R} \Rightarrow \text{FMOC-NH-CH}_2\text{COO-R} + \text{H}_2\text{O}$$

Fmoc-glycine paper ester

(In presence of DIC, HOBt, and catalyst)

The water finishes up reacting with the DIC, which is a dehydrating agent. Following removal of the Fmoc group, a second amino acid active ester is spotted on the paper in a poorly volatile solvent to create the spots on which the individual peptides will be constructed. The unreacted amino groups covering the rest of the

paper must then be acetylated, and the Fmoc group on the second linking amino acid removed to provide paper with spots which contain available amino groups for peptide synthesis, and the rest of the paper where amino groups are now absent.These spots can be visualized by adding a small amount of the dye bromophenol blue in a non-aqueous solvent. Bromophenol blue is yellow in solvent where it cannot ionize or donate a proton to a suitable acceptor–like an amino group, but when it ionizes it turns the familiar blue colour. Treatment of the paper reveals spots where the presence of amino groups both bind bromo-phenol blue and turn it blue, and show that such amino groups are not available over the rest of the paper (Figure 2.2, and 2.3). These spots also provide a convenient aid for spotting amino acid derivatives for synthesis.

Synthesis consists of cycles of addition of appropriate amino acid active esters to each spot (Figure 2.3), allowing reaction to proceed, capping of any un-reacted amino groups by acetylation, de-protection of the N-terminal Fmoc and re-treatment with bromophenol blue ready for the next cycle of addition. Where peptides of different lengths are to be synthesized, peptides are acetylated as they are finished. At the end of synthesis, the N-terminal Fmocs are removed and the N-terminal amino groups acetylated, and the side-chain protecting groups, which have remained intact during synthesis, are removed by treatment with trifluoroacetic acid (TFA). Following washing the paper is ready for use. This work was preformed under the expert supervision of Dr P.Corran, Immunology unit, LSHTM.

In order to improve the antigenic properties of the three mimotopes, the same SPOTs technique was used to systematically substitute each amino acid the in the 8mer peptide sequence with all possible naturally occurring amino acids.

Figure 2.1

Treatment of the paper reveals spots where the presence of amino groups both bind bromophenol blue and turn it blue.

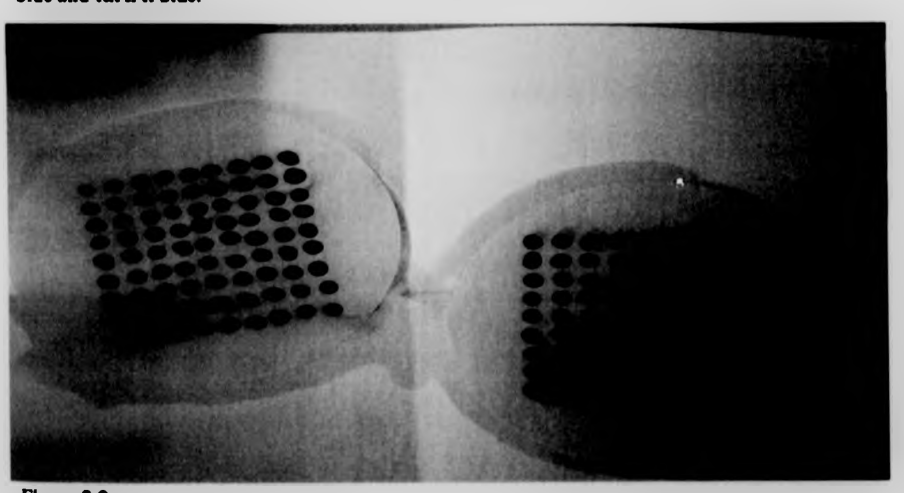

Figure 2.2

Treatment of the paper reveals spots where the presence of amino groups both bind bromophenol blue and turn it blue, and show that such amino groups are not available over the rest of the paper.

These spots also provide a convenient aid for spotting amino acid derivatives for synthesis.

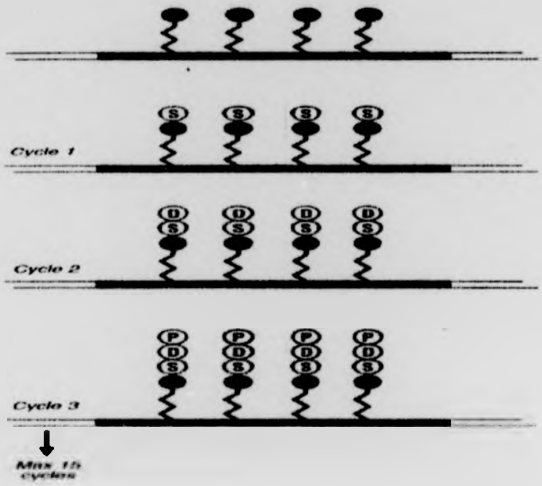

Figure 2.3

Synthesis of SPOTs consists of cycles of addition of appropriate amino acid active esters to each spot.

2.2.4.2 Synthesis of mimotopes by solid phase synthesis

Peptides representing the identified mimotope sequences were synthesized as described in the solid phase peptide synthesis section (2.1.2) by Ms. M.Long in Immunology unit, LSHTM.

2.2.4.3 Coupling mimotopes to KLH

Sephadex PD-10 G25 was soaked overnight in an excess of phosphate buffered saline (PBS). A disc of 3MM filter paper to fit the syringe barrel was inserted as a bed support. The column was allowed to run dry and a second disc of filter paper was place on the column top. A solution 1 % of BSA was prepared and. 0.5 ml was added to the Sephadex column and the eluate was collected. The column was washed with 0.5 ml PBS, and collected into the same fraction. The column was then eluted with 1 ml aliquots of PBS, and the eluate collected into 20 separate fractions. An aliquot of each fraction was assayed for protein using Bradford's reagent

Sulfo-MBS (m-Maleimidobenzoyl-N-hydoxysuccinimide ester) was allowed to warm to room temperature, and was washed into the KLH solution with a pipette and rotated until dissolved (at room temperature for 1 h). The column was allowed to dry and KLH coupling mixture carefully pipetted into the middle of the filter paper disc and allowed to run into the column top. 1 ml aliquots of PBS were added to the column, and allowed to drain into the column each time before adding the next aliqoate. 20 ml fractions were collected. 5µl of each fraction was transferred into the wells of a flat-bottomed titre plate and 200 µl of Bradford reagent diluted 1:1 with water was added to each well. Protein-containing fractions turned bright blue and protein emerged in fractions 6-7 (Figure 2.4). An appropriate aliquot of the KLH solution was added to each of the peptides (pH between 7.2 and 7.8) and rotated at 4°C overnight (or for a minimum of 2h at ambient temperature) and excess peptide removed by dialysis against PBS.

2.2.4.4 Coupling mimotopes to Biotin

The avidin-biotin interaction is the strongest known non-covalent biological recognition between protein and ligand. The bond formation between biotin and avidin is very rapid and once formed is unaffected by most extremes of pH, organic solvents and other denaturing agents. Optimal antigen binding capabilities can be realized by using a biotin derivative that has an extended spacer arm, thus reducing steric hindrance. The spacer arm also improves the complex formation of biotin with the deep biotin binding site of avidin. This affords an increase in sensitivity of detection. EZ link-NHS-LC-LC-Biotin was used as a biotin analogue with extended spacer arms of approximately 22.4 Å and 30.5 Å in length, the chemistry of EZ link-NHS-LC-LC-Biotin is similar to that of Sulfo-NHS-LC-LC-Biotin reaction and to that of other NHS esters. The biotinylation reaction was carried out in the absence of organic solvents such as DMSO or DMF. The reaction was carried out at 4°C, reaction mixture pH values of 7-9. The mimotopes were still on the resins and therefore, biotinylation was carried out manually in the cartridge. The resins were weighed and soaked in DMF for 10 minutes and left to drain. 20% DMF/pip was added twice for 3, and 7 minutes allowing the liquid drain between each addition. This was followed with a wash in DMF for 10 minutes. The Biotin analogue was weighed (4 fold more than the weight of each mimotope), dissolved and DMF and stirred in DMF for 10 minutes. Then it was added to the cartridge for 1 hour and drained. A wash with DMF was preformed 10 times, then 4 washes with propan-2. The cartrdridges then were dried in the desiccator over night and the final mimotopes were weighed and cleaved as described previously.

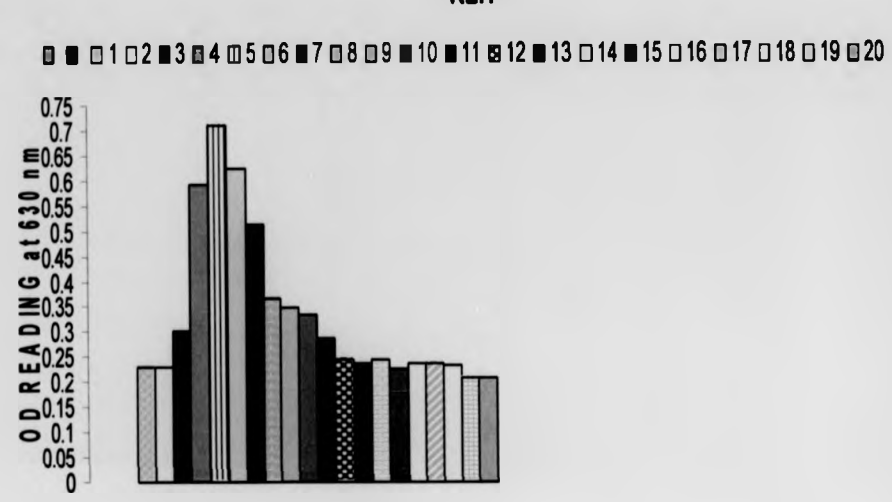

Figure 2.4

Detection of KLH Protein-containing fractions using Bradford reagent.

KlH was collected in 20 fractions and was detected in fraction 5-7. The OD shown is the maen of 2 OD readings.

2.3 Handling of human sera

All procedures involving manipulation of human serum was carried out in a class I microbiological safety cabinet (Envair, UK).

The human sera used in the work described in this thesis were from two sources;

1. Serum from healthy adults working in the Department of Infectious and Tropical Diseases, and 5 other serum samples obtained from individuals working elsewhere in LSHTM.
2. Sera were received from Prof. Peter Aaby from babies from Guinea-Bissau, taken as part of an ongoing programme of research on RSV. Two or three samples were obtained from each child. The first serum sample was taken at 6 months of age before any observed infection with RSV and is considered a pre-infection sample; post-infection samples were taken at 9 or 18 months or both for some children. Serum samples from 49 children were received of which 20 have so far been analyzed (12 of these had RSV before six months of age). Samples were analyzed coded, but no information about the detailed clinical status of these children was obtained.

2.3.1 Animals and immunization

Animals

Groups of four week old inbred female BALB/c *(H-2d)* mice were purchased from the Medical Research Council, Mill Hill, London, United Kingdom.

Animals were housed for one week before use in a pathogen-free animal facility at the Biological Services Unit at LSHTM. The animals were kept throughout the experiment in the same unit and were checked for the appearance of any disorder. Any animal showing sign of disease was excluded from the experiment.

Immunization of mice:

Previous studies in the laboratory had shown that the optimal route to produce the best serum anti-peptide antibody response was found to be the sub-cutaneous route.

Immunization with linear peptides

All mice were sub-cutaneously co-immunized with 50μg of the linear peptide and 50μg of TTB –peptide (T-cell helper epitope from measles fusion protein) in complete Freund's adjuvant (CFA). The animals were boosted by the same route with the same dose of the peptide alone in_incomplete Freund's adjuvant (IFA) three weeks later. Sera from the animals were collected from each mouse from the retroorbital venous plexus under halothane anesthesia before boosting and then 20 days after the boost. The ability of each peptide to induce serum antibody was assessed by direct peptide ELISA as described previously. Sera from the animals from each group were obtained before the start of the experiment and used as a negative control for reactivity with both RSV Ag and the tested peptide in every assay.

Immunization with mimotopes:

Mice were sub-cutaneously immunized with 50μg of the peptide in CFA, and boosted three weeks later by the same route with the same dose of the same peptide in IFA. Sera from individual animals were collected prior to the boost and then 20 days after the post the boost. The ability of each peptide to induce a serum antibody response was assessed by direct peptide ELISA.

2.4 Enzyme Linked Immunosorbent Assay (ELISA)

2.4.1 Production of negative human sera

Sera from a healthy working adults working in Immunology unit, LSHTM were selected to produce the anti- RSV negative control serum. Immunolon 2 HB 96-well microtitre plates (Dynex) were coated overnight at 4°C with RSV Ag at a concentration of 5μg/ml in carbonate bicarbonate.

Then sera was added and incubated for 3 hrs at 37°C, antigen and antibody complex was allowed to form. Supernatant was then removed and spun down to remove any Ab/Ag complex that was not absorbed stuck to the plate. Then reactivity of the normal sera plus the absorbed sera was measured and reduction in the antibody reactivity was observed in the absorbed sera compared to the normal serum.

2.4.2 ELISA for human anti- peptide IgG antibodies

Immunolon 2 HB 96-well microtitre plates (Dynex) were coated overnight at 4°C with synthetic peptide at a concentration of 5μg/ml in carbonate bicarbonate buffer 0.1M (pH 9.6) at 50(μl/well). Plates were then washed four times with the washing buffer: PBS, 0.1% Tween 20 pH 7.2 (PBS-Tw). Any remaining binding sites were blocked with blocking buffer (PBS, 0.1 % Tween 20, 1% BSA at 37°C for 2 hours. Samples were tested in duplicate at a starting dilution of 1/200 for the children's samples and at 1/100 for adult sera. After incubation at 37°C for 45minutes, plates were washed and goat anti-human IgG peroxidase conjugate was added. After a further 45 minutes at 37°C, the plates were washed and 50μl /well substrate was added to each well. This consisted of a freshly made solution of 0.004% hydrogen peroxide and 0.5mg/ml o-phenylenediamine in phosphate-citrate buffer (0.2M sodium orthophosphate and 0.1M citric acid, pH 5.5). The reaction was stopped after by the addition of 50μl/of 2M sulphuric acid. Plates were then read in the ELISA reader (Dynatech MR500) at 490

nm the reactivity of serum with each peptide was expressed for each sample as the optical density value A_{490}.

2.4.3 ELISA for human IgG anti- RSV antibodies

Immunolon 2 HB 96-well microtitre plates (Dynex) were coated overnight at 4°C with RSV antigen obtained from Biogenesis Ltd, UK at a concentration of 5μg/ml in carbonate bicarbonate buffer 0.1M (pH 9.6) at 50μl/well. The RSV antigen was prepared from the RSV long strain on a monolyer of MA cells. The virus was purified from cells by low speed centrifugation then collected by high speed centrifugation. The ELISA was performed as described in 2.5.1.

2.4.4 ELISA for human anti- RSV S1S (M1) - SDS (M2) mimotopes.

ELISA plates were coated with two mimotope peptides representing a conformational epitope from RSV F protein (S1S and SdS) identified by screening the solid phase peptide library with anti-RSV Mab19 (Chargelegue, Obeid et al. 1998). Mimotopes were coated at 5μg/ml in carbonate bicarbonate buffer 0.1M (pH 9.6) at 50μl/ well. The ELISA was performed as described in 2.5.1.

2.4.5 Standardizing ELISA for human samples

The optimal conditions for the ELISA assay to screen human sera for reactivity with the overlapping peptides of the F protein of RSV was determined by the use of different ELISA plates (Nunc, Immunolon 2, and 4) , as well as by varying the percentage of Tween 20 in both the blocking buffer and the diluents. Also, the use of the gelatin was compared to the use of BSA as a blocking agent in the blocking buffer or the diluents. The optimal conditions for the analysis of human sera by ELISA as a result of these experiments were as follows: Immunolon 2 HB 96-well microtitre plates (Dynex), 1% BSA in the blocking buffer with 0.1% Tween 20, 0.25% BSA in the diluents with 0.1% Tween 20.The reactivity of the optimal dilution of the human

sera for use in ELISA was determined by checkerboard titration ELISA with RSV Ag on the plate.

2.4.6 Controlling the assay

A pool from all the nine adult anti-RSV positive human sera was used to produce a positive control. This pool was checked for its reactivity with RSV antigen. Amongst the nine adult human sera titrated against RSV Ag, one with the lowest reactivity with the RSV antigen was chosen for use negative control by absorption of the antibody with RSV antigen. The Ag-Ab complexes were allowed to form and the spun down at 3000 rpm. The reactivity of this negative control was compared to that of the original serum sample. Total IgG also was determined for the sample before and after absorption.

2.5 Cell culture

2.5.1 Growing RSV

RSV (A2 strain) stock was grown in HEp-2 cells in MEM-10 FCS + 0.2 p/s. The titre of RSV stock was estimated by a plaque assay and expressed as the log10 of the reciprocal of the dilution giving 1 PFU as follows :When the cells reached about 25% confluence (or up to 50%), then were washed in serum-free MEM. Virus stock was diluted to 0.1 PFU /cell or less in final volume of 3.5 ml (approx $1x10^6$ pfu/ flask) and incubated at 37°C for 90 min. The medium was redistributed every 10 minutes by rotating the flask through 90°. Fresh MEM 10% was added and the flask incubated for 48 hours. Medium was then removed and spun down. The pellet was re-suspended in 10 ml of spent medium and returned to the flask. 40 ml of MEM (with 2% FCS was added and the flask incubated for 24 hours. When the cells were at least 75% confluent (if not, they were incubated for another day). The medium was then collected and remaining cells rinsed with a few ml PBS, poured off and added to the supernatant. Adherent cells were then re-

suspended with trypisin/ EDTA and mixed with the supernatant. Cell debris was removed by spinning at 3000rpm for 15 mins and aliquots of 2ml were immediately prepared and put into cryoutubes on ice and stored in liquid N_2.

2.5.2 RSV titration assay

HEP-2 cells were grown overnight in 96-well flat-bottom culture plates (Nunc) having 5×10^4 cells in each well. The medium in each well was removed and serial dilutions (10 fold) of the virus in maintenance medium (mm) containing 2% FCS were added (50µl/well). The plates were incubated at 37 °C for 2 hours in 5% CO_2 to allow the adsorption of virus to the cells. The inoculum was replaced with 0.75% carboxymethylcellulose (CMC Sigma) in growth medium containing 10% FCS and plates were incubated at 37°C for four days. Plates were observed daily for the appearance of cytopathic effect (plaque formation). The infected and mock infected monolayers were fixed in formaldehyde in phosphate-buffered saline (1ml/well) at room temperature for one hour. The fixed monolayer was washed with distilled water and stained with 0.1% crystal violet for 30 minutes. The plates were washed with distilled water again and dried in a hot room and the titre was calculated.

CHAPTER 3

THE IDENTIFICATION OF LINEAR B-CELL EPITOPES FROM THE F PROTEIN OF RSV USING ADULT SERA.

3.1 Introduction

Re-infection with RSV can occur in both adults and children, but adults re-infected with RSV are rarely symptomatic and usually display symptoms only as severe and long-lasting as a common cold (Hall, Geiman et al. 1976; Hall, Hall et al. 1978; Hall, Walsh et al. 1991). Although RSV is being recognized as a crucial pathogen in young children and in the elderly population, there are few published studies that document immunity to RSV in the largest risk population (healthy adults and young children). RSV infection in adults was first reported more than 40 years ago by Hamry and Procknow, they reported the recovery of RSV isolates from 15 medical students; the infections were described as causing only upper respiratory tract illness with an average duration of 10.3 days (Hamre D 1966). Subsequent descriptions of RSV infection in adults have focused mainly on high risk populations, such as elderly individuals and those with underlying diseases (Hall, Long et al. 2001). The early work on RSV adult infection was confirmed by a more recent study from Hall etal.2000, 2960 healthy adults were evaluated for having RSV infection. The surveillance was carried out over 20 years and included epidemiological and clinical observations when RSV was active in the community. The study included families of hospitalized children living in the city, students, medical personnel and residents. 7% of the2960 adults evaluated acquired RSV infections which suggest that RSV is relatively a common infection among adults. The mean duration of illness was 9.5 days (Hall, Long et al. 2001).The widespread nature of RSV infections and the consistent, sizeable number of yearly outbreaks of RSV, suggest that RSV infections also may be frequent in healthy employed adults (Falsey and Walsh 2000).

Nevertheless, it appears that the immune response to RSV does play a role in mediating the severity of the clinical symptoms of the infection and therefore it is possible the quality of the antibody and cellular responses to the infection may play a role in determining whether or not there is a repeat infection.

3.1.1 Adult humoral response to RSV

Following primary infection, anti-RSV antibodies decline to low or undetectable levels within a year (Welliver, Kaul et al. 1980). Higher titres of antibody are produced and persist longer after a second infection. Thus, there is a pattern of increasing levels of serum antibody with successive infections which parallels the accumulative acquisition of resistance to severe illness (Stott and Taylor 1985).

There are few published studies that document immunity to RSV in the elderly. It is not clear if all older adults or only specific groups such as elderly with underlying disease have reduced humoral immunity. Neutralizing anti-RSV antibody titres in 20 healthy older adults were significantly lower and demonstrated greater diversity than those in younger subjects (Falsey and Walsh 1998). Several reports indicate that all older adults have measurable RSV-specific IgG in their serum and that most infected individuals will develop a fourfold or greater increase in IgG (Falsey and Walsh 1992). Results from this study suggest that the increased morbidity and mortality observed in older persons with RSV infection is not due to a key defect in the humoral immune system. No decrease in quantitative or qualitative measures of RSV serum antibody was observed among these subjects. In addition, no differences in complement – enhanced neutralizing titres were seen, suggesting that there were no age-related differences in IgG isotypes (Falsey, Walsh et al. 1999).

A possible role of serum anti-RSV IgE in pathogenesis was suggested. The study analyzed anti-RSV IgE in the blood of 220 RSV cases among adults with different various complications of bronchitis. A correlation between high levels of

anti- RSV IgE and the presence of bronchospasm in patients was revealed in this study (Falsey and Walsh 1992). In a study by Noah and Becker 2000, 10 healthy non-smoking adults were inoculated nasally with RSV and a daily nasal lavage was taken to assess viral load and chemokine profile. Only 3 out of the 10 subjects developed RSV (Noah and Becker 2000). In a more recent study, RSV was nasally inoculated to 32 healthy adult volunteers. The subjects were followed for 21 days with daily monitoring of their health. In addiation a nasal lavage sample was taken daily and used for viral detection. 18 of the 32 adults became infected as detected by both viral shedding and Ag detection. The 18 adults showed URTI symptoms and disruption of normal middle ear function (Buchman, Doyle et al. 2002).

All these studies and observations from adult infection with RSV highlight the importance of investigating the immune response to RSV in adults. Thus, it is possible that some adults either develop: Full immunity to RSV in adulthood which could explain why infection with RSV happens only in half of any adult population following intranasal challenge with RSV (Noah and Becker 2000; Buchman, Doyle et al. 2002) and the development of milder disease following re-infection with RSV in adulthood. Consequently, the identification of antigenic regions in the F protein of RSV that induce antibody responses in adults could contribute to our understanding of this so-called partial immunity. In addition, identifying such regions could provide information that could help in the design of a vaccine that protect against RSV therefore, in this chapter, sera from 10 healthy adults working at the London School of Hygiene and Tropical Medicine were used to screen for both linear and conformational B-cell epitopes on the fusion protein of HRSV.

3.2 Results

3.2.1 Reactivity of the pooled human sera and the absorbed human negative serum

For all the experiments presented in this chapter, the availability of an anti-RSV negative control serum was crucial. It was decided to produce this negative control serum in-house. Plates were coated with RSV Ag (Biogenesis) at 5 µl /ml and used to screen the anti-RSV reactivity of human pooled sera, human absorbed serum and human normal serum before absorption (see section 2.4.1) (Figure 3.1).Results showed that as expected, there was a significant reduction in the reactivity of the absorbed serum compared to that seen in the serum before absorption. And accordingly, a cut off value of O.D. 0.2 was used for all human sera

3.2.2 Identification of antigenic epitopes of F protein using overlapping synthetic peptides

55 overlapping peptides covering the whole of F protein were used as solid-phase antigens on ELISA plates, to screen for linear epitopes recognized by adult polyclonal human sera. The reactivity of pooled polyclonal sera to RSV Ag was used a positive control, and the human serum absorbed with RSV Ag was used as negative control. Following an initial screening of the 55 peptides with one serum sample, all of the nine healthy adult serum samples were screened for reactivity with the 55 peptides for reactivity at a dilution of 1/100 (Appendix1-3). Peptide six was excluded from the study since in preliminary experiments, it showed cross-reactivity with the secondary antibody used (goat anti-human IgG peroxidase conjugate). The 9 human sera reacted positively with several peptides at 1/100. A number of peptides were recognized by all the nine adult sera with high levels of reactivity (greater than 2x the cut off).These were peptides: 4, 8, 11, 14,18,24,36, and 41 (Figure 3.3). Other peptides were recognized at a lower level of binding by the majority of the nine human samples (e.g.

p28). Peptides that were recognized by only some of the sera, or had a binding of less than 2 x the cut off, were not included in the final analysis of the data (See appendix). A summary of reactivity of the sera with the peptides is given in Table 3.1.The frequency of peptide recognition by the 9 healthy human sera of the 55 peptides is shown in Figure 3.2.

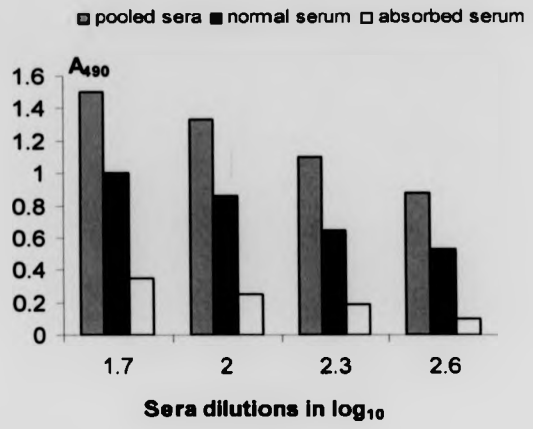

Figure 3.1

Reactivity of the pooled human sera and the absorbed human negative sera.

The human pooled sera was found to bind strongly to the RSV Ag, the absorbed anti-serum to RSV was shown to have a very significantly reduced reactivity with RSV Ag compared to the normal pooled serum. The OD shown is the maen of 2 OD readings.

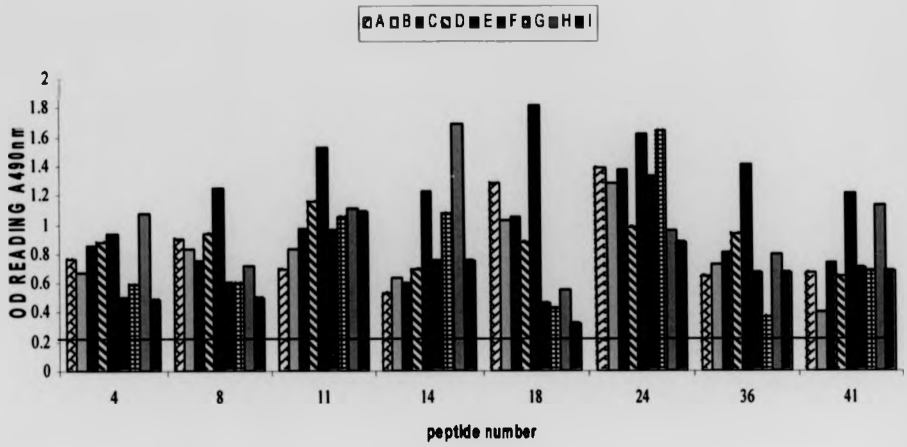

Figure 3.2

Reactivity of the adult human sera with the 8 peptides, with the highest OD readings. Sera samples were run at 1/100 dilution. Sera were from 9 healthy individuals and are indicated as A-H. * Cut/off was at 0.2 OD reading at 490 nm. The OD shown is the maen of 2 OD readings.

Table 3.1

Reactivity of sera with peptides (OD at 1:100 dilution)

Peptide number								
Sera sample	**P4**	**P8**	**P11**	**P14**	**P18**	**P24**	**P36**	**P41**
A	0.764	0.907	0.692	0.537	1.28	1.389	0.653	0.668
B	0.674	0.876	0.831	0.637	1.03	1.28	0.761	0.41
C	0.855	0.75	0.969	0.596	1.055	1.37	0.8	0.74
D	0.875	0.89	1.158	0.695	0.883	0.987	0.921	0.653
E	0.936	0.829	1.527	1.23	1.811	1.62	1.304	1.209
F	0.532	0.604	0.956	0.748	0.462	1.324	0.67	0.703
G	0.593	0.6	1.053	1.075	0.423	1.643	0.64	0.682
H	1.073	0.809	1.114	1.689	0.552	0.956	0.8	1.133
I	0.522	0.607	1.09	0.748	0.323	0.876	0.665	0.682

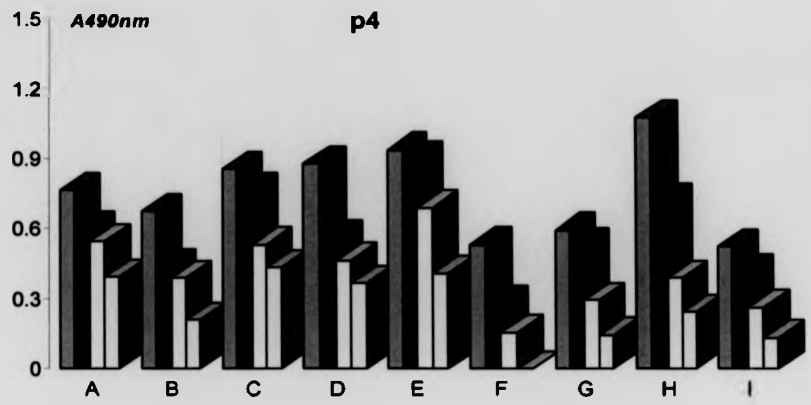

Figure 3.3.a

Reactivity of the nine human adult sera with peptide 4 The OD shown is the maen of 2 OD readings

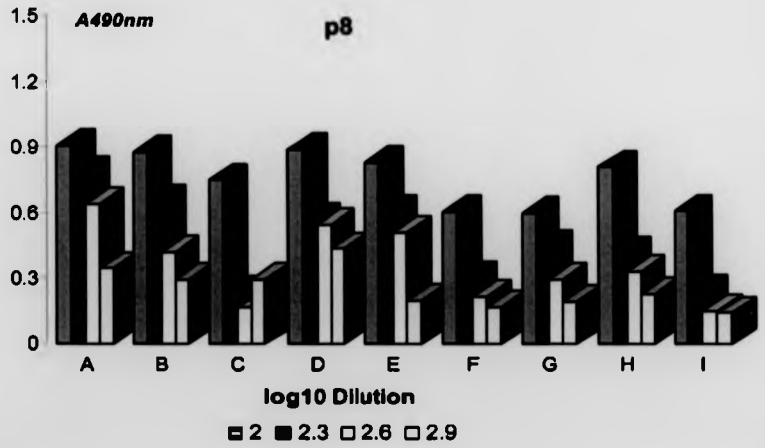

Figure 3.3.b

Reactivity of the nine human adult sera with peptide 8 The OD shown is the maen of 2 OD readings

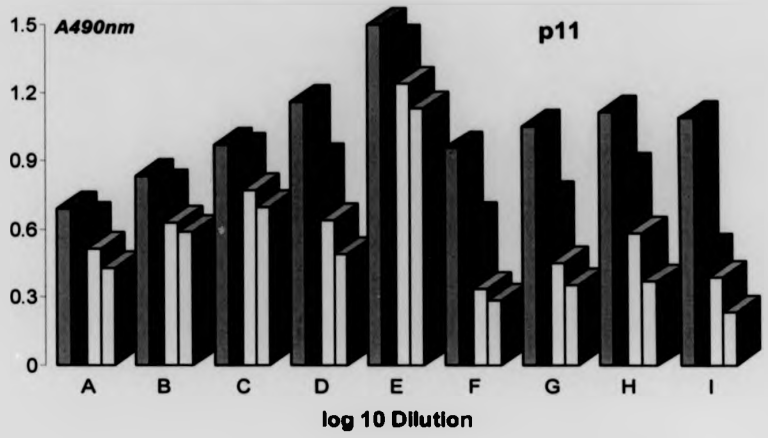

Figure 3.3.c

Reactivity of the nine human adult sera with peptide 11 The OD shown is the maen of 2 OD readings

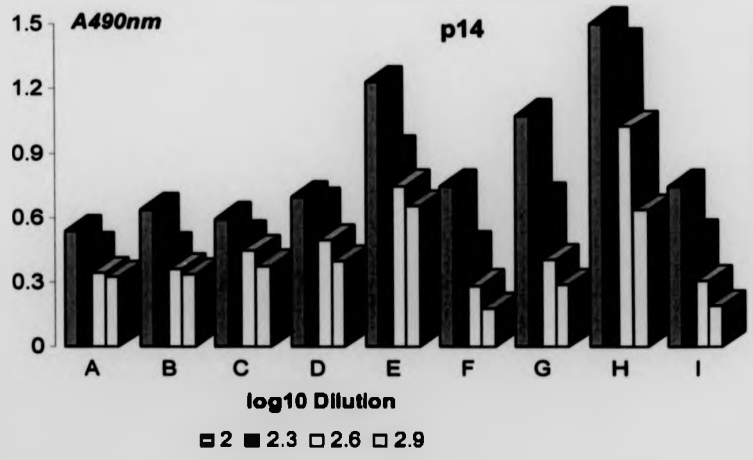

Figure 3.3.d

Reactivity of the nine human adult sera with peptide 14. The OD shown is the maen of 2 OD readings

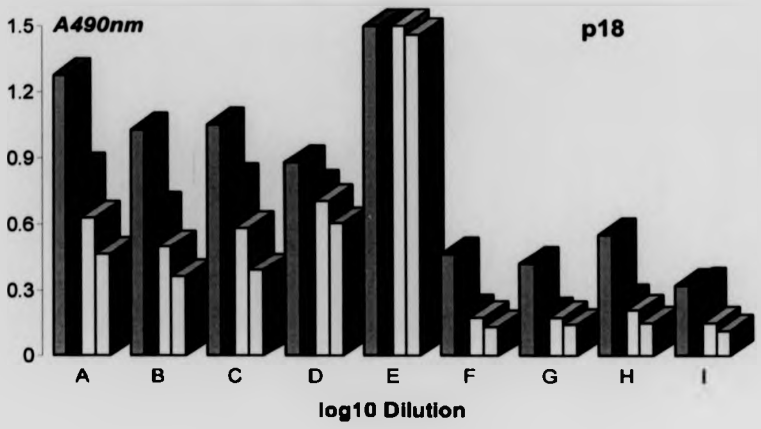

Figure 3.3.e

Reactivity of the nine human adult sera with peptide 18. The OD shown is the maen of 2 OD readings

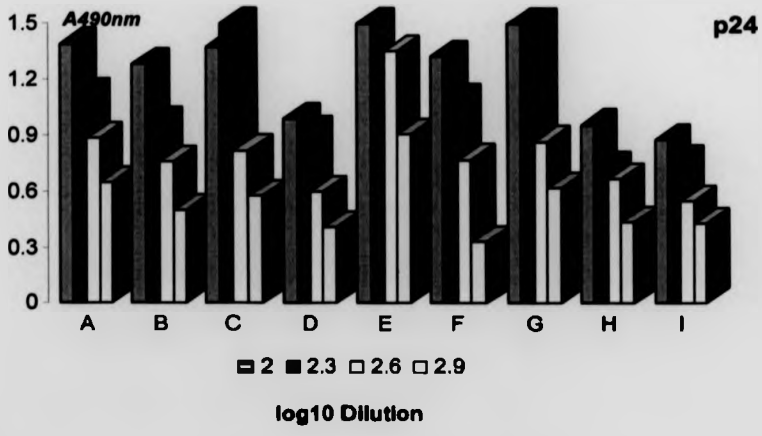

Figure 3.3.f

Reactivity of the nine human adult sera with peptide 24. The OD shown is the maen of 2 OD readings

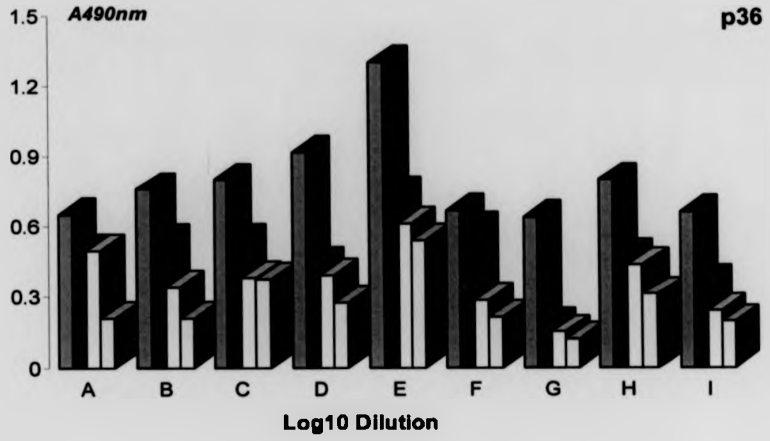

Figure 3.3.g

Reactivity of the nine human adult sera with peptide 36. The OD shown is the maen of 2 OD readings

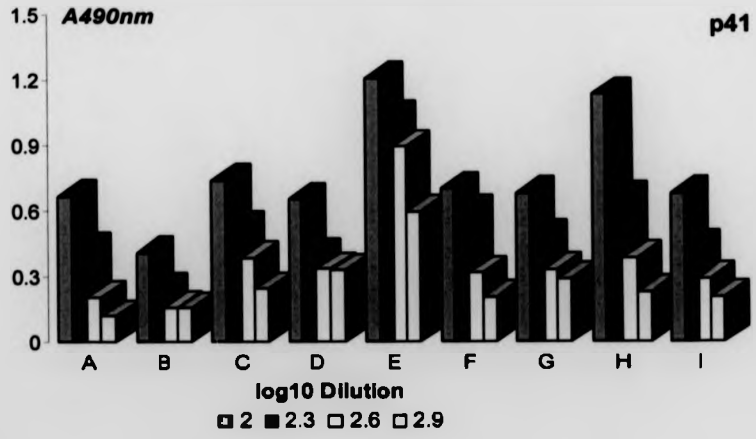

Figure 3.3.h

Reactivity of the nine human adult sera with peptide 41. The OD shown is the maen of 2 OD readings

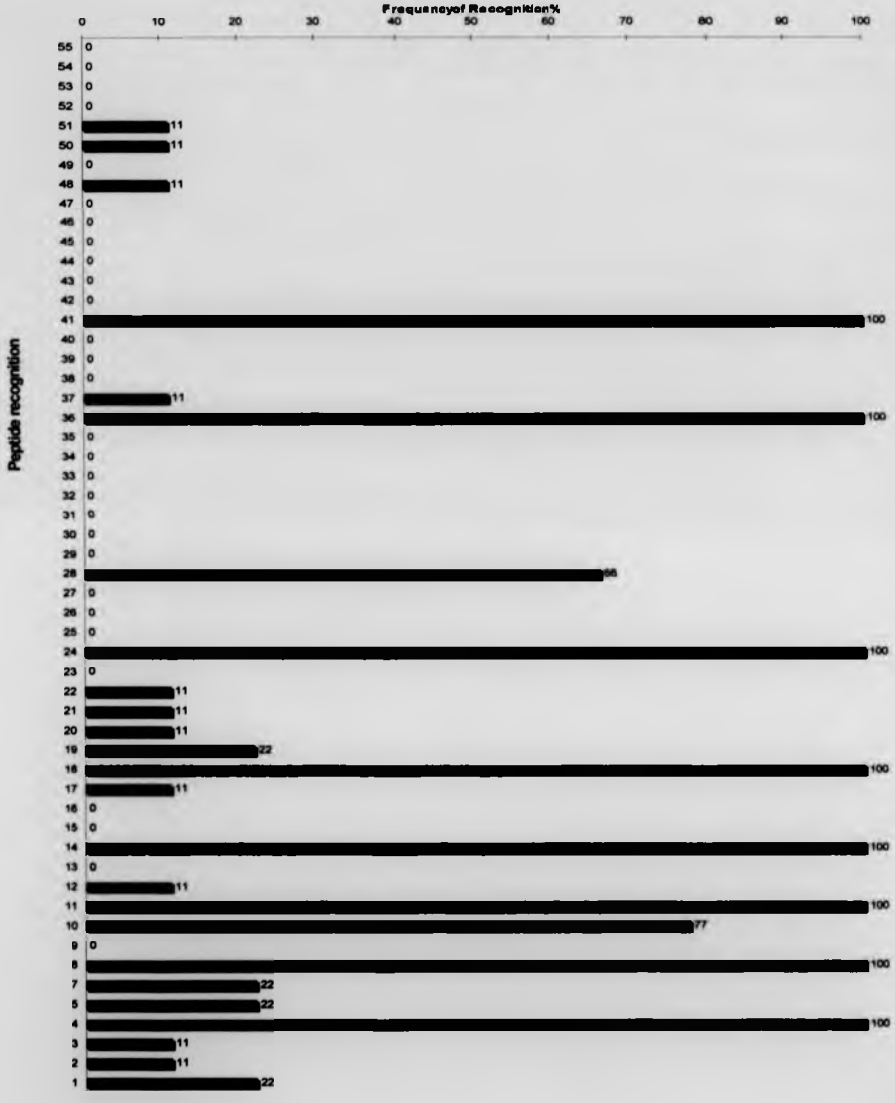

Figure 3.4

Frequency of peptide recognition by the 9 healthy human sera with the 55 peptides

3.2.3 Discussion

The immune response to RSV plays a role in mediating the severity of the clinical symptoms of the infection and therefore it is possible that the quality of the antibody and cellular responses to the infection may play a role in determining whether or not there is a repeat infection. But adults re-infected with RSV are rarely symptomatic and usually display symptoms only as severe and long-lasting as those for a common cold. Little information about the role of immunity to RSV exists in the major part of the population; the rate of RSV infection in persons with no underlying compromising conditions (healthy working adults) is difficult to determine, considering the variability of exposure among adults at work and home. Both the significant gaps in our understanding of RSV infection in adults, and the difficulty of diagnosing RSV in adults, highlights the importance of studying RSV immunology in this group of individuals.

The role of antibodies in immunity to RSV remains not fully understood and in this context, screening of the reactivity of the human sera with the 55 RSV-F protein peptides as linear B-cell epitopes plus their reactivity with the mimotopes identified from the F protein of RSV could be useful in identifying immune dominant epitopes involved in inducing protective antibody responces against RSV. The results obtained from screening the sera of laboratory personnal working on RSV showed a clear profile for peptide recognition. Eight of the 55 peptides were strongly bound by all adult sera: peptides 4, 8, 11, 14, 18, 24, 36, and 41 (Figure 3.2). All nine (100%) sera recognized the following peptides in the order of decreasing OD values; 11>24>14>36>18>41>4>8. 77% of the sera recognized peptide 10. 66% of the sera recognized peptide 28. Peptides 1/5/7/19 were recognized by 22% of the sera. It is possible that these 8 peptides most frequently recognized by the sera may represent linear epitopes of the F protein that could be important in the development of a

protective antibody response. Accordingly it was decided to test their immunogenicity in animal studies (see chapter 6). If these linear epitopes could produce protective antibody in animals this would suggest that they could be candidates for vaccine development.

CHAPTER 4

REACTIVITY OF SERA FROM GUINEA BISSAU CHILDREN WITH LINEAR PEPTIDES FROM RSV F-PROTEIN

4.1 Introduction

Infectious diseases are considered to be the major reason for morbidity and mortality in very young infants in developing countries. RSV is certainly the most important cause of viral acute LRI in both developed and developing countries (Simoes 1999). It also accounts for 20-25% hospitalization of infants in developing countries (Wright 1996). Children with a cough or difficult breathing and fast breathing would meet the current world health organization definition of pneumonia used by health workers at health care facilities in developing countries. Weber et al. showed that RSV LRI led to 8-fold and 4-fold increases in subsequent wheezing and non-wheezing LRI in the following 2 years compared with control subjects (Simoes 1999). In a more recent study, Openshaw et al tested the effect of age at first infection on re-infection in mice. Mice were infected at day 1, 4, or 8 weeks of age then challenged at 12 weeks. Neonatal mice challenged with RSV showed increased inflammatory cell infiltration (Th2 cells and eosinophilia) plus increased weight loss. The delay in priming the led to enhanced IFNγ production and the clinical profile of the disease was also less severe. (Culley, Pollott et al. 2002)

In developing countries, health care workers often do not use a stethoscope to distinguish between wheezing and non-wheezing LRI, and perceptible wheeze is the only criterion used in some of these situations. However, wheeze that is easy to hear

present only in the more severely ill children with wheezing LRI, and its recognition by health care workers is often poor (Simoes 1999).

There are three main trategies for controlling RSV infections (Weber MW 1999).

i) Case management

ii) Prevention through vaccination

iii) Identification of risk factors which might be altered through interventions.

4.1.1 The influence of maternal antibody on immunity to RSV in African children

In a study carried out in the Gambia, designed to determine the influence of placental malaria infection and maternal hyper-gamma-globulinemia on trans-placental antibody transfer, 213 mother-baby pairs were recruited at delivery in the labour ward. Only 51.1% of the study population had placental malaria infection 24.7% had active infection, whereas 49 (45%) and 33 (30.3%) had active chronic infection and chronic infections, respectively. Mothers with placental malaria infection transferred significantly lower levels of IgG1, IgG2 to their newborns than did mothers without placental malaria infection. Placental transfer of antibodies against HSV-1, RSV, and VZV was a lot lower in the placental malaria-positive mothers/baby pairs than in the placental malaria negative pairs. Results from this study confirm data from similar studies in Brazil and Malawi (Okoko, Wesumperuma et al. 2001).

In a study carried out in rural Mozambique during the 20 months of hospital-based surveillance (October 1, 1998, to May 15, 2000), 4589 children were admitted, all children <5 years old admitted to the ward fulfilling the WHO criteria for LRTI, a nasopharyngeal aspirate (NPA) was collected. Serum samples were collected also from infants with RSV infection < 1 year of age and from uninfected controls to

assess the effect of serum antibodies on the incidence of RSV infection in. Anti-RSV antibodies were assessed by a membrane fluorescent antibody test (MFAT) for (IgG) antibodies and by a neutralizing antibody test. IgG anti-RSV antibodies were of higher prevalence and at higher levels in the control group compared to the infected case group, indicating an important role for IgG antibodies in protection. IgA RSV antibodies were also measured by MFAT. IgA anti-RSV antibody prevalence was very low in patients and controls, signifying that the detected IgG RSV antibody in both groups was of maternal origin. The results emphasize the theory of maternal vaccination in the control of RSV in very young children that represent the highest risk group (Roca, Abacassamo et al. 2002).

Evidence from laboratory studies and clinical trials strongly suggests a dominant role for antibodies in protection against re-infection. The relative protection of infants from virus-associated lower respiratory tract (LRT) disease in the first weeks or months of life is related to the level of passively-acquired maternal antibodies to RSV (Crowe 1996). Data from passive-transfer studies in rodents have demonstrated the protective effect of polyclonal immune sera and neutralizing RSV monoclonal antibodies (MAbs) in the LRT (Crowe 1996). Virus replication in the LRT was inversely correlated with the serum RSV-neutralizing-antibody titre following passive transfer. While in contrast, the presence of serum neutralizing RSV antibodies usually has little effect on virus replication in the upper respiratory tract (URT) (Crowe 1996). However, high titres of serum neutralizing antibody against RSV do protect the LRT against RSV infection, as shown in animal studies, epidemiological observations, and most recently, in clinical trails of RSV hyper immune globulin (RSVIG) passively transferred to high-risk infants (Dudas and Karron 1998). High titres of maternally-derived RSV antibody (as measured by IgG enzyme-linked immunosorbent assay [ELISA] or virus neutralization), in maternal

blood or cord blood have been shown to correlate inversely with the incidence of RSV infection (Dudas and Karron 1998) and with the severity of RSV pneumonia in the first 6 months of life (Dudas and Karron 1998). The rate of re-infection with RSV and the rate of LRI at the time of re-infection also correlate inversely with the level of serum neutralizing antibody against RSV following primary infection in young infants (Dudas and Karron 1998). The effect of the administration of RSVIG in high-risk infants was measured in a randomized study, and it was shown that those who received monthly doses of 750mg/kg had significant reductions in the rate and severity of LRI as measured by the need for hospitalization, days spent in hospital,and days spent in intensive care (Dudas and Karron 1998).

Primary infection with RSV does not always evoke an immune response that will protect the lower respiratory tract, since RSV-associated LRI can occur in young children experiencing their second episode of RSV (Dudas and Karron 1998). The lack of protection may be explained in part by group specificity, since children with primary RSV infection develop a neutralizing antibody response in serum more frequently and of greater magnitude to the infecting strain than to the heterologous RSV strain. The identification of an ideal response of young infants to primary infection with RSV has important implications for vaccine development, because it suggests that more than one dose of vaccine will probably be needed to induce sufficient levels of RSV neutralizing antibody in this age group (Dudas and Karron 1998).

A prospective community cohort was set up in several districts in the capital of Guinea-Bissau. More than 2000 houses were included in the study. Around 1300 children were followed with weekly morbidity reports and NPA (nasopharyngeal aspirate) when they have WHO criteria for LRI.

The WHO definition of LRTI was defined as children who presented with cough, nasal secretion or difficulty in breathing and who had one or more of the following: chest indrawing, crackles, wheezing, or increased respiratory rate (WHO 1991)

The outpatient clinic at the hospital and the health centre is screened for severe cases. Blood samples have been taken from cases (and controls as well as mothers of cases and controls) at time of infection but the diagnosis was based on NPA. Most of these children were included in a community study of a two-dose measles immunization schedule (6+9 months) and have had blood samples taken at 6, 9 and 18 months.

The following samples were selected for the work discussed in this chapter:

- Children with a pre-primary infection sample (6 months) and a post-infection sample (at 9 or 18 months or both). In all cases, the RSV was diagnosed by antigen detection.

- Children with two episodes of RSV and two post-infection samples: 11 had the first episode before 6 months and one or two further episodes after 6 months but before 18 months. 6 children had episodes between both 6-9 and 9-18 months (Figure 4.1).

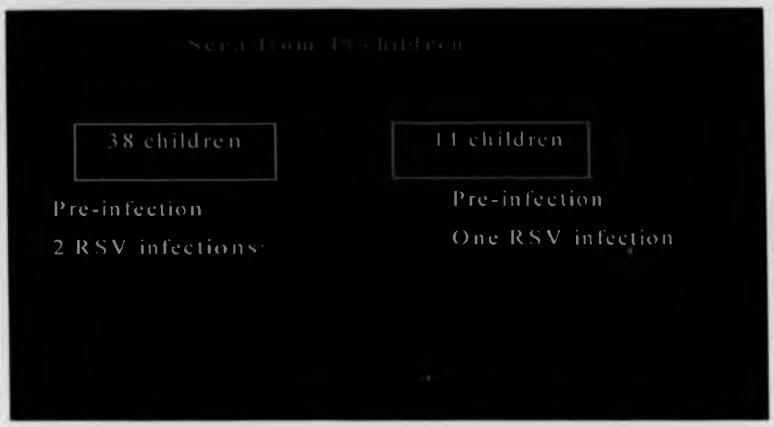

Diagram showing the number of children samples used in the study and how the samples were divided.

The hypothesis to be tested in the work described in this chapter is that during the first infection with RSV, incomplete immunity is developed as a measure of the production of an antibody response that is not directed to the protective epitope or epitopes. Furthermore, in order to attempt to answer the question of why individuals can be re-infected with RSV, the panel of synthetic 55 overlapping peptides covering the whole of the fusion protein was screened with sera from children with sequential infections with RSV to determine whether or not there were differences in the pattern of peptide recognition in sera at different stages of exposure to RSV.

4.2 Results

4.2.1 Recognition of the F peptides by sera from Guinea Bissau

In order to answer the question of why individuals can be re-infected with RSV, an attempt was made to determine whether or not there were differences in the pattern of peptide recognition by human sera at different stages of exposure to RSV. ELISA plates were coated with the 55 peptides representing the whole of the fusion protein. Since the sera were from infants, very limited volumes were available and thus the samples were used at 1/200 dilution. The reactivity of each serum was determined as the mean of the optical density of the wells containing the specified peptide minus the OD value obtained with empty, blocked wells with the samples from each child. An adult human serum pool was used as a positive control, an RSV absorbed human serum was used as a negative control.

Analysis of the binding of peptides by sera from the children at various stages of infection showed that in children with pre and first infection samples only, peptide 4 was recognized by 45% of the pre-infection sera. Binding of peptide 4 was seen in 18% in the primary sera after exposure to RSV infection. Peptide 48 on the other hand, was recognized by 46% of the post infection sera and peptide 14 was recognized by 72% of both pre -infection and first infection samples (Figure 4.2).

In samples from children with both primary and secondary infection, peptides 19, 20, 32 and 48 were recognized by 50-54 % of the secondary infection sera. Peptide 14 in was recognized by 66% of the pre-infection serum samples, 81% of the sera obtained following the primary infection with the virus, and 73% of the sera following the secondary infection with the virus. Peptide 4 was recognized by approximately 40% of the sera at all stages of exposure to RSV (Figure 4.3).

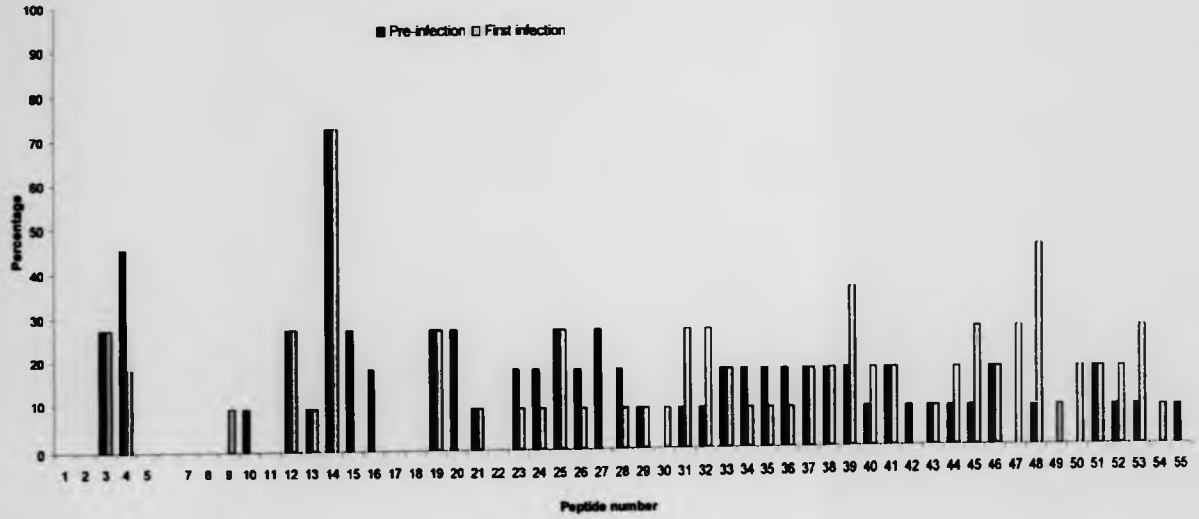

Figure 4.2

Percentage of the binding of peptides recognized by sera from RSV infected infants. Data shown are from sera taken from children prior to exposure and following primary RSV infection. Sera were run at 1/200 dilution.The OD shown is the maen of 2 OD readings.

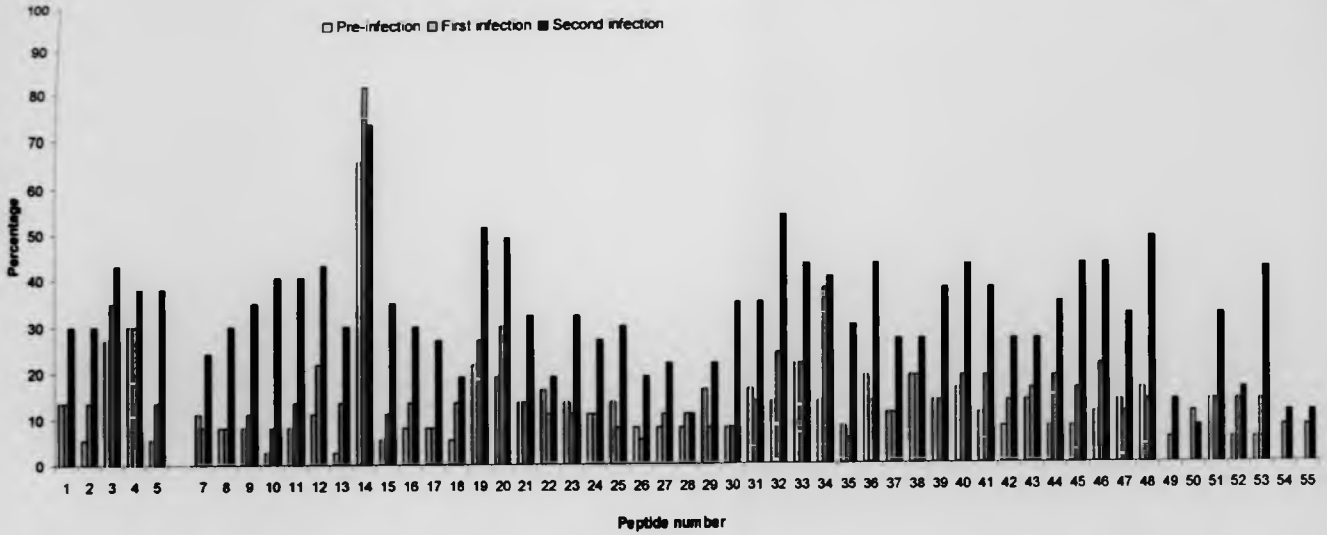

Figure 4.3

Percentage of the binding of peptides recognized by sera from RSV infected infants.Data shown are from sera taken from children prior to exposure and following primary and secondary RSV infection. Sera were run at 1/200 dilution. The OD shown is the maen of 2 OD readings.

4.2.2 Recognition of peptides 4 and 14 by sera from children

Peptides 4 and 14 were the most frequently recognized peptides among all the serum samples tested. Therefore, the reactivity of the sera to these two peptides was analyzed in details and related to the child's state of exposure to the virus. The reactivity of sera from children with pre-and first infection samples was not high and only two samples had demonstrable reactivity to peptide 4 as shown in Figure 4.4. However, in sera from children with 2 repeated infections with RSV, peptide 4 was recognized by 16 (42%) of the samples.

In an attempt to determine if the presence of antibodies to peptide 4 was associated with prevention of a repeated infection, the reactivity of sera with peptide 4 at various stages of exposure to the virus was analyzed. Sera from children 1, 23, and 32 reacted with peptide 4 in the pre-infection samples but serum taken following the first infection and the second infection did not recognize the peptide. Sera from child 1 had no detectable anti-RSV antibodies which again suggest that the child was not actually infected. In the case of child 23, the post-first infection sample had high antibody reactivity against RSV but the second post-infection sample was not available.

Sera from both child number 5 and 25 had reactivity with peptide 4 following the first infection. No anti-RSV Ag reactivity was detectable, again suggesting that these children were not infected. Sera from children number 26, 40, and 44 had anti-peptide antibodies in both pre-infection, and post-first infection samples but no reactivity to the peptide in the second post infection samples. However the sera did havedetectable anti-RSV antibodies.

Peptide 14 was recognized by the pre, first and second- infection samples from most individuals, and in most of the sera, OD values for binding of peptide 14 were high at all stages of exposure to the virus. Approximately 78% of the sera taken from post-first infection bound significantly to the peptide. However, highest values of binding

were shown in sera taken post-second infection (Figure 4.6, and 4.7).

The question of whether the presence of antibodies to peptide14 was associated with prevention of a repeated RSV infection was also addressed. It was noticed that only child number 34 had reactivity to peptide 14 in the pre-infection samples but not in the post-first infection sample, while the reactivity of this serum to RSV Ag was high.

Among children from whom samples from two repeated infection were available, child 1, 28, and 40 had reactivity to peptide 14 in their pre- and first infection samples but not in the sera from the second post-infection samples. Furthermore, none of the second post-infection samples had anti-RSV antibodies.

4.2.3 Recognition of peptide 19

In children sera with only pre- and first infection samples, peptide 19 was recognized by 27% of the sera both prior to and when exposed to the virus for the first time.

In infected children sera from which 2 samples were available, peptide 19 was recognized by 21% of the sera in pre-infection samples and by 27% of the sera following their first exposure to the virus. The reactivity of the sera with peptide 19 increased to 51% following the second infection with RSV.

4.2.4 Recognition of peptide 20

It was noticed that only in the pre-infection samples and not in the post-first infection samples was peptide 20 recognized. However, in children with two episodes of RSV infection, 21% of the pre-infection samples 30% of the post-first infection samples and 51% of the samples following second exposure to RSV were reactive to with peptide 20

4.2.5 Recognition of peptide 32

In pre-infection sera, 9% were reactive with peptide 32 and 27% of post-first infection sera were reactive with the peptide. While in sera from children both first and second infection samples, reactivity with peptide 32 was 13% in pre-infection samples, 24% in first infection samples and 54% following second infection.

4.2.6 Recognition of peptides 48

In pre-and first infection samples, the proportion of sera reactive with peptide 48 was 9%, and 45%, respectively while in both first, and second infection sample reactivity of the sera with peptide 48 was 16% of pre-infection samples, 13% of first infection samples and 50% in sera with second post infection samples.

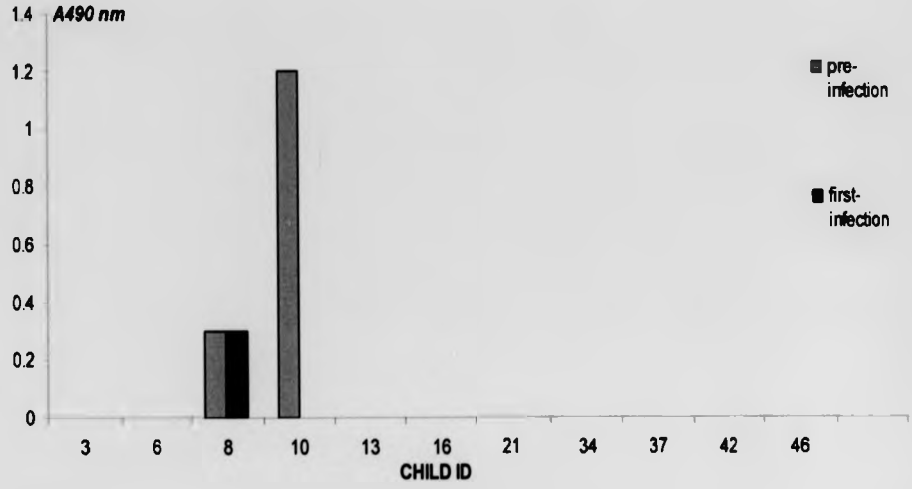

Figure 4.4

Recognition of peptide 4 by sera from children with pre-and first infection samples only.

OD values shown are the OD at 490 nm of a 1:200 dilution. The OD shown is the maen of 2 OD readings

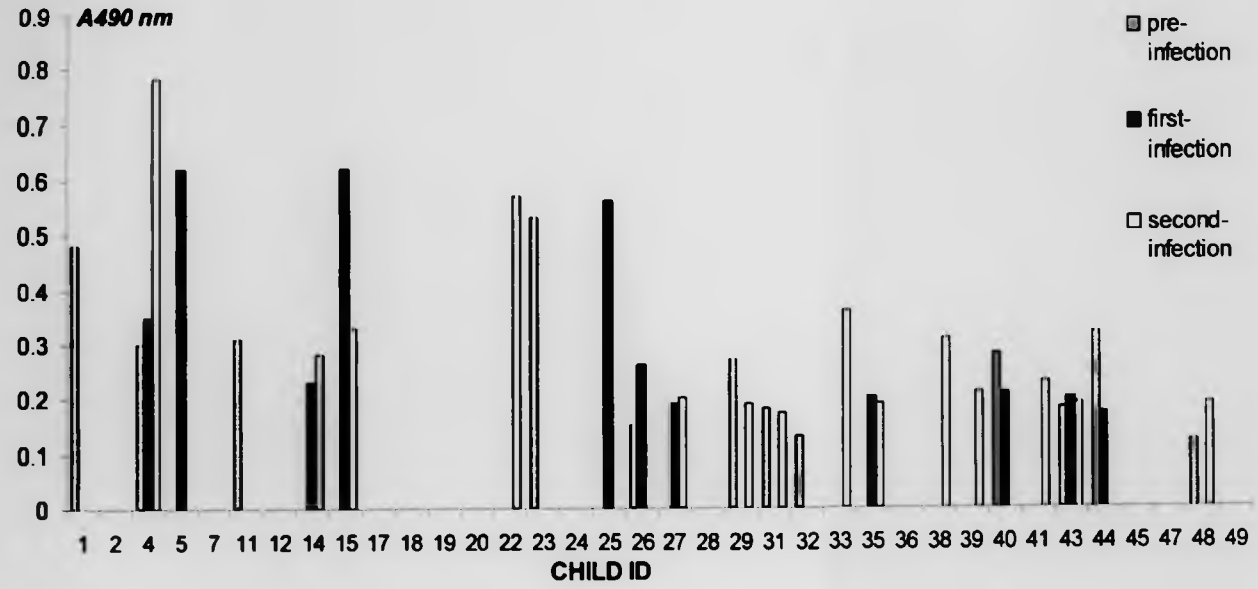

Figure 4.5

Recognition of peptide 4 by sera from children with 2 repeated infections. OD values shown are the OD at 490 nm of a 1:200 dilution. The OD shown is the maen of 2 OD readings

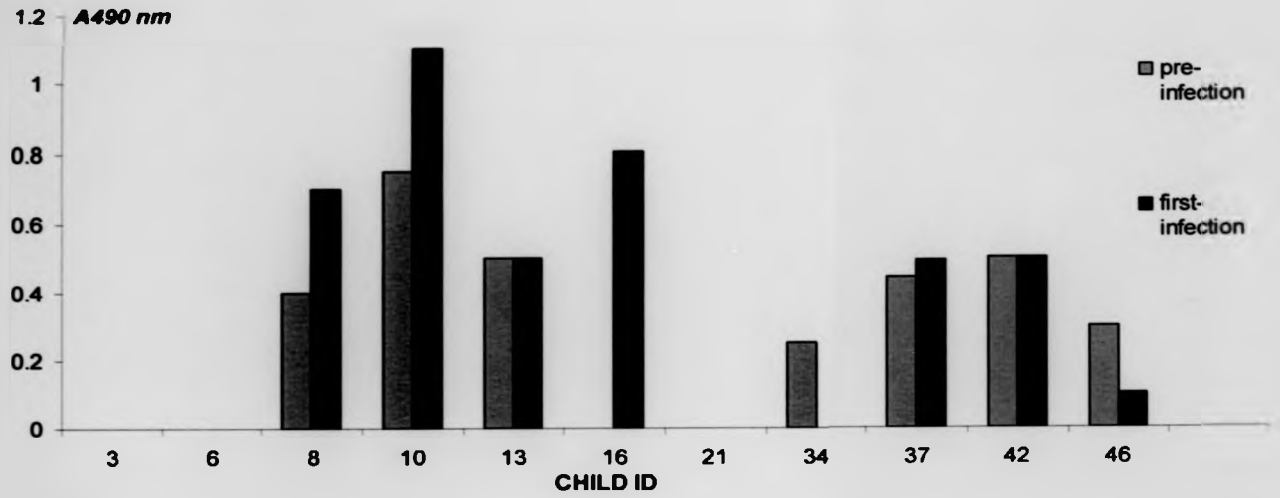

Figure 4.6

Reactivity of peptide 14 by sera from children. With pre-and first infection samples only. OD values shown are the OD at 490 nm of a 1:200 dilution. The OD shown is the mean of 2 OD readings

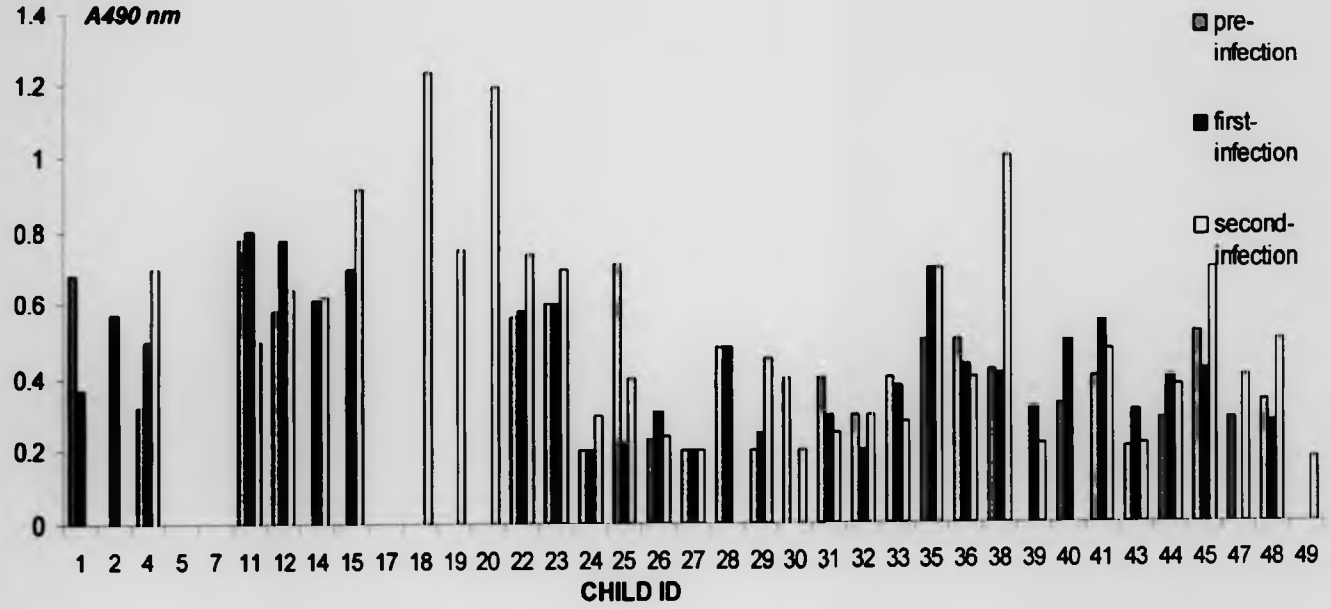

Figure 4.7

Recognition of peptide 14 by sera from children with 2 repeated infections. OD values shown are the OD at 490 nm of a 1:200 dilution. The OD shown is the maen of 2 OD readings

4.2.7 Reactivity of sera from children with the RSV antigen

The reactivity of sera with RSV antigen was also tested at the same time using the commercially available RSV antigen (Biogenesis). The reactivity of each serum with RSV Ag was determined as the mean of the optical density of the wells containing the RSV antigen minus the OD recorded with empty, blocked wells with samples from each child (Table 4.1). Twelve of the children's sera that were taken prior to exposure to RSV infection had reactivity with RSV antigen. This could mean that an active RSV infection had started around the time the sample was taken. Therefore, these samples were screened to determine whether or not IgM anti-RSV antibodies could be detected (Figure 4.8). 4 children (8, 10, 11, and 30) out of the 12 that were suspected to have an active RSV infection had detectable IgM anti-RSV binding (OD ranged from 0.14-0.22) in the pre-infection serum sample (6 months of age) (Figure 4.8). Other observations can be drawn out here:

- Child 3: Sera were obtained after only one episode of RSV. There was no recognition of any of the peptides either in the pre-infection sample or in the sample following primary infection. Furthermore, these sera had no detectable reactivity with the RSV antigen in the ELISA.
- Child 9: No peptides were recognized by the pre, or post first–infection serum samples from this child. However, serum from the post second-infection strongly recognized peptide 48. These results are consistent with those from the RSV ELISA, which showed the absence of reactivity with RSV in pre-infection and post first infection sera. Post second infection serum showed reactivity with RSV at twice the background level.
- Child 19, and child 29: Sera from these children at the pre-infection and post first infection stage did not have reactivity to either RSV antigen or to any of the peptides, but sera taken post-second infection did react with peptide 14 and

with the RSV antigen.

4.2.8 Sequence analysis of the identified peptides:

A comparative study using the computer program Swiss-Prot/protein blast p was performed in order to confirm the identified peptides (Table 4.2). The program uses (Swiss-Prot/protein database on the web) search sequence databases, to evaluate similarity scores, and identify periodic structures based on local sequence similarity. The peptides had 100% identity with their native protein which confirms their origin. Some of the first matched sequences are shown in (Table 4.3), and all the rest are shown in the appendix section (Appendix 4-15). None of the peptides had similarity with any human proteins, with the exeption of peptide 41 which had similarity with Cytochrome c, which is a small, highly conserved protein involved in mitochondrial electron transport. This may suggest that the peptide could not be of use as a vaccine and this needs further invistigation However, a few peptides had sequence similarity with other unrelated proteins, the most significant ones are listed in (Table 4.4).

Child number	Pre-infection	Post-first-infection	Post-second-infection
1			
2			
3			N.A
4			++++
5			
6			
7		+	
8	+	+++	N.A
9			++
10	+	+	N.A
11	++	++	
12			
13			N.A
14	N.A		+
15			
16			N.A
17			
18			
19			
20			
21	N.A	N.A	N.A
22	+++		+++
23	+	++++++	N.A
24			++++++
25			
26	+	+++++++	+++
27	+++		++++
28			N.A
29			++++
30	++	+	N.A
31	+++	++	++
32			
33	+++	++	+++++++++
34		+++++	N.A
35		++	
36	+		++++
37	++		N.A
38			+++++++++
39	Lost		++++++++
40			
41			++++++++
42	+++		N.A
43			
44			++++++
45			++++++
46			N.A
47		N.A	++++++
48			+++++++++
49	N.A		++++++

Table 4.1

Reactivity with RSV antigen of sera from children.

Each sample was tested at 1/200. The reactivity with RSV Ag was determined as the optical density of the background (cut off) subtracted from the OD in wells with the RSV antigen. Results are illustrated in the table as + = cut off and N.A= sample not available.

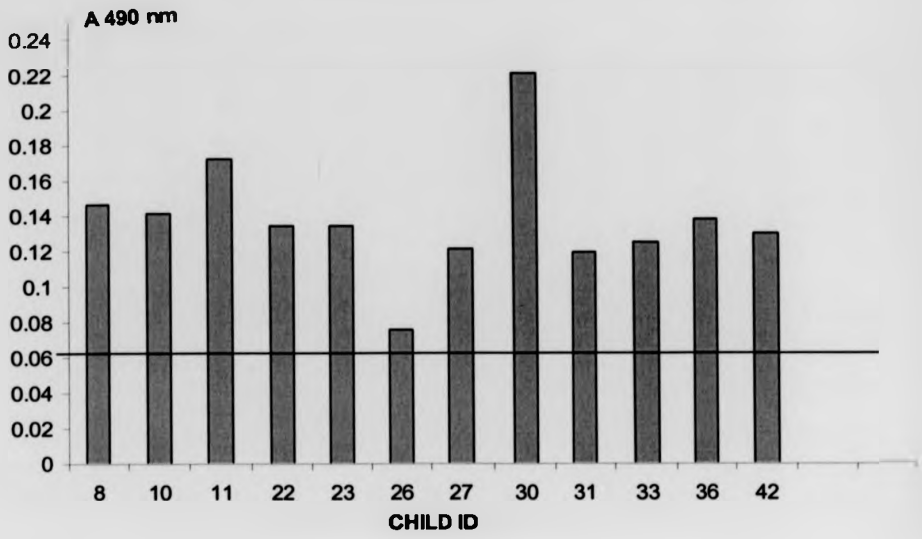

Figure 4.8

IgM level in pre-infection serum samples.

Samples were tested at 1/200 dilution. IgM anti-RSV reactivity with RSV was expressed as the OD of the sera in the well containing the RSV Ag minus the OD obtained after addition of the secondary antibody to an empty blocked well. The cut off in this assay was the OD of the secondary antibody to an empty blocked well. The OD shown is the maen of 2 OD readings.

Peptide	Sequence	
4	WYTSVWYTSVITIE LSNIKE	aa52-66
8	ELQLLMQST PPTNNR	aa92-106
11	AKKTNVTLS KKRKRR	aa122-136
14	VAVSKVLHL EGEVNK	aa152-166
18	VLDLKNYID KQLLPI	aa192-206
19	QLLPIVNKQ SCSISN	aa202-216
20	CSISNIETV IEFQQK	aa212-226
24	LTNSELLSL INDMPI	252-266
32	ICLTRTDRG WYCDNA	aa332-346
36	SLTLPSEIN LCNVDI	372-386
41	CTASNKNRG IIKTFS	aa422-436
48	ISQVNEKIN QSLAFI	aa492-506

Table 4.2

Sequences and amino acid position for the mapped peptides.

Peptide	Identities	Accession number	Definition	Isolate
4	15/15 - 100%	AAM68160	fusion glycoprotein [Human respiratory syncytial virus]	HRSV
8	15/15-100%	P03420	Fusion glycoprotein precursor [Contains: Fusion glycoprotein F2; Fusion glycoprotein F1]	HRSVA2
		VGNZA2	cell fusion glycoprotein precursor - human respiratory syncytial virus (strain A2).	HRSVA2
		AAB59858	fusion glycoprotein [Human respiratory syncytial virus	HRSVA2
11	15/15 - 100%	B28929	cell fusion glycoprotein - human respiratory syncytial virus (type A).	HRSVA
14	15/15 - 100%	P23728	Fusion glycoprotein precursor [Contains: Fusion glycoprotein F2; Fusion glycoprotein F1].	BRSV RB94
		VGNZBS	cell fusion glycoprotein precursor - bovine respiratory syncytial virus (strain RB94).	BRSV RB94
		BAA00798	fusion protein [Bovine respiratory syncytial virus].	BRSV
18	15/15 - 100%	AAM68160	fusion glycoprotein [Human respiratory syncytial virus	HRSVA
19	15/15 - 100%	AAM68160	fusion glycoprotein [Human respiratory syncytial virus	HRSVA
20	15/15 - 100%	AAM68160	fusion glycoprotein [Human respiratory syncytial virus	HRSVA
24	15/15 - 100%	P23728	Fusion glycoprotein precursor [Contains: Fusion glycoprotein F2; Fusion glycoprotein F1].	BRSV RB94
		VGNZBS	cell fusion glycoprotein precursor - bovine respiratory syncytial virus (strain RB94).	BRSV RB94
		BAA00798	fusion protein [Bovine respiratory syncytial virus].	BRSV
32	15/15 - 100%	P23728	Fusion glycoprotein precursor [Contains: Fusion glycoprotein F2; Fusion glycoprotein F1].	BRSV RB94
		VGNZBS	cell fusion glycoprotein precursor - bovine respiratory syncytial virus (strain RB94).	BRSV RB94
		BAA00798	fusion protein [Bovine respiratory syncytial virus].	BRSV
36	15/15 - 100%	B28929	cell fusion glycoprotein - human respiratory syncytial virus (type A).	
41	15/15 - 100%	P23728	Fusion glycoprotein precursor [Contains: Fusion glycoprotein F2; Fusion glycoprotein F1].	BRSV RB94
		VGNZBS	cell fusion glycoprotein precursor - bovine respiratory syncytial virus (strain RB94).	BRSV RB94
		BAA00798	fusion protein [Bovine respiratory syncytial virus].	BRSV
48	15/15 - 100%	AAM68160	fusion glycoprotein [Human respiratory syncytial virus	HRSVA

Table 4.3

RSV sequences producing significant alignments with the identified peptides.

Peptide	Identities	Accession number	Definition	Isolate
4	9/14 -64%	NP_716069	aconitate hydratase 2-	Shewanella oneidensis MR-1
	9/13 -69%	P19584 A31389 AAA23204	Beta-amylase, thermophilic precursor (1,4-alpha-D-glucan maltohydrolase	Thermoanaerobacterium thermosulfurigenes
	8/14 -57%	NP_454772 CAD01317.1 NP_804047 AE0522 AAO67896.1	aconitate hydratase 2	Salmonella enterica CT18 and Ty2
	8/14 -57%	NP_285814.1 NP_308149.1 B90644 B85495 AAG54422.1 BAB33545.1	aconitate hydrase B/ Escherichia coli	O157:H7 EDL933 and O157:H7
8	7/7-100%	CAF89628	unnamed protein product	Tetraodon nigroviridis. taxon:99883
	8/10 -80%	NP_985668 AAS53492.1	AFR121Wp.	Eremothecium gossypii -ATCC 10895
	7/7-100%	AAL88727	similar to Homo sapiens (Human). HERC2 protein [Dictyostelium discoideum].	Dictyostelium discoideum
	45-100%	Multiple	hypothetical protein	Multiple organisms
11	9/11-81% 8/9-88% 8/10-80%	EAA61925 NP_705854 NP_588327.1	hypothetical protein AN9092.2 hypothetical protein hypothetical protein	Aspergillus nidulans FGSC A4 Plasmodium falciparum 3D7 Schizosaccharomyces pombe
	8/10-80%	CAE01390	tuber borchii white collar	Tuber borchii (whitish truffle)
	8/9-88%	EAA17525	AT hook motif, putative	Plasmodium yoelii yoelii
	8/9-88%	CAA29838.1	pyrG product	Aspergillus niger
	8/9-88%	DCASON	orotidine-5'-phosphate decarboxylase	Aspergillus niger
41	8/9-100%	EAA20535	hypothetical protein	Plasmodium yoelii yoelii
	8/10-80%	Multiple	cytochrome c	Multiple human and chimpanzee

Table 4.4

Non-RSV sequences producing significant alignments with the identified peptides

4.3 Discussion

Evidence from laboratory studies and clinical trials strongly suggests a dominant role for antibodies in protection against re-infection. The relative protection of infants from virus-associated lower respiratory tract (LRT) disease in the first weeks or months of life is related to the level of passively-acquired maternal antibodies to RSV. Since the presence of specific anti-F antibodies plays a major role in immunity against RSV, the identification of the immunogenic epitopes recognized by sera from infected individuals could be very useful in vaccine development against RSV, and this study was performed to identify these epitopes using a panel of peptides representing the F protein. The pattern of peptide recognition varied according to the state of exposure to the virus. When the data were analyzed with the sera treated as 3 different groups that had different exposure to RSV infection regardless of them being from the same children, no clear pattern could be observed. Nevertheless, the following points can be drawn from an analysis of the results:

- In all children, sera obtained following the second infection were likely to show peptide binding activity.
- Peptides 4 and 14 were recognized strongly by sera from both adults and children.
- Peptide 4 was recognized by 45% of the pre-infection samples. This value drops to 18% in sera of children that had been exposed to RSV infection. In addition, sera from adults also had reactivity to this peptide. Taken together, these observations indicate that reactivity with this peptide in the children is maternally-derived antibody and suggest that the peptidesis not likely to represent a protective epitope.

- As discussed in section 4.2, peptides 19, 20, 32, and 48 were recognized by 50-54% of the post-second infection serum samples but antibodies were also present in both pre- and post- first infection samples. This could again suggest that these peptides may not represent protective epitopes.

Several factors complicated the analysis of the data:

- The negative controls available were the so–called pre-primary infection samples which are from children who did not have clinical RSV before 18 months of age - but they may well have had sub-clinical infection which could explain the presence of antibodies to RSV in these sera.
- The reactivity of some of the sera either negatively or positively with RSV Ag was not related to the presence of antibodies to peptides, this caused a confusion in interpreting the data.
- The presence of maternally-derived antibodies could have also interfered with the results obtained since maternal antibody has been shown to interfere with how babies respond to RSV. In a study carried out in a rural area of the Gambia, mothers with placental malaria infection transferred significantly lower levels of IgG1 and IgG2 to their newborns than did mothers without placental malaria infection. Placental transfer of antibodies against HSV-1, RSV, and VZV was a lot lower in the placental malaria-positive mother/baby pairs than in the placental malaria negative pairs. Only 51.1% of the study population had placental malaria infection (Okoko, Wesumperuma et al. 2001).

To exclude the possibility of the presence of cross-reacting antibodies from other infections, a comparative study using the computer program Swiss-Prot/protein blast p was also performed in order to identify any similarity between our reactive peptides with any other human proteins. No homology was detected with any human protein.

Nevertheless, analysis of the location of the identified peptides from both the children and the adult sera on the F protein reveals some interesting points for discussion as shown in Table 4.16, and Figure4.9. Both peptide 4 and 8 are located in the F2 region of the fusion protein which is the most divergent fragment of the F molecule among all the antigenic groups of HRSV. The F2 has the bulk of the potential sites for N glycosylation in the F protein (Lopez, Bustos et al. 1998) which makes it very difficult to map epitopes within this region. Therefore it is quite interesting that sera from both adult and children did have reactivity towards peptide 4 and 8, and especially since a protective CTL epitope has been identified in peptide 8.

Peptide 11 shares the sequence KKRKRR at the C-terminal end of F2, the most likely the site of proteolytic processing of the F_0 precursor. Most interestingly, peptide 11 contains 15 amino acids of the pep27 fragment which has been shown to be released between the two cleavage sites as a separate peptide (Zimmer G 2001), (Calder, Gonzalez-Reyes et al. 2000). However, its function is still unknown.

Peptide no	Location on the F protein	aa position
4	F2	52-66
8	F2	92-106
11	F2 (cleavage-peptide)-Pep 27	122-136
14	S-S bridge/ F1	152-166
18	F1	192-206
19	F1/Fusion peptide	202-216
20	F1	212-226
24	F1	252-266
32	F1	332-346
36	F1	372-386
41	F1	412-426
48		492-506

Table 4.9

Location of peptides in the F-protein that are recognized by sera from both children and adult

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CHAPTER 5

THE ANTIGENICITY AND IMMUNOGENICITY OF MIMOTOPES FROM THE F PROTEIN OF HRSV

5.1. Introduction

5.1.1 Concept of mimicry.

Antibodies can be directed to both linear and conformational epitopes. Linear epitopes of an immunogenic region of the protein are represented by continuous amino acid sequences. Conformational epitopes on the other hand, are made up of amino acid sequences brought together by the folding of the protein and are thus discontinuous in nature (Partidos and Steward 2002).

It is likely that the majority of antibody responses to viral proteins are directed towards conformational rather than to linear B-cell epitopes (Steward 1997; Olszewska, Obeid et al. 2000). Since conformational epitopes are very difficult to predict from the primary amino acid sequence and are perhaps impossible to produce by conventional peptide synthesis, peptide vaccines developed thus far have been restricted to amino acid sequences representing linear B-cell epitopes. However, the development of phage display libraries and solid phase combinatorial peptide libraries has provided a means for mimicking conformational epitopes. These libraries can be screened with monoclonal antibodies, polyclonal sera or receptor molecules on various cell types that are specific for conformational structures. In this way, bacteriophage or resin beads expressing peptide sequences that mimic the shape of the epitope that induced its production *in vivo* can be identified.

Library- derived peptides that have been isolated by affinity selection and those that mimic these complex epitopes are therefore called *mimotopes.* It was the pioneering work on the specificity of antibody-antigen interaction by M. Geysen that led to the

introduction of the term *mimotope*. He defined the term to mean a "molecule able to bind to the antigen combining site of an antibody molecule, not necessarily identical with the epitope inducing the antibody, but an acceptable mimic of the essential features of the epitope" (Geysen, Rodda et al. 1986). Although atomic identity might explain the same antigenicity displayed by an epitope and mimotope, this molecular mimicry may also arise because both mimotope and epitope bind to different subsites within the same paratope. It follows therefore, that a mimotope may react with an antibody directed to the native epitope without actually binding to the same paratope as the native epitope. In this case, anti-mimotope antibodies will not cross-react with the native epitope (El Kasmi, Fillon et al. 2000).

Mimotopes have to be antigen- specific and at the same time immunogenic in order to qualify as potential vaccine candidates. The ability of a mimotope to induce antibody responses against the native epitope that it mimics is also challenging in that, in addition to the structural features of the mimotope, host factors such as antigen presentation, genetic background and MHC haplotype influence the induction of immune responses and control antibody affinity and specificity. Mimotopes can be used to induce a defined antibody response towards a specific epitope where immunization with the antigen is either not possible or induces an inappropriate antibody response. If as seems likely, epitopes that induce virus neutralizing antibodies are of a discontinuous nature, the use of mimotopes could be considered as a new generation of specific sub-unit vaccines against viruses.

Several mimotopes have been characterized and have been shown to generate antigen–specific neutralizing and protective antibodies when used as immunogens (e.g. against RSV fusion protein). A protective neutralizing murine monoclonal anti-RSV antibody (mAb 19) was used to screen a solid phase combinatorial peptide library (Taylor, Stott et al. 1992). Five peptides were selected but none reacted with

mAb 19 when used as free peptide, nor in a peptide ELISA, and none were able to inhibit the binding of mAb 19 to the RSV antigen in a fluid phase assay. The five peptides were re-synthesized as peptide–TG resin complexes. Two of the mimotopes, S1 and S2, reacted strongly with mAb 19 when presented as resin-bound peptides and S1, but not S2, reacted strongly with the monoclonal antibody when synthesized as SPOTs peptides. In an attempt to improve the binding of mAb 19 with the two mimotopes, single amino acid substitutions at each position of the 8-mer were performed. 3 sequences were derived in this way from mimotope S1 that showed increased binding with the monoclonal (S1S, S1K, and S1P). These three mimotopes were synthesized as multiple antigenic peptides (mAPs) and when BALB/c mice were co-immunized with the mAPs together with the promiscuous T-helper epitope from measles virus fusion protein, (aa 288-302) specific anti-peptide antibody was induced. These anti- peptide antibodies inhibited the binding of mAb19 to RSV antigen and neutralized the virus with similar titres to those found in sera from RSV-infected animals (Chargelegue, Obeid et al. 1998).

In a similar approach using molecular mimicry, a phage display library was used to select mimotopes for HBsAg using human sera. Four mimotopes (¢ 13, ¢ 14, ¢ 17 and ¢ 30) were selected by screening the phage library with sera from selected patients and the ¢ 35 mimotope was identified using a monoclonal antibody (Meola, Delmastro et al. 1995). Sera from C57BL/6 and BALB/c mice, two groups of rabbits immunized with these mimotopes and sera from patients reacted with the mimotope. Sera from healthy control individuals did not react. Furthermore, the anti-mimotope sera reacted with HBsAg.

Similar protective mimotopes have been identified in the F protein of measles virus (MV). A panel of six mimotopes of an epitope recognized by a monoclonal antibody to the F protein of MV was identified using a solid-phase 8-mer random combinatorial

peptide library. The 8-mer mimotopes selected from the library were bound by the monoclonal antibody with different relative affinities using an inhibition immunoassay. Affinity of the binding ranged from 1.1×10^2 to 1.26×10^6 M^{-1}. The six peptides were not immunogenic on their own, but following co-immunization of BALB/c mice with the individual mimotopes and a T-helper epitope peptide from the fusion protein (F) of MV, anti-mimotope antibodies were induced with affinities for their homologous peptides ranging from 8.9×10^5 to 4.5×10^7 M^{-1}. However, only one of the anti-mimotope antibodies (to mimotope 2) cross-reacted with MV in an ELISA and inhibited MV plaque formation (Shaw, Stanley et al. 1993; Obeid, Partidos et al. 1995) (Partidos and Steward 1992; Olszewska, Obeid et al. 2000) (Steward 1997).

A panel of four mimotopes of an epitope recognized by a highly protective monoclonal antibody against *Schistosoma mansoni* (152-66-9B) was obtained by screening the solid-phase 8mer random peptide library (Arnon, Tarrab-Hazdai et al. 2000). Three of the four mimotopes (p28, p29 and p30) were efficiently recognized in an *in vitro* radioimmunoassay by the monoclonal antibody and by sera from infected mice. One mimotope, (p30) induced *in vitro* proliferation of primed lymphocytes. When the mimotopes were conjugated to bovine serum albumin (BSA) and the conjugates used to immunize C57Bl/6J mice, only the p30-BSA induced antibodies that were effective at complement-mediated killing of schistosomula (Arnon, Tarrab-Hazdai et al. 2000).

The potential for the use of the mimotopes extends to fields other than synthetic vaccines. The approach has been used to identify and mimic receptors or binding sites such as TNF-α and chemokine receptor CCR5 which works as a co receptor for macrophage (m)-tropic (or R5) strains of HIV-1.Two mAbs to CCR5 , were used to screen a cysteine constrained phage library in a search for mimotopes of CCR5.Two mimotopes were identified. Both mimotopes and synthetic peptides

reacted with the corresponding antibody, synthetic peptides inhibited antibody binding to the phagotope. The synthetic peptides corresponding to the phagotopes also showed a CD4-dependent reactivity with gp 120 of a primary-m-tropic HIV-1 isolate (Konigs, Rowley et al. 2000).

In an attempt to mimic the conformational characteristics of the TNF-α receptor binding site, a phage library displaying 15-mer random peptide sequences as fusions at the amino terminus of the coat protein p III was tested with recombinant human TNF-α bound onto polystyrene beads. 40 randomly selected phage clones were amplified and tested for their ability to inhibit mouseTNF- α. Only 5 clones inhibited the apoptotic effect of TNF-α *in vitro*. Sequencing of their peptide inserts yielded 2 sequences which did not exhibit any homology with the primary structures of the human or the mouse TNF-α 55 kDa and 75 kDa receptors. The two mimotopes were then synthesized and tested for their ability to inhibit the cytotoxic activity of TNF-α onto L929 cells. The mimotope corresponding to clone DEF was effective in inhibiting both the mouse of TNF-α, and the human TNF-α. The mimotope corresponding to the phage clone B13 had lost its activity (Chirinos-Rojas, Steward et al. 1998).

BSW17 is a mouse anti-human IgE monoclonal antibody, which recognizes a receptor bound–IgE without inducing inflammatory mediator release (Rudolf, Vogel et al. 1998). Immune complexes of IgE and BSW17 did not bind to the IgE receptor (FcϵRI). Isolation of a mimotope for BSW17 was achieved only when a random nona-peptide library in which the peptides were displayed as circular loops held together by a disulphide bond was used. The amino acid sequence of the BSW17 mimotope was not present within the primary sequence of the IgE molecule. Furthermore, reduction of the disulphide bonds destroyed the reactivity of BSW17 with the mimotope, which confirms its conformational nature. Binding inhibition studies carried out using

synthetic peptides and phage-displayed mimotope confirmed the specificity of the mimotope as an epitope for BSW17. Immunizing rabbits with mimotope induced anti-IgE antibodies which were inhibited by BSW17 (Rudolf, Vogel et al. 1998).

The identification of mimotopes can also provide reagents that can be used for diagnostic assays.

To identify a candidate diagnostic for Lyme disease (LD),biotinylated IgG samples from ten positive and ten negative sera were used to probe a a 12-mer random phage display library and 17 peptides were selected. Four of the peptides reacted well with all the ten positive sera and very weakly, or not at all, with the negative sera. These four peptides therefore may well be of value as diagnostic antigens (Kouzmitcheva, Petrenko et al. 2001).

In systemic lupus erythematosus (SLE), a peptide mimic was selected using patients' sera to screen a phage display library. The peptide identified was bound by both anti-dsDNA and anti-ssDNA antibodies from SLE sera, suggesting that the mimotope shares an antigenic structure with both ssDNA and dsDNA and that, SLE anti-DNA antibodies are cross-reactive. Therefore, the SLE mimotope has potential for use as a diagnostic candidate and/ or therapeutic agent (Sun, Fong et al. 2001).

Because of the complexity and low abundance of epitopes presented on tumours, the identification of these epitopes is very difficult, particularly since the proteins concerned are unknown. Therefore, mimotopes may have therapeutic potential in cancer immunotherapy. A combinatorial peptide library has been used to identify two CTL mimotopes specific for the EL4 mouse lymphoma. Two peptides were identified that mimic an H-2D^b and an H-2K^b restricted CTL epitope. Immunization of mice with the mimotopes mixed with incomplete Freund's adjuvant elicited CTLs that lysed EL4 cells, but immunization with the mimotopes had no therapeutic effect on tumors that existed already. Both of the mimotopes needed a T-

helper epitope to be included in the immunization to induce a CTL response and immunization of H-2D^b mice resulted in the induction of a protective immune response against challenge with EL4 cells (Tirosh, el-Shami et al. 1999).

PS (Polysaccharide) conjugates are now being used to induce anti-polysaccharide antibodies and this approach is the basis of the some very effective anti-bacterial vaccines (Valadon, Nussbaum et al. 1998). Conjugating PS to a carrier protein converts the antigen from a thymus independent antigen (TI) to a thymus dependent antigen (TD). A major limitation to the use of carbohydrate –based vaccines is the difficulty of designing the oligosaccharides that are known to induce protective antibodies. As a result, research has been focused on identifying peptide mimics of carbohydrate antigens and to use these as surrogate antigens for use as vaccines (Valadon, Nussbaum et al. 1998).

Mab 2E9 is a human monoclonal antibody to GXM (Cryptococcus *neoformans* capsular polysaccharide glucuronoxylomannan). 2E9 has shown to be protective in animal models of cryptococcosis and shares an idiotype (Id) with antibodies to GXM found in human sera. 2E9 was used to screen a peptide library and this resulted in the identification a peptide mimic of the GXM epitope it recognized (P13). Immunization of BALB/c mice with P13 presented as three different conjugates (coupled to BSA, tetanus toxoid (TT), or to BSA2) resulted in the production of three populations of protective antibodies:anti–GXM antibodies; anti-P13 antibodies and antibodies reactive with both P13 and GXM (Valadon, Nussbaum et al. 1998).

There are several more mimotopes that have been shown to be useful as potential vaccine candidates and these are listed in Table 5.1.

5.1.2 RSV Human monoclonal antibody (RF2)

Problems with the use of murine Mabs in humans derive from their short circulating half-life when used therapeutically and the association with serious serum sickness or type I II hypersensitivity, particularly in situations where a second administration of the antibody is needed. Murine Mabs also have limited functional activity in humans, which reduces their effectiveness in viral clearance. By comparison, human Mabs function optimally in man since they are predominantly non-immunogenic, and they have longer serum half- lives than their murine counterparts. Thus, producing MAbs from B cells from individuals who have appropriate anti-viral immunity could provide a potentially valuable source of therapeutic antibodies (Chamat, Walsh et al. 1999).

For the experiments described in this chapter, a human monoclonal antibody (RF2) specific for a conformational epitope on human RSV was kindly provided by Dr.P.Brams, IDEC Pharmaceuticals, San Diego California. The antibody was isolated from a spontaneous abdominal tumour in a severe combined immunodeficient mouse (SCID) that had been reconstituted with human spleen cells from a single donor with idiopathic thrombocytopanic purpura and then boosted with F protein of RSV. The tumour was shown to have developed spontaneously as *EBV*–transformed human B cells. The tumor cells expanded rapidly in culture were stable for more than 18 months and secreted high levels of specific human anti-RSV monoclonal antibody. Antibodies were purified from supernatants by adsorption to a protein A Sepharose 4B column and the protein A-bound IgG was eluted with citrate buffer, pH 3.5. RF2 was of the human (γ_1,κ) isotype subclass and recognizes a conformational epitope as it requires both 48K and 23K disulphide-linked fragments of the F protein for recognition when immunoblotted (Chamat, Walsh et al. 1999).

In the work described here, Mab RF2 was used to screen a solid phase combinatorial peptide library in an attempt to identify mimotopes of the

conformational epitope recognized by the antibody. The library was synthesized using the Novasyn TG resin and consisted of $2.8x10^6$ 8-mer peptides (containing all natural amino acids except cysteine) per gram of resin (Hsu, Shaw et al. 1995) as described in Chapter 2.

5.1.4 The biological significance of antibody affinity

Affinity is the term that defines the quality of the binding between an Ag and its paratope (a single binding site on the antibody molecule) and it determines the ability of the antibodies to form a stable complex with the antigen. When a B cell encounters antigen, it starts a clonal expansion process producing cells that share the same B-cell receptor (BCR) and subsequently plasma cells that produce antibodies with the same antigen-binding site. Affinity maturation occurs during the development of the antibody response as a result of mutation of the genes of the variable region in the DNA encoding the hyper variable regions that result in the production of cell receptors of better or increasing affinity for selection by antigens to produce a higher affinity antibody response.

It was Heidelberger and Kendall in 1935 who first demonstrated the progressive change in the quality of the antibody response to albumin which occurs with time after immunization. They observed the rapid production of high affinity antibody in secondary responses. There is now a substantial body of literature that demonstrates the superiority of high affinity antibody compared to low affinity antibody in a number of biological reactions and indicates the importance and biological significance of affinity maturation. It is very clear that the affinity markedly influences the biological function and effectiveness of antibodies. Therefore, the production of high affinity antibody is an advantage to the host, since immunity provided by such antibody is more effective than that provided by a lower affinity response.

As the production of antibody responses of high affinity is likely to be more effective *in vivo* than antibodies of low affinity, certain diseases may be associated with the production of antibody of a particular affinity. Early work that supported this view was the observation that low affinity antibody production was associated with susceptibility to chronic immune complex disease in mice (Steward 1976) (Steward 1979) (Steward 1979) (Steward 2001) (Partidos and Steward 2002).

Evidence obtained subsequently from several laboratories as demonstrated the association of the production of an antibody response of a particular affinity with the susceptibility to disease.

5.1.5 Affinity maturation

The observed rapid production of high affinity antibody in secondary responses to the antigen and the greater biological effectiveness of the high affinity antibodies compared to low affinity antibodies suggests that a mechanism of selection does exist.

Two theories to account for affinity maturation have been proposed:

The first theory was that early after immunization, a large amount of free antigen is present in the circulation which binds to high affinity antibodies resulting in their removal from the circulation, leaving only low affinity antibodies will be able for binding . As antigen becomes limiting, free high affinity antibody becomes available in the circulation. According to this theory, the affinity characteristic is the same throughout the period of response to the antigen and the observed differences in affinity following immunization arise simply from the selective adsorption, by antigen, of the highest affinity antibody.

The second theory for affinity maturation is that early in the response to antigen when there is a lot of antigen present, all antigen-specific antibody cell receptors can bind antigen irrespective of their affinity. Thus, the average affinity of the antibody produced by these cells will be low. As antigen becomes limiting, the

higher affinity receptors have an advantage over those of lower affinity to bind the limited antigen and are thus stimulated. Low affinity receptor-bearing cells are not triggered to produce antibody and the result is that the affinity of the antibody response increases. Thus in the early response, antibody producing cells produce low affinity antibody and later, predominantly high affinity antibody is produced. This later theory was supported by results derived from experiments measuring the affinity of antibodies produced by lymphoid cells from immunized animals *in vitro* in the absence of antigen. Cells obtained early after immunization produced lower affinity antibodies than the antibodies produced by cells obtained from animals later after the immunization. It was also demonstrated that rabbits immunized with low doses of antigen produced higher affinity antibody in vitro than did cells from animals immunized with higher antigen doses.

The process of B-cell affinity maturation takes place in the germinal centers. After a B cell encounters antigen, it migrates to the primary follicles and begins a rapid mitosis producing clones of cells synthesizing the same B-cell receptor BCR and, eventually, secreting antibodies with the same binding site. This is associated with somatic or / and point mutations of the Ig genes, those occurring in the DNA encoding the hypervariable regions may generate a binding site with increased affinity for its epitope. The result is affinity maturation, the production of antibodies of progressively increasing affinity for the antigen.

Mimotope identified	Reference
Hepatitis B virus envelope protein	(Meola, Delmastro et al. 1995)
Measles fusion protein	(Steward, Stanley et al. 1995)
Plasmodium falciparum circumsporozite surface protein	(Stoute, Ballou et al. 1995)
Hepatitis A virus capsid protein	(Mattioli, Imberti et al. 1995)
Hepatitis C virus	(Prezzi, Nuzzo et al. 1996)
Respiratory Syncytial Virus fusion protein	(Chargelegue, Obeid et al. 1998)
Binding site of the TNF-alpha receptor	(Chirnos-Rojas et al , 1997)
Measles virus haemagglutinin	(El Kasmi, Deroo et al. 1999)
CTL mimotope of Lymphoma E4	(Tirosh, el-Shami et al. 1999)
anti- IgE	(Rudolf, Vogel et al. 1998)
Chemokine receptor CCR5 binding site of gp 120	(Konigs, Rowley et al. 2000)
Schistosoma mansoni	(Arnon, Tarrab-Hazdai et al. 2000)
dsDNA of (SLE)	(Sun, Fong et al. 2001)
Diagnostic mimotope of LD	(Kouzmitcheva, Petrenko et al. 2001)
Surrogate Ag of carbohydrates in vaccine	(Monzavi-Karbassi, Cunto-Amesty et al. 2002)

Table 5.1

Summary of some of the published research on mimotopes

5.2 Results

5.2.1 Reactivity of Human monoclonal anti-RSV (RF2) with RSV antigen (RSV/Ag)

Before using the antibody RF2, its reactivity with RSV antigen was confirmed using an ELISA. RF2 was titrated against the RSV antigen (Biogenesis) starting at a 1:100 dilution of 64 mg/μl antibody. The results showed that RF2 gives a consistent OD reading of between 1.6-1.8 at A 490 *n*m at dilutions of 1/100-1/10000 and the titre of this sample dilution was $\log_{10}$ 7.7 (Figure 5.1)

5.2.2 Reactivity of human monoclonal antibody RT-2 with a panel of 55 overlapping peptides of the F protein of RSV.

The ability of RF2 to bind to linear peptides from RSV F was assessed by ELISA using a panel of 55 overlapping peptides representing the F protein of RSV. The antibodies were used at a starting dilution of 1/100 and a human serum that had been absorbed with the RSV/ Ag was used as a negative control. The results obtained show that RF2 did not recognize any of the 55 linear peptides, (OD values < 0.2 at dilutions of 1:100) which appear to confirm that it recognizes a conformationally determined epitope on the protein (data not shown).

5.2.3 The sequence of the mimotopes selected from screening the peptide library with RF2

Following the screening of the combinatorial peptide library with antibody RF2, three strongly-stained beads were obtained and the amino acid sequence was determined by micro-sequencing. The sequences obtained were as follows:

Mimotope RH 1 **YPKRKHAD**

Mimotope RH 2 **IHRAKRKW**

Mimotope RH 3 **VKPKFVYM**

5.2.4 Reactivity of RF2 with the mimotopes RH1, RH2, RH3 in ELISA

Mimotopes RH1, RH2, RH3 were synthesized as described in Chapter 2 and RF2 was tested against the three mimotopes in an ELISA (Figure 5.2) at the same dilution as was used for the screening of the peptide library. RF2 was used at a starting dilution of 1/100 and ELISA plates were coated with the three mimotopes at 5mg/ml in carbonate/bicarbonate buffer. None of the mimotopes showed binding by the antibody that was significantly above background levels when they were bound to the solid phase in ELISA.

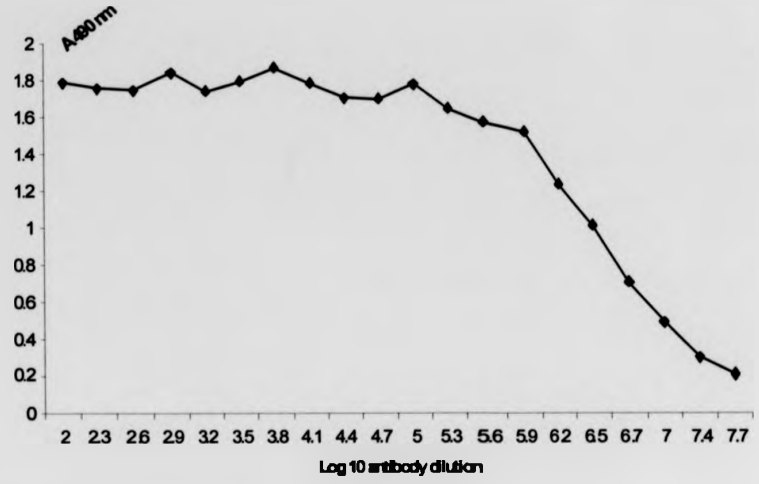


Figure 5.1

Binding of human monoclonal anti-RSV RF2 to RSV antigen in ELISA. The OD shown is the maen of 2 OD readings

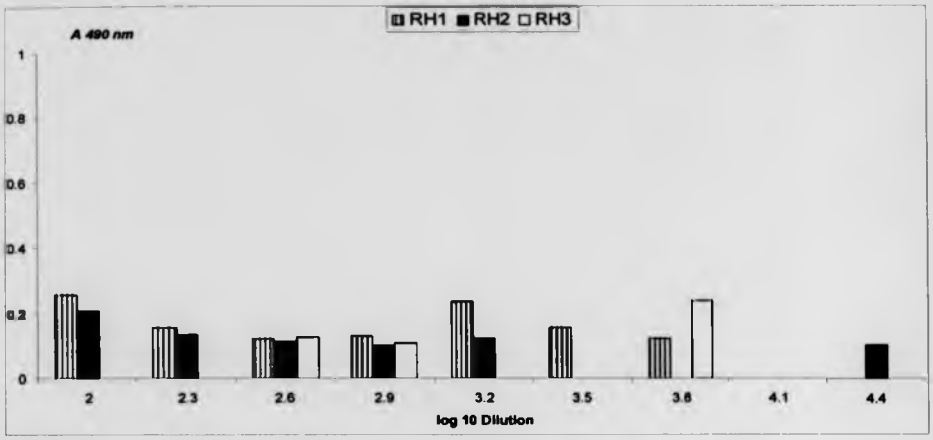


Figure 5.2

Binding of the three mimotopes RH1, 2, and 3 by RF2 in ELISA.

RF2 was used at a starting dilution of 1/100 and ELISA plates were coated with the three mimotopes at 5mg/ml in carbonate /bicarbonate buffer. The OD shown is the maen of 2 OD readings.

5.2.5 Reactivity of adult human sera with mimotopes of conformational epitopes of the F protein

In the studies described here, two monoclonal antibodies were used to screen a random solid phase peptide library to identify mimotopes of the conformational epitopes:

1. The murine monoclonal anti-RSV F antibody (Mab 19) and
2. The human anti-RSV F antibody (RF2).

Two mimotopes recognized by Mab 19 (M1 and M2 originally called S1 /S2 (Chargelegue, Obeid et al. 1998) and three mimotopes recognized by Mab RF2 (RH1, RH2, and RH3) identified in this study.

5.2.5.1 Reactivity of human anti-RSV sera with mimotopes of Mab 19 (M1, M2)

The panel of nine adult human sera, were used in ELISA assays to determine their ability to recognize the mimotopes M1 and M2. The sequence of mimotope M1 was HWS1SKPQ and that of mimotope M2 was HdWS1SKPQ. They thus differ only in that M2 has a D-amino acid at position 2 (Chargelegue et al, personal communication) Sera were used at 1/100 dilution and samples were run in duplicate. The results (Figure 5.3) show that all the sera bound to both M1 and M2 but the binding to M2 was slightly higher.

5.2.5.2 Reactivity of human anti-RSV sera with mimotopes recognized by the human Mob RF2 (RH1, 2, 3)

The nine adult human sera were used in ELISA assays to determine their ability to recognize the mimotopes of the conformational RSV F epitope recognized by the human monoclonal anti-RSV antibody RF2 (RH1, RH2, and RH3). Doubling dilutions of the sera were used, starting at 1/100 dilution and samples were run in duplicate. Two of the human adult sera (D and I) bound mimotope RH2 with an OD

that was twice the cut off and one adult serum (I) bound to the mimotope RH1 with a value higher than cut off at 1/200 dilution. None of the adult human sera bound to mimotope RH3 with OD values that were greater than 2 times the cut off (Figure 5.4).

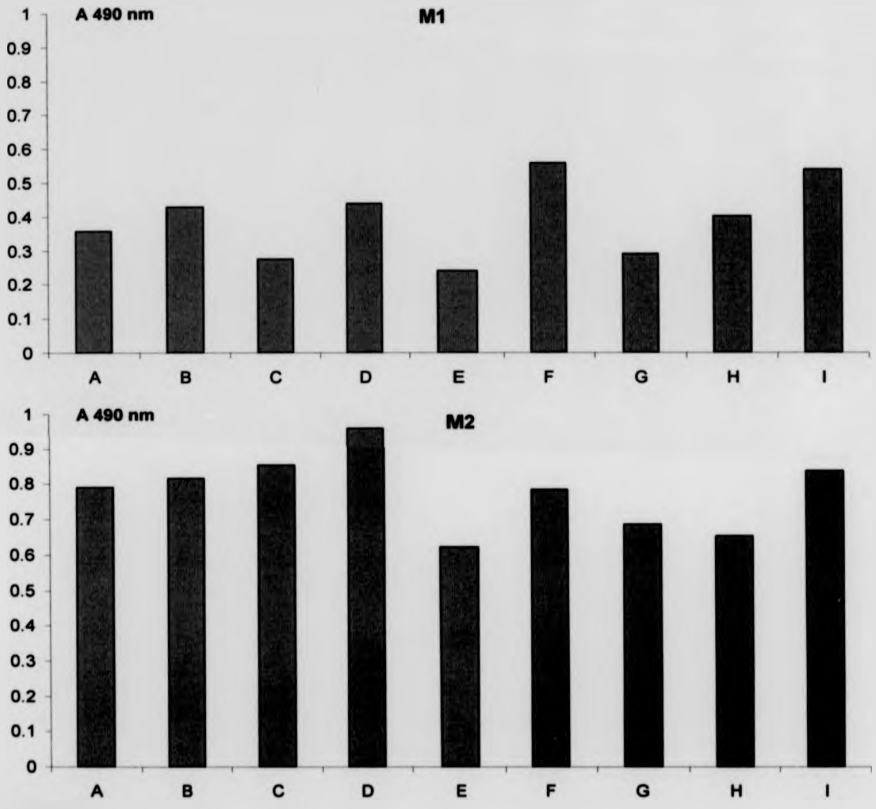


Figure 5.3.

Reactivity of human anti-RSV sera with mimotopes of Mab 19. The OD shown is the maen of 2 OD readings. Sera were used at a dilution1/100

Figure 5.3.a. Binding of M1 mimotope of RSV by 9 of the human adult sera

Figure 5.3. b. Binding of M2 mimotope of RSV by 9 of the human adult sera

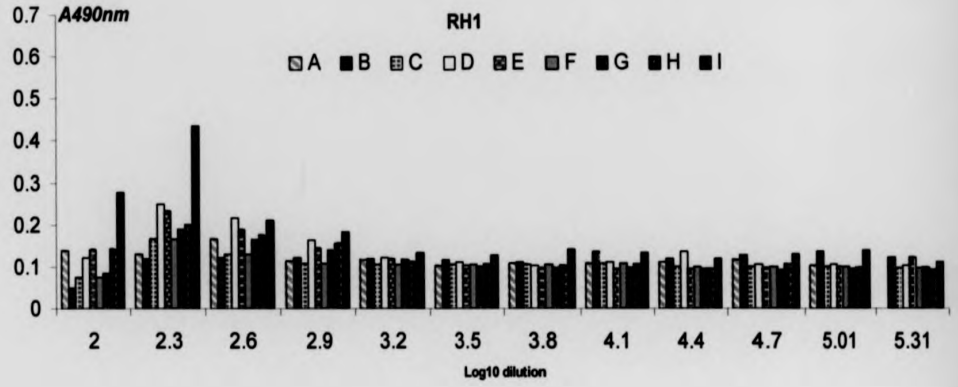


Figure 5.4.a

Binding of RH1 mimotope of RSV by 9 of the human adult sera. The OD shown is the maen of 2 OD readings.

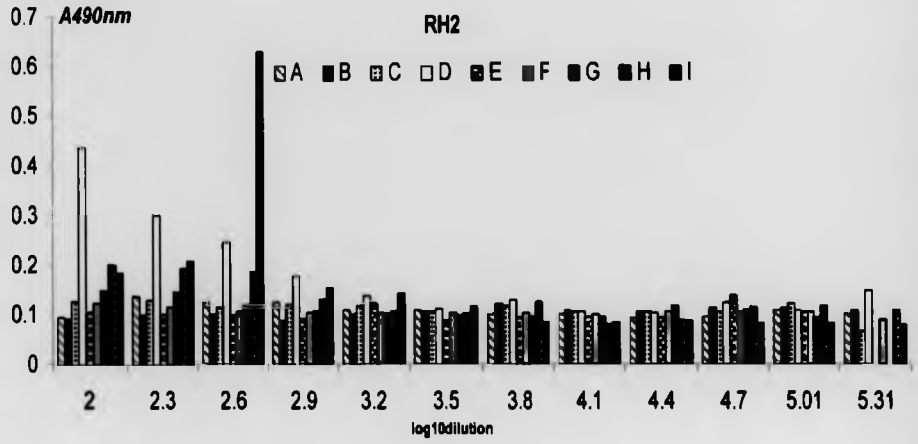


Figure.5.4. b

Binding of RH2 mimotope of RSV by 4 of the human adult sera The OD shown is the maen of 2 OD readings.

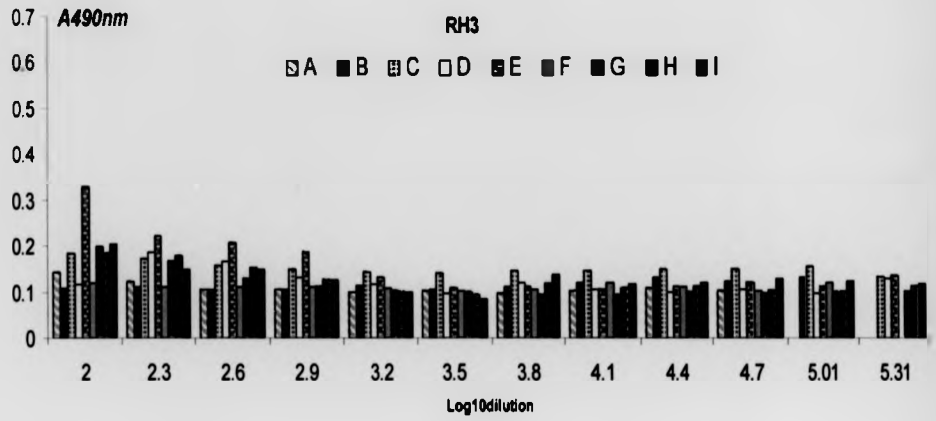


Figure 5.4.c

Reactivity of human anti-RSV sera with mimotope RH3 with the nine adult human sera The OD shown is the maen of 2 OD readings.

5.2.6 SPOTs synthesis of mimotopes RH1, RH2, RH3

In an attempt to enhance the binding of RF2 to the 3 mimotopes selected by the combinatorial library the three mimotope sequences were re-synthesized using the SPOTs system in which each amino acid in the mimotope sequences was replaced by glycine. 8 peptides were generated for each mimotope and their reactivity with RF2 at a dilution of 1/10,000 was assessed. The OD data were analyzed with the (image J) software.

Substitution of the amino acids in mimotope RH1 with glycine did not negatively affect binding of RF2 to any of the generated peptides. However, the results in Figure 5.5 and 5.6 suggest that replacement of amino acids in peptides 5, 6, 7 (arginine 4, lysine 5, histidine 6) with glycine actually enhances the binding of RF2 to RH1.

Substitution of the amino acids in mimotope RH2 with glycine shows that arginine 3, alanine 4, and lysine7 are important contact amino acids for the binding of RF2 to RH2, since replacement of these amino acids inhibited or reduced binding activity of RF2 to peptides 13, 14, and 17.

When valine 6, tyrosine7, and methionine 8 were replaced with glycine in mimotope RH3, binding of RF2 to peptides 25, and 26 was lost and the binding of RF2 to RH3 to peptide 27 was reduced.

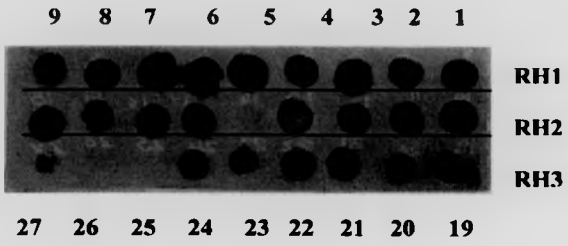


Figure 5.5

RH1, RH2, RH3-mimotopes synthesized as Spots peptides screened with RF2 Mab

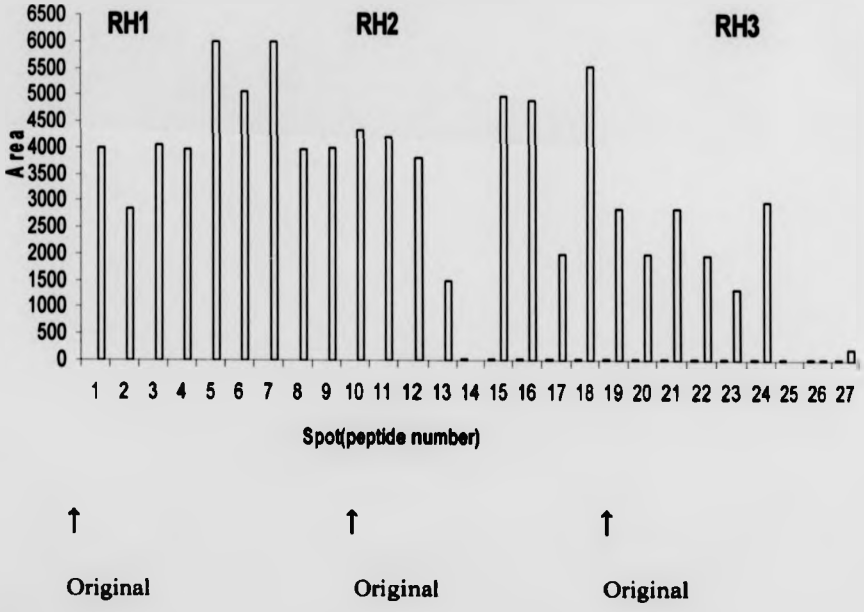


Figure 5.6

Reactivity of RF2 to spots expressed as area-

The histogram was generated from data shown in Figure 5.5.

Spot number	*Sequence*
1	**IHRAKRKW**
2	GHRAKRKW
3	IGRAKRKW
4	IHGAKRKW
5	***IHRGKRKW***
6	***IHRAGRKW***
7	***IHRAKGKW***
8	IHRAKRGW
9	IHRAKRKG
10	**YPKRKHAD**
11	GPKRKHAD
12	YPGRKHAD
13	***YPKGKHAD***
14	***YPKRGHAD***
15	YPKRKGAD
16	YPKRKHGD
17	***YPKRKHAG***
18	YPKRKHAD
19	**VKPKFVYM**
20	GKPKFVYM
21	VGPKFVYM
22	VKGKFVYM
23	VKPGFVYM
24	VKPKGVYM
25	***VKPKFGYM***
26	***VKPKFVGM***
27	***VKPKFVYG***

Table 5.2

The amino acid sequences of each spot (peptide).

The original mimotope sequences are highlighted. Sequences of the peptides that were affected by the amino acid substitution are in bold and italic font.

5.2.7 Systematic substitutions of amino acids in RH1, 2, and 3.

Preliminary results (Figures 5.5 and 5.6) from glycine substitutions in the RSVF mimotopes highlighted how a single amino acid replacement in the peptide sequence could influence the binding of Mab RF2 either by enhancing, reducing, or even inhibiting the binding. Therefore, in order to identify the contact residues in mimotopes RH1/2/3, a series of peptides was generated from each of the RH1/2/3 mimotope sequences in which each amino acid was systematically replaced by all the possible amino acids at each amino acid position. The original mimotope was repeatedly included among the substituted peptides as a positive control. A human monoclonal antibody to HIV-1 gp120 (GP68) was used to screen all the SPOTs assays as a negative control. Results were first analyzed visually as shown in Figures 5.7, 5.9, and 5.11, where the results are represented in the form of graded grey scale boxes  . The black box represents the strongest binding and the white box represents the lowest binding achieved between the Mab and the synthesized mimotope according to visual analysis. The sequences of the original and the substituted peptides are listed in Figures 5.8, 5.10, and 5.12. Results in parallel were analyzed using Total Lab Array, a software product that was developed specifically for analyzing microtitre plate images, grided arrays, dots and slot blots. The software defines the area of each spot, calibrates their quantity of material and produces measurement data. It reads each individual digital pixel within the area of the tested defined spot and then expresses the data as a total volume of the individual spot according to the density of the staining and also as a total volume. The spot representing the negative control was defined and this volume was subtracted from the total volume of the tested spots. The cut-off value was considered to be the highest volume achieved among the spots representing the original mimotope sequence. Accordingly, any value below this cut off represents a reduction of the binding, and

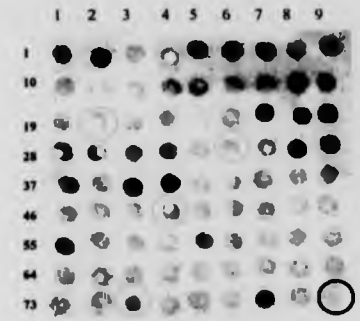
values twice the cut off represent enhancement of the binding. This analysis of the data was dependent on both the visual assessment and the use of the total array software.

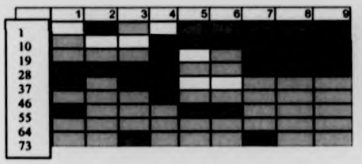
RH1

Results from the systematic substitution in each position were not consistent with the results obtained with the glycine substitutions of RH1 shown previously (Figure 5.5, and 5.6). In the experiments here, a negative effect on binding of RF2 to RH1 was only observed when arginine 4 was substituted with glycine (peptide 66) whereas in the previous experiments, this showed enhancement of the binding. However, earlier results were confirmed with the enhancement of binding seen when histidine 6 was replaced with glycine in peptide 107 (volume reading was equal to the volume of the average positive peptide tested). This confirms the data shown in Section 5.2.4. The substitution of tyrosine 1 showed an increase in the binding of RF2 to RH1 when it was replaced with cysteine (peptide 2), lysine (peptide 10), tyrosine (peptide 17), valine (peptide 18).

A reduction of RF2 binding to RH2 was seen when tyrosine 1 it was replaced with glutamic acid (peptide 4), methionine (peptide 11), and asparagine (peptide 12). An enhancement of the binding was observed when proline 2 was substituted with phenylalanine (peptide 25), arginine (peptide 35), serine (peptide 36), tryptophan (peptide 39) and tyrosine (peptide 40).Whilst the binding was inhibited when proline 2 was replaced with either aspartic acid (peptide 23), glutamic acid (peptide 24), or aspargine (peptide 32). Substitutions of leucine 3 did not result in enhancement of binding. In fact, when leucine 3 was replaced with alanine (peptide 41) or cysteine (peptide 42). A further 7 out of the 20 peptides showed a decrease in the level of binding by RF2 (47, 48, 50, 53, 54, 57, and 58) in which leucine 3 was substituted. Similar results were obtained for substitutions of arginine 4 where 10 out of 20

peptides (61, 63, 67, 68, 69, 70, 71, 72, 76, and 78) showed a decreased binding by RF2.





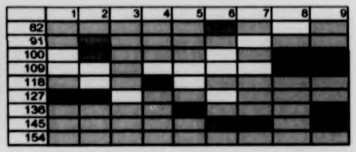


Figure 6.
RH1a

Figure7.
RH1 b

Figure 5.7

Systematic substitution in RH1.

Circles in yellow indicate the original sequence of the mimotope; red circles represent the negative control (unrelated sequence)

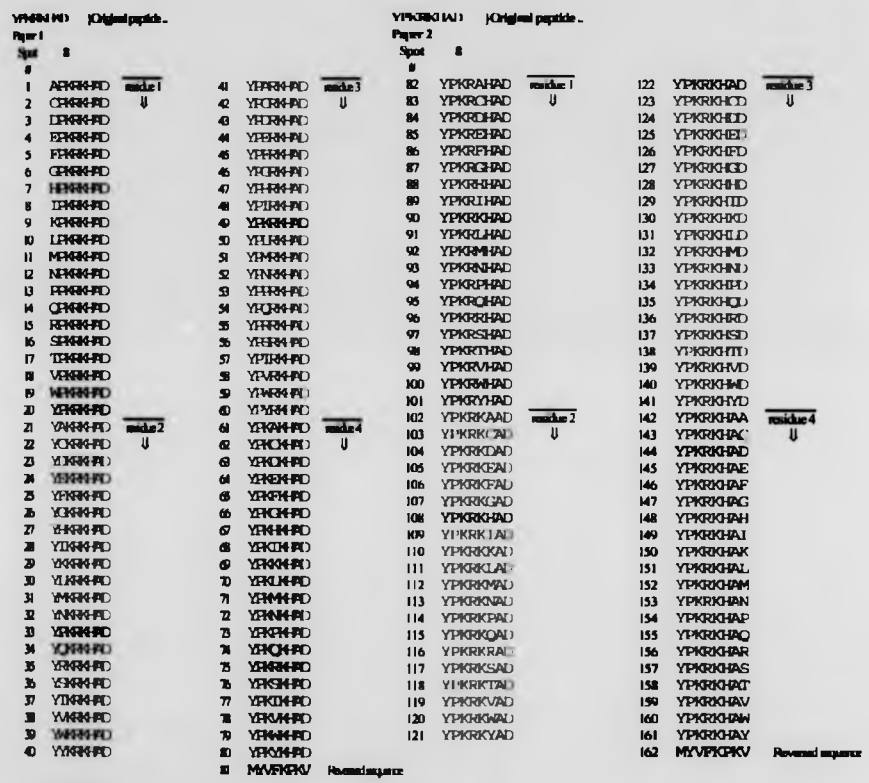


Figure 5.8

The sequences of the original and the substituted peptides in mimotope RH I

RH2

Earlier data (Section 5.2.4) indicated that arginine 3, alanine 4 and lysine 7 are important contact residues for the binding of RF2 to RH2. Results shown in this section confirm that lysine 7 is an important contact residue for RH2 as the replacement of this amino acid inhibited the binding of RF2 to the substituted peptides (123,125,133, and 136). The binding by RF2 was also inhibited in the following peptides (83,104,105,106,113,142,149,153,156) which may indicate that lysine 5; arginine 6, lysine 7, and tryptophan 8 are contact residues for RH2 although by visual inspection, an improvement of the binding by RF2 to several newly generated peptides can be observed. However, when total array software analysis was carried out, the cut off was quite high, and subsequently only peptides 9, 102, and 103 were considered to have improvement in the binding to RF2 where isoleucine1 was replaced with lysine or arginine 6 was replaced with either alanine or cysteine (Peptide 9,102,103). It was very difficult to determine which peptides showed a reduction in the binding by RF2, again because of the high cut off value.

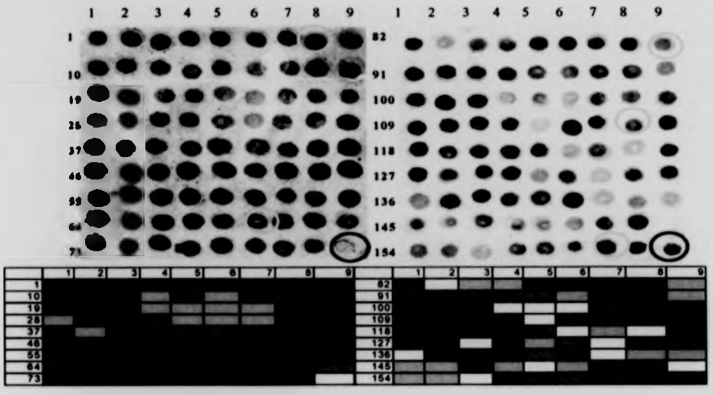


Figure 34
RH2 a

Figure 35
RH2 b

Figure 5.9

Systematic substitution in RH2.

Circles in yellow indicate the original sequence of the mimotope; red circles represent the negative control (unrelated sequence)

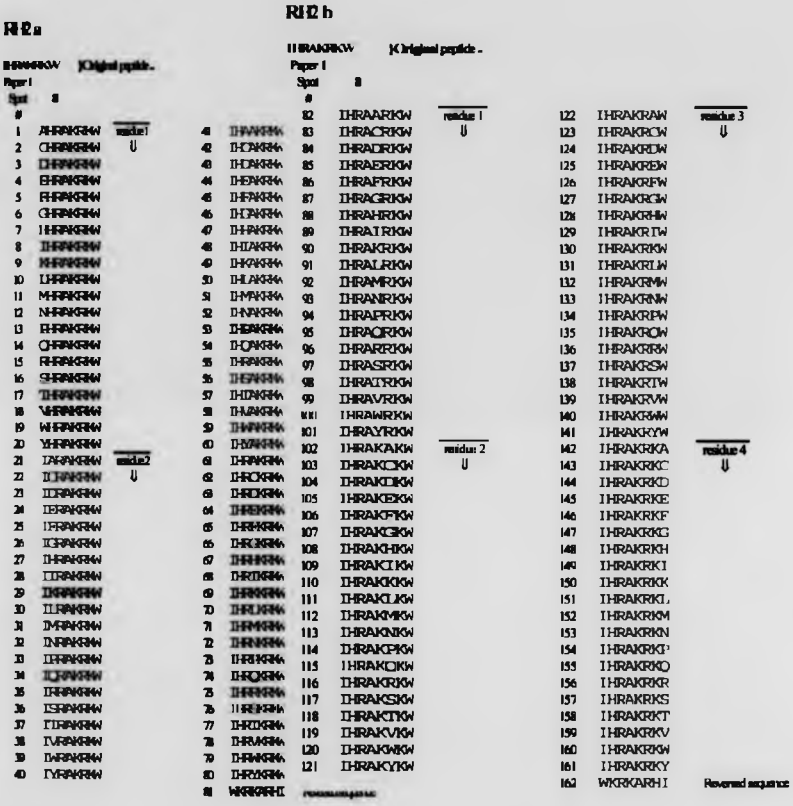


Figure 5.10

The sequences of the original and the substituted peptides in mimotope RH2

RH3

Replacement of valine1 by histidine (peptide 7), asparagine (peptide 12), serine (peptide 16) resulted in the enhancement of the binding of RF2 to the newly RH3 generated peptides. It should be noted, however, that when valine1 was substituted by any other amino acid, the binding to RF2 was inhibited. Substitutions of lysine2 in RH3 resulted in enhancement of the binding only when it was replaced with phenylalanine (peptide 25), or tryptophan (peptide 39). All other substitutions in position 2 either reduced or blocked the binding of RF2 to mimotope RH3.

At position 3 (proline), substitution with either aspartic acid (peptide 43), methionine (peptide 51) or asparagine (peptide 52) resulted in an increased binding by RF2. All other substitutions at that position either blocked or decreased the binding. Any amino acid substitutions at lysine4 reduced the binding of RF2 to RH3 with the exception that replacement of lysine4 with threonine (peptide 77) enhanced the binding between RF2 and the generated peptide. Peptides generated from substitutions made between positions 5 to position 8 were excluded from the final analysis because a possible technical fault in the synthesis process.

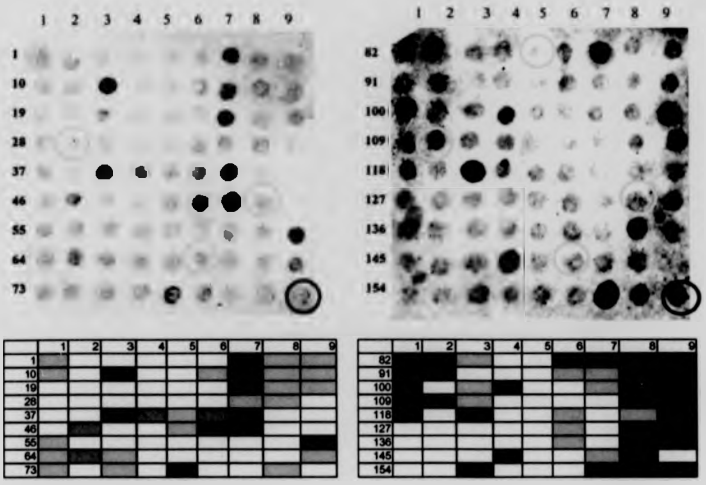


Figure.5.11

Systematic substitution in RH3.

Circles in yellow indicate the original sequence of the mimotope; red circles represent the negative control (unrelated sequence)

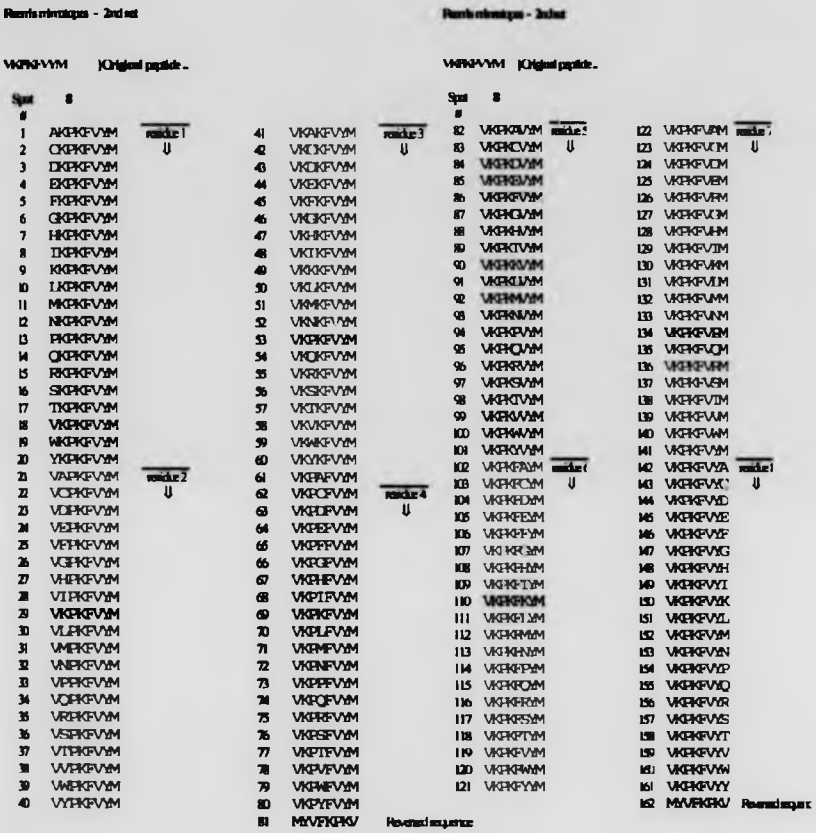


Figure 5.12

The sequences of the original and the substituted peptides in mimotope RH3

Original sequence	Substituted peptide sequence
RH1-Original sequence YPKRKHAD	RH1 (Substituted peptide sequence)
	RH1a YWKRKHAD
	RH1b YYKRKHAD
	RH1c YPKRKSAD
RH2-Original sequence IHRAKRKW	RH2 -Substituted peptide sequence)
	RH2a KHRAKRKW
	RH2b IHRAKAKW
	RH2c IHRAKCKW
RH3-Original sequence VKPKFVYM	RH3 (Substituted peptide sequence)
	RH3a VKDKFVYM
	RH3b VKNKFVYM
	RH3c VKPKFWYM

Table 5.3

The sequence of the mimotopes that showed increased reactivity with RF2 as a result of amino acids substitutions.

5.2.9 Discussion

The reactivity of the panel of nine adult human sera with the two mimotopes (M1, and M2) recognized by murine (Mab 19) was assessed by ELISA. The results showed that 5 of the 9 sera bound to M1 with OD value that was 2 times the cut off (0.2). Sera from all the 9 adult sera recognized M2 with significantly higher OD values than that seen with the binding to M1. The reactivity of all the 9 human sera with M2 ranged between 3 times the cut off and 5 times the cut off (0.6-1 OD A_{490nm}). Suggesting that mimotope M2 may represent a potential candidate epitope for inclusion in a vaccine. This possibility was investigated by experiments described in Chapter 6.

The nine adult human sera were also used in ELISA assays to determine their ability to react with mimotopes RH1, RH2, and RH3 recognized by the human monoclonal anti-RSV antibody (RF2). Two of the human adult sera bound mimotope RH2 with an OD 2 times the cut off and one adult serum bound to the mimotope RH1 with a value higher than cut off at 1/200 dilution. None of the adult human sera bound to mimotope RH3 at a value 2 times the cut off. Work described in Chapter 6 tests the immunogenicity of the three mimotopes.

Analysis of the binding the adult sera to the mimotopes showed that mimotopes M1, M2, RH1, and RH2 could represent epitopes that may have importance in generating a protective antibody response. However, when the reactivity of RF2 to the detected mimotopes was tested in ELISA no binding was detectable. This is possibly due to the conformation they adopt on binding to the solid phase in the ELISA plates. In order to improve the binding of RF2 to the identified mimotopes by presenting them in a more appropriate conformation to RF2, they were synthesized in the SPOTs system.

The substitution of amino acids in the sequences of the detected mimotopes was performed in two stages. In the first stage, each amino acid in the 8-mer peptide

sequence was replaced with glycine. In the second stage, every amino acid in the 8-mer sequence was systematically replaced by all 20 possible amino acid substitutions. In RH1 the results showed that proline 2 is potentially an important contact residue in the RH1 sequence for the binding by RF2, since replacing proline 2 with either aspartic acid (peptide 23), glutamic acid (peptide 24), or asparagine (peptide 32) resulted in inhibition of binding. Replacement of histidine 6 with glycine (peptide 107) resulted in an enhancement of the binding of RF2 to the peptide. The substitution of tyrosine 1 also resulted in an increase in the binding by RF2 when it was replaced with cysteine (peptide 2), lysine (peptide 10), tyrosine (peptide 17), and valine (peptide 18).

Data from the amino acid substitution in RH2 indicated that that lysine 7 is a potential contact residue for RH2 as the replacement of this amino acid inhibited the binding of RF2 to peptides (123,125,133, and 136). The binding of RF2 was also blocked in the following peptides (83, 104, 105, 106, 113, 142, 149, 153, 156) which highlight that lysine 5; arginine 6, lysine 7, and tryptophan 8 may be contact residues for RH2 although the visual inspection could falsely show improvement of the binding of RF2 to several newly generated peptides (data summarized in Table 5.3).

Data obtained from substituting amino acids in the RH3 mimotope sequence indicated that residues 1-4 may be important contact residues where only in few peptides there was an increase in the binding. Unfortunately due to a technical mistake during the synthesis, all the substitutions made from position 5-8 were excluded from the analysis of the data and therefore nothing can be concluded here about these substitutions.

Although the results from the experiments described here support the suggestion that amino acid substitution in the mimotopes could enhance their potential as vaccine candidates, difficulties exist in assessing the data. The observation that the

binding results with certain peptides were not consistent is a particular problem. These inconsistencies could have arisen from the relative insensitivity of the assay. However, the synthesis of so many peptides is very labour-intensive and the possibility of errors in the procedure cannot be ruled out. The data generated from these studies are summarized in Tables 5.4; 5.5 and 5.6.

Following the successful identification of mimotopes bound by the human monoclonal anti-RSV antibody RF2, it was planned to make substitution of each of the amino acids in their 8-mer sequence to look for enhancement binding by RF2. Amino acid substitutions of the mimotopes in the SPOTs system indicated that such changes affected binding by the monoclonal antibody and this was observed by an increase in density of the colour. (As measured by increased colour in the SPOTs system) (Table 5.3). It is important to know whether this increase in binding is a reflection of an increase in antibody affinity. Several methods are available for the measurement of antibody affinity. In each method, a system is set in which antigen and antibody are allowed to come to equilibrium. Data from these systems are analyzed by applying the Law of Mass Action to give the equilibrium (affinity) constant K. For this work, it was proposed to use the technique of surface plasmon resonance (SPR) to determine the kinetics and affinity constant of the interaction of Mab RF with peptide mimotopes using the Biacore. The mimotopes were to be mono-biotinylated, and will be bound to streptavidin (Sigma) immobilized onto the CM5 sensor chip on which the legends would be coupled by streptavidin –biotin coupling.

However, although preliminary experiments were preformed, time did not allow sufficient work to be performed to merit inclusion in this chapter.

Mimotope	Peptide number	(Amino acid + original position	New amino acid substituted
RH2	83	Lysine 5	Cysteine
	104	arginine 6	aspartic acid
	105	~	glutamic acid
	106	~	Phenylalanine
	113	~	Asparagine
	123	Lysine 7	Cysteine
	125	~	glutamic acid
	133	~	Asparagine
	136	~	arginine
	142	Tryptophan 8	Alanine
	149		Isoleucine
	153		Asparagine
	156		Arginine

Table 5.3

Summary of amino acids substitutions in RH2

Mimotope sequence									
Amino acids Used		Y	P	K	R	K	H	A	D
	A			↓	↓				
	C			↓			↓	↓	↓
	D	↓				↓	↓	↓	
	E						↓	↓	
	F						↓	↓	
	G				↓		↓	↓	
	H			↓	↓	↓			↓
	I			↓	↓	↓			
	K				↓		↓		
	L				↓	↓	↓	↓	
	M	↓			↓		↓	↓	
	N	↓			↓		↓	↓	
	P			↓			↓	↓	
	Q			↓			↓	↓	
	R			↓			↑ H	↓	
	S						↑ H		
	T	↑		↓			X		
	V			↓			↓	↓	
	W						↓		
	Y								

Table 5.4

Summary of the effects of amino acid substitutions in mimotope RH1 using the total array software.

(↑) means enhancement in the binding by RF2. (↓) is reduced binding compared to the original mimotope. . X -indicate a total block of the binding .No sign means that the volume was equal to the average volume calculated. H is high.

	Mimotope sequence								
Amino acids Used		I	H	R	A	K	R	K	W
	A		↓	↓	↓				
	C		↓	↓					
	D		↓	↓					
	E		↓	↓					
	F		↓						
	G		↓						
	H								
	I		↓						
	K		↓	↓					
	L								
	M			↓					
	N	↓	↓	↓	↓				
	P	↓	↓						
	Q	↓	↓						
	R	↓	↓						
	S	↓							
	T								
	V		↓						
	W						↓		
	Y								

Table 5.5

Summary of the effects of amino acid substitutions in mimotope RH2 using the total array software.

(↑) means enhancement in the binding by RF2. (↓) is reduced binding compared to the original mimotope. . X -indicate a total block of the binding .No sign means that the volume was equal to the average volume calculated.

Amino acids Used		V	K	P	K	F	V	Y	M
	A			↓	↓				
	C	X	X	↑	↑				
	D	X	X	↑	↓				
	E	X	X	X	↑				
	F	X	↑	X	↓				
	G	X		X	↓				
	H			↑	↓				
	I	↑	↓	X	↓				
	K			X	↓				
	L	X	↓	X	↓				
	M	X	X	↑	↑				
	N	↑ VH	X	↑	↓				
	P	X	↓		↓				
	Q	X	↓	↓	↓				
	R	X	↓	↓	↓				
	S	↑	X	↓	↑				
	T	↑	↓	↓					
	V		X	↓	↓				
	W	X	↑	↓	↓				
	Y	X	↑	↓					

Table 5.6

Summary of the effects of amino acid substitutions in mimotope RH3 using the total array software.

(↑) means enhancement in the binding by RF2. (↓) is reduced binding compared to the original mimotope. X -indicate a total block of the binding .No sign means that the volume was equal to the average volume calculated.

CHAPTER 6

ASSESSMENT OF THE IMMUNOGENICITY OF LINEAR AND CONFORMATIONAL EPITOPES FROM THE F PROTEIN OF RSV

6.1 Introduction

Although the severity of RSV disease declines with repeated infection, previous infection with the virus does not prevent disease and immunity remains incomplete. Virus- specific neutralizing antibodies, when administered prophylactically have been shown to protect against infection and disease in the mouse model of RSV and in infants (Prince, Horswood et al. 1985). In animal models, a vaccine inducing neutralizing antibodies provided long term immunity (Prince, Horswood et al. 1985). Also in early infancy, levels of maternally–derived antibodies correlated with protection from RSV infection (Englund 1994). Therefore, developing an epitope-based synthetic peptide vaccine that includes appropriate epitopes to induce virus-neutralizing antibodies against RSV would be a valid approach to developing a potential vaccine for human use. The conserved F protein is one of the two major protective antigens of RSV (the other is the G protein) and induces protective antibody responses (Wertz, Stott et al. 1987). Therefore identification of epitopes defined by such protective antibodies would provide important information for the design of an effective epitope-based vaccine.

The potential of synthetic peptides as vaccines has been recognized for over 30 years. Anderer (1967) was the first to show that synthetic peptides can induce antibodies with virus-neutralizing properties. Most synthetic peptides are good immunogens when presented appropriately to the immune system, and there is usually

no difficulty in raising antibodies against them by standard immunization procedures. For example, work by Muller (Muller, Plaue et al. 1990) showed that immunization with free peptides of a length of 14-25 residues could lead to moderate antibody response after 2-3 injections. However in order to obtain anti-sera of adequate titre, 4-5 injections were usually necessary.

Synthetic peptide vaccines have several potential advantages including:

- They are inexpensive to produce;
- They are easy to manufacture and purify;
- They are stable and thus there is no need for cold chain maintenance;
- They are not of a biological nature and are thus non –infectious;
- They are of a chemical nature and thus have genetic and structural stability.

Experimental peptide-based vaccines have been widely studied in the past. The effectiveness of linear peptides to induce humoral immune response and significant protection against infection in animals has been documented in measles. Partidos et al. showed that a chimeric peptide representing two copies of a T-cell epitope from the F fusion protein of measles virus (aa 288-304) coupled via a glycine-glycine spacer to a B-cell epitope also from F protein (aa 404-414) was immunogenic in several inbred strains of mice. This peptide (TTB) induced high titres of peptide-and virus-specific antibodies (Partidos, Stanley et al. 1991).

Obeid et al.1995 tested the ability of immunization with TTB to induce a protective immune response against intra-cerebral challenge with neuro-adapted strains of MV and canine distemper virus (CDV) in mice and showed that TTB induced a protective response against challenge with both MV and CDV. A significant reduction in mortality was demonstrated together with the absence of acute clinical encephalitis and histological absence of acute encephalitis. In TTB-immune mice, challenged with MV, a significant reduction in viral load in the brains was seen in

comparison to that in the non-immunized but challenged control mice. In a subsequent study, a chimeric peptide composed of one copy of the T-cell epitope and one copy of the B-cell epitope (TB), failed to induce a protective response. It was suggested that the differences in protective efficacy observed following immunization could be due to higher affinity of the antibody being induced by the TTB peptide than that produced following immunity with the TB peptide. Also, differences in the immunoglobulin G subclass of the anti-peptide antibody responses were observed, which could also have contributed to the differences in protection seen (Obeid, Partidos et al. 1995).

In a later study by the same group Partidos et al.1997, passive transfer of anti-TTB antibody to naïve animals protected against MV-induced encephalitis. Immunization with the TTB peptide was shown to induce high affinity antibodies against the B-cell epitope 404-414. Immunization with the TT peptide covalently linked to a CTL epitope (aa52-aa60) from the MV nucleo-protein, induced strong CTL responses in mice (Partidos et al., 1997). Further work from the same group using sera from humans containing anti-MV antibodies (Atabani et al, 1997) identified an antigenic region from the F protein representing peptide aa 388–aa 402 (p32) that was located within the highly conserved, cysteine-rich domain of the F protein of paramyxoviruses. The entire 15-amino-acid sequence was required for high-affinity interaction with anti-MV antibodies. The peptide p32 was shown to be immunogenic in two strains of mice and induced anti-peptide antibodies that cross-reacted with MV and neutralized the virus *in vitro*. In addition, antibodies to p32 conferred significant protection against MV-induced encephalitis when passively transferred to naive mice. Data from Obied et al. and those from other groups highlight that synthetic peptide vaccines have potential for use in young infants to overcome the suppressive effect of maternally-transmitted anti-RSV antibodies. A cocktail of peptides from the H protein

of (MV) was able to induce anti-MV responses in neonates even in the presence of maternally-derived anti-MV antibodies (Obeid and Steward 1994).

These studies, using defined peptides, whether linked to a carrier or used together with an adjuvant, highlight the ability of small linear peptides to be immunogenic. However, it is generally accepted that B-cell epitopes that induce protective antibody responses are usually conformational rather than linear in nature and thus in the work described in this chapter, an attempt will be made to mimic such a structure in the F protein of RSV. The immunogenicity of these mimotopes will be assessed together with the immunogenicity of linear B-cells epitopes were identified using human sera.

In Chapters 3 and 4, several linear B-cell epitopes were identified using the sera from 9 adults and 49 infants. Peptides 4, 8, 11, 14, 18, 24, 36, 41 were strongly recognized by the 9 adult sera. Analysis, of the binding of peptides by sera from the babies at various stages of infection showed that in sera from children with pre- and first infection samples only, peptide 4 was recognized by 45% of the pre -infection sera and this value dropped to 18% in the primary sera after infection. Peptide 48 was also recognized by 46% of the sera and peptide 14 was recognized by 72% in both pre -infection and first infection. In samples from children with both primary and secondary infection. Peptides 19, 20, 32 and 48 were recognized by 50-60% of the secondary infection sera. In children with samples of 2 post infection sera with RSV Peptide 14 was recognized by 66% of the pre-infection serum samples, by 81.4% of the sera following the primary infection with the virus and by 73% of the sera following the secondary infection with the virus. The pattern of peptide recognition varied according to the state of exposure to the virus but in all children, sera obtained following the second infection showed the highest levels of peptide binding and peptides 4 and 14 were recognized strongly by sera from both adults and children.

In the work described in Chapter 5, a human monoclonal antibody (RF2) that recognizes a conformational epitope on the fusion protein of HRSV was used to screen a combinatorial peptide library to identify mimotopes of this conformational epitope. These mimoptopes were identified (RH1, RH2, RH3). On the basis of these results, both the linear B-cell epitopes and the mimotopes identified were used *in vivo* experiments to assess their ability to induce anti-peptide antibodies and to determine if these antibodies cross-react with RSV.

6.2 Results

6.2.1 Antibody response following immunization with linear peptides

The immunogenicity of the linear peptides: 4, 8, 11, 14, 19, 20, 24, 32, 36, 41, and 48 was tested individually in groups of four of BALB/c mice. In all experiments, mice were sub-cutaneously co-immunised with 50μg of the linear peptide and 50μg of TTB –peptide (to provide T-cell help) in complete Freund's adjuvant (CFA).

Previously, Partidos et al. had shown that simple co-immunization of a non-immunogenic B-cell epitope with a peptide representing a Th epitope can induce antibody to the B-cell epitope. The co-immunization did not require covalent linkage of the two peptides and this approach has being used frequently in the lab therefore was adopted in this study (Partidos, Obeid et al. 1992). The animals were boosted by the same route with the same dose of the peptide alone inincomplete Freund's adjuvant (IFA) three weeks later. Sera from the animals were collected from each mouse before boosting and then 20 days after the boost. The ability of each peptide to induce antibody was assessed by direct peptide ELISA as described previously. Sera from the animals from each group were obtained before the start of the experiment and used as a negative control for reactivity with both RSV Ag and the tested peptide in every assay. In addition, two positive controls were included: 1) sera from mice that had been intranasally immunised and challenged with RSV, and 2) RF2, the human monoclonal anti -RSV F antibody (see Chapter 2).

In preliminary studies, mice were immunised with peptides 11, 24, and 36, without including TTB as a source of T-cell help. Peptide 11 induced an antibody response (Figure 6.1) with titre of 2.2 for sera from mouse 1 and 2, and 1.9 for serum from mouse 3. No response was detected in any mouse even following boosting with the peptides 24, and 36 (data not shown). Therefore, in all subsequent immunisations,

the individual peptides were co-immunised with peptide TTB for the induction of T-cell help.

Antibody response following immunization with peptide 4

IgG antibodies to peptide 4 were not detectable in the sera of the mice either after the first immunisation or after the boost, nor was there detectable reactivity with the RSV antigen either before or after the boost (data not shown).

Antibody response following immunization with peptide 8

Detectable levels of anti-peptide antibody were observed against peptide 8 at a dilution of 1/10 in all mice and sera from two mice 1, and 4, had titres of $\log_{10}1.6$ and 2.2, respectively (Figure 6.2). However, none of the sera from the four animals immunised with peptide 8 reacted with RSV Ag on the plate.

Antibody response following immunization with peptide 11

Sera from three of the four immunized animals (2, 3, 4) showed antibody binding that was greater than the negative control at a dilution of 1/10, and two of the sera had titres of 2.5 and 2.8, respectively (Figure 6.3.a). However, none of the peptide-immunized mice produced antibodies that cross-reacted with RSV (Figure 6.3.b).

Antibody response following immunization with peptides 14/19/ 20/ and 32

None of the sera from immunized with the peptides 14, 19, 20, and 32 had detectable anti-peptide or anti-RSV responses even after boosting (data not shown).

Antibody response following immunisation with peptide 24

Mice immunised with peptide 24 did not produce detectable anti-peptide antibodies. Interestingly, sera from animals 2 and 4 showed cross reactivity with the RSV antigen with an OD value at a dilution of 1/10 that was twice that of the control negative serum (Figure 6.4).

Antibody response following immunisation with peptide 36

Sera from mice immunised with peptide 36 did not show reactivity against the immunising peptide either from the second bleed (data not shown) or in the final bleed. However, as with sera from peptide 24 immunised mice, sera from 3 mice immunised and boosted with peptide 36 (animals 1, 2,4) cross -reacted with the RSV antigen with an OD value at a dilution of 1/10 that was twice that of the control negative serum (Figure 6.5).

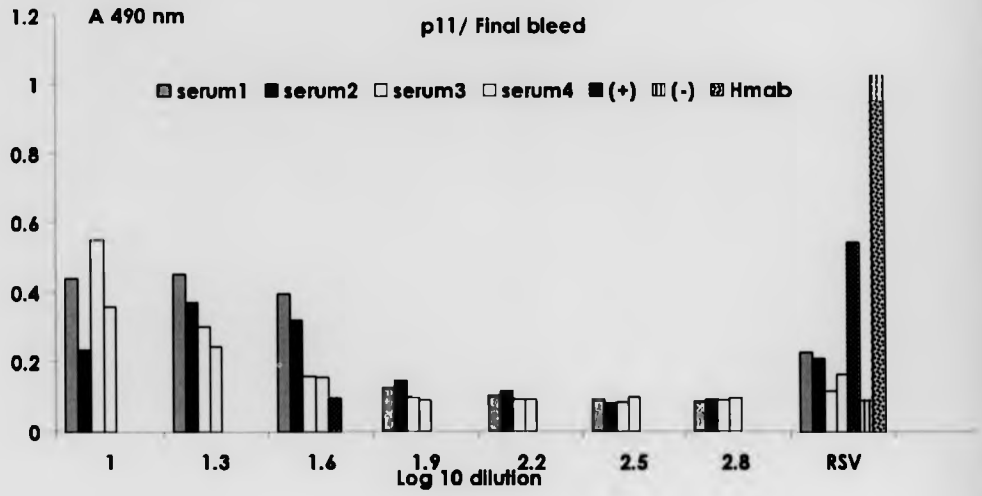


Figure 6.1a

Immunoreactivity of sera from mice immunized with peptide 11 following immunization in the absence of TTB as source of T-cell help. The OD shown is the maen of 2 OD readings.

Antibody binding is shown as OD values at A_{490}. At various $\log_{10}$ dilutions of sera from individual mice. Data shown are from the final bleed following boosting with the peptide only.(+) is the positive control used which is sera from mice challenged three times with RSV intranasally/(-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

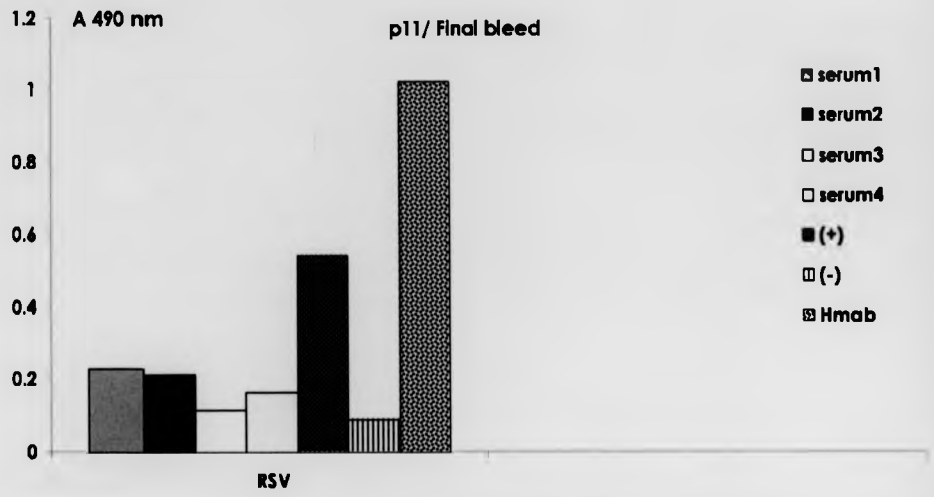


Figure 6.1 b

Reactivity of sera at 1:10 dilution with RSV antigen in the plate. The OD shown is the maen of 2 OD readings.

Data shown are from the final bleed following boosting with the peptide only.

(+) is the positive control pooled serum from mice challenged three times with RSV intranasally/. (-) is the negative control serum

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

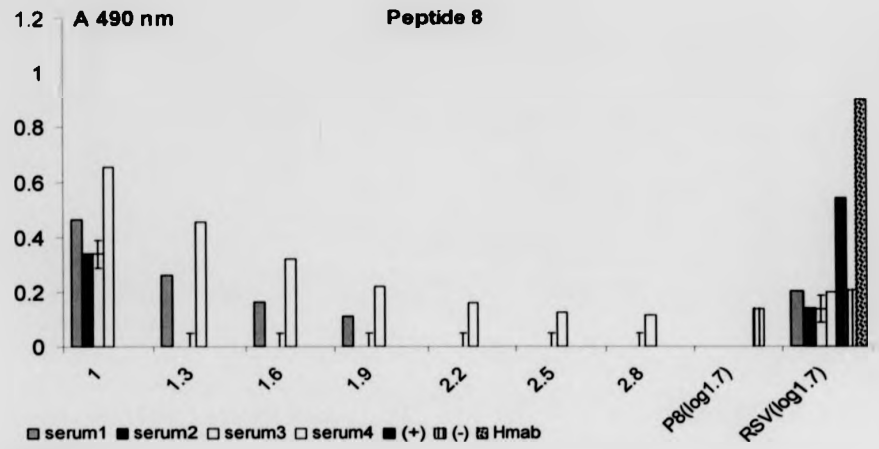


Figure 6.2

Reactivity of sera from mice immunized with peptide 8 against peptide 8 in ELISA. The OD shown is the maen of 2 OD readings.

Antibody binding is shown as OD values at A 490 at various log 10 dilutions of sera from individual mice. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/(-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

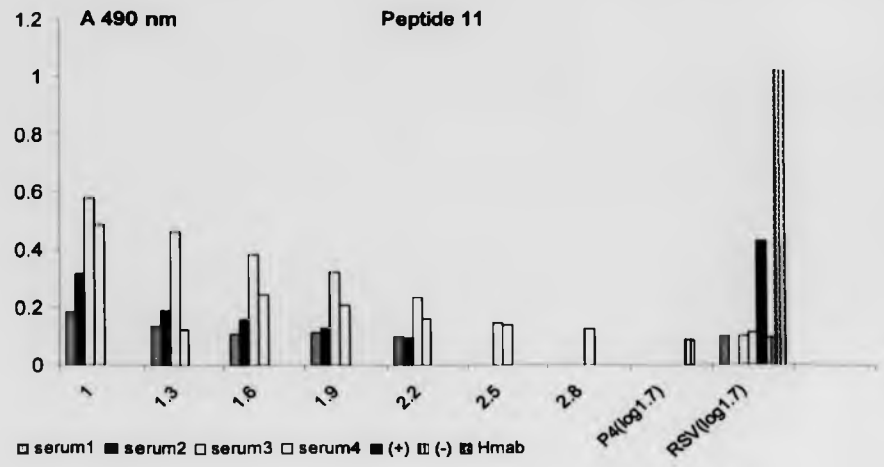


Figure 6.3.a

Reactivity of sera from mice immunized with peptide 11 against peptide 11. The OD shown is the maen of 2 OD readings.

Anti peptide antibody binding is shown as OD values at A_{490}. At various log_{10} dilutions of sera from individual mice. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

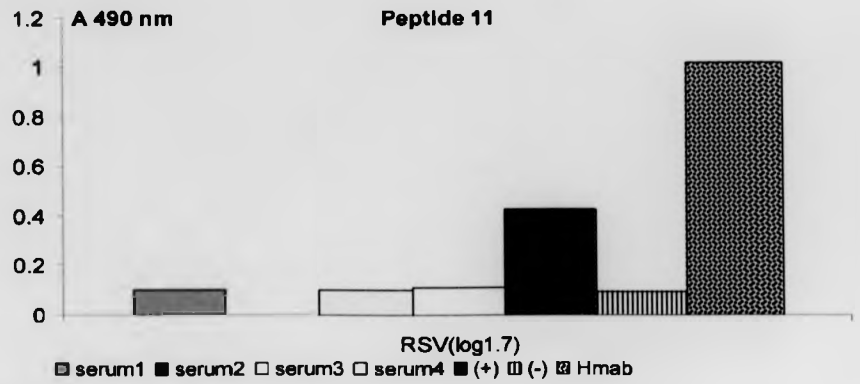


Figure 6.3.b

Reactivity of sera at 1:10 dilution with RSV antigen in the plate. The OD shown is the maen of 2 OD readings.

(+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

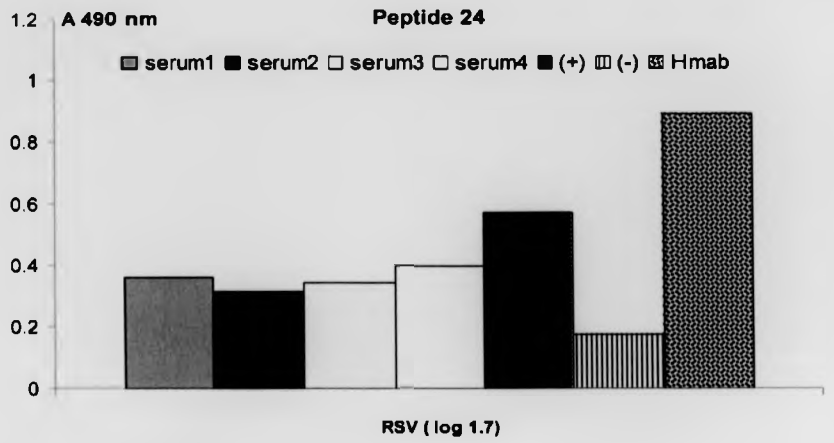


Figure 6.4

Reactivity of sera from mice immunized with peptide 24 against RSV antigen. The OD shown is the maen of 2 OD readings.

Antibody binding is shown as OD values at A_{490}. At a serum dilution of 1:10. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

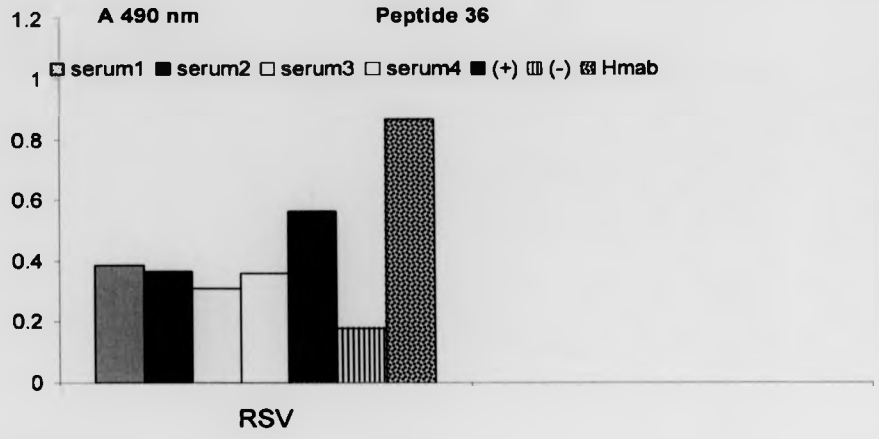


Figure 6.5

Reactivity of sera from mice immunized with peptide 36 against RSV antigen. The OD shown is the maen of 2 OD readings.

Antibody bindings shown as OD values at A_{490} at a 1:10 dilution of the sera. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

Antibody response following immunization with peptide 41

Mice immunized with peptide 41 did not produce detectable anti-peptide antibodies either following priming or boosting. However, sera from the final bleed of mouse 4 and mouse 1 showed reactivity with RSV antigen. The serum from animal number 4 bound RSV antigen with an OD value that was three times the control negative serum, and serum from mouse 1 bound the RSV antigen with an OD value twice that of the negative control (Figure 6.6).

Antibody response following immunization with peptide 48

As with mice immunized with peptide 24, 36, and 41, mice immunized with peptide 48 did not produce detectable anti-peptide antibodies either following priming or boosting. However serum from the final bleed of one mouse (number 1) immunized with peptide 48 showed reactivity with RSV antigen with an OD value that was three times the negative control value (Figure 6.7).

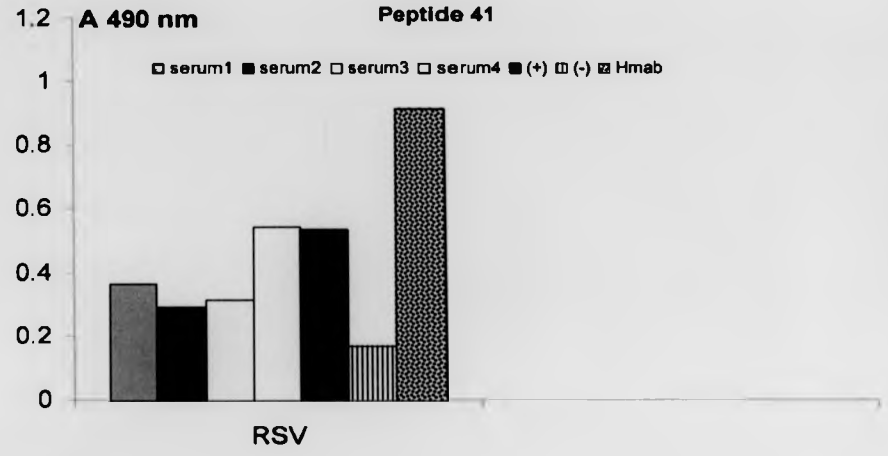


Figure 6.6

Reactivity of sera from mice immunized with peptide 41 against RSV antigen. The OD shown is the maen of 2 OD readings.

Antibody binding is shown as OD values at A_{490} at a 1:10 serum dilution. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

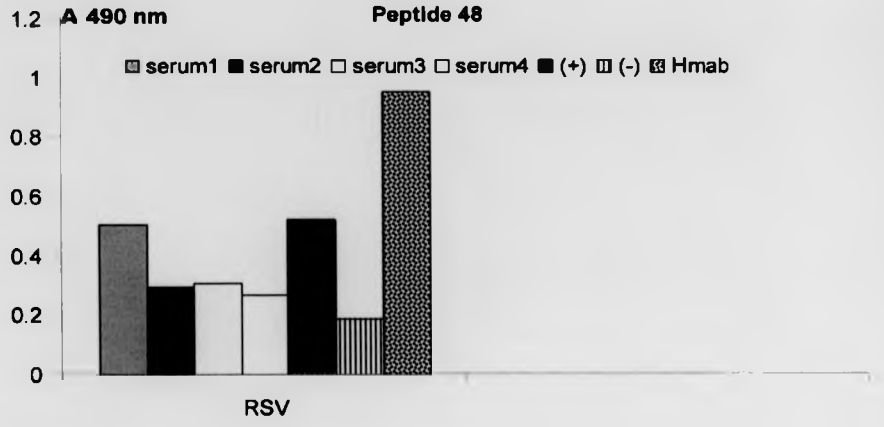


Figure 6.8

Immunoreactivity of sera from mice immunized with peptide 48 against RSV. The OD shown is the maen of 2 OD readings.

Antibody binding is shown as OD values at A_{490} at a 1:10 serum dilution. (+) is the positive control used which is sera from mice challenged three times with RSV intranasally/ (-) negative sera

Represents the HMab RF2 which is used as a control for the assay for binding to RSV

6.2.2 Competitive inhibition assay using mouse anti-peptide 8 sera.

In order to verify that mouse anti-peptide 8 antibody indeed recognized the peptide that induced it, an inhibition assay was carried out. Plates were coated with peptide 8 overnight at a 5μg/ml as described in Chapter 2.

Half log peptide dilutions starting at 2mg/ml were prepared freshly and added to a dilution of the mouse serum and incubated for 3 hours. The mixture was added to each well of the peptide coated plate. As shown in Figure 6.9, peptide 8 in solution inhibited the binding of anti-peptide 8 antibodies to peptide in the ELISA; a decrease of 0.2 OD was observed with serum from animal 1, and a decrease of 0.1 OD with sera from mice 2 and 3.

6.2.3 Competitive inhibition assay using anti-peptide 11 sera.

A competitive inhibition assay was carried out using the anti-peptide antibody induced by the immunization with peptide 11 as described above for peptide 8.

The presence of peptide 11 free in solution inhibited the binding of anti-peptide 11 antibody to peptide 11 in ELISA as shown in (Figure 6.10) ; a decrease of 0.2 OD was observed with serum from mouse 3, and a decrease of 0.1 OD from mouse 4 and the inhibition was is in a concentration-dependent in sera from both mice.

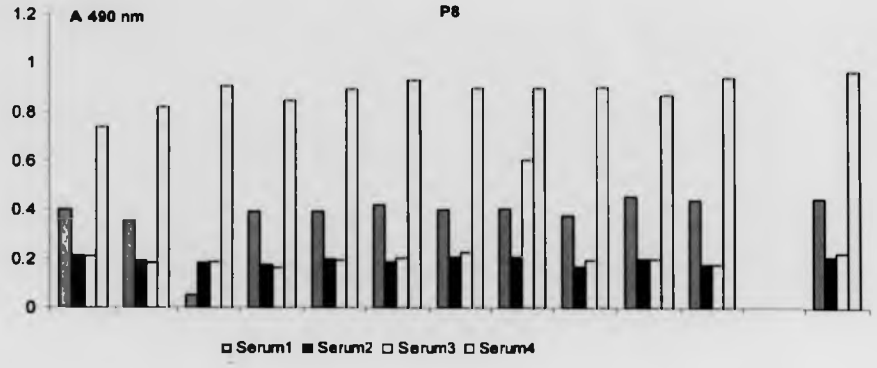


Figure 6.9

Competitive inhibition ELISA using peptide 8 and mouse anti-peptide sera. The OD shown is the maen of 2 OD readings.

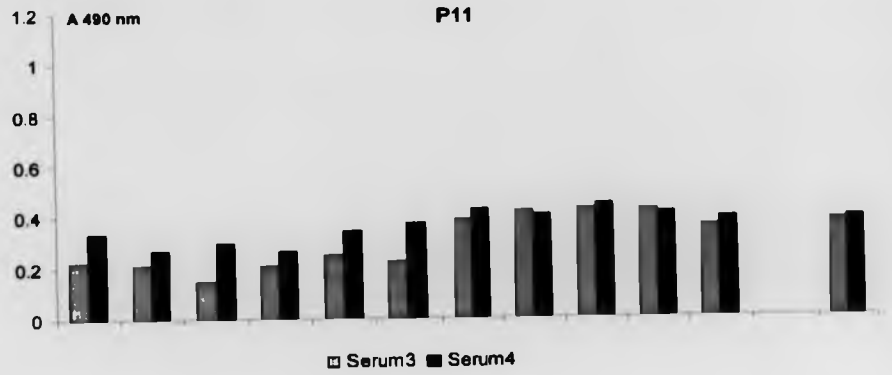


Figure 6.10

Competitive inhibition ELISA using peptide 11 and mouse anti-peptide sera. The OD shown is the maen of 2 OD readings.

6.2.4 Competitive inhibition assay using RSV antigen.

To determine of that the binding of the mouse anti-peptide serum with RSV in ELISA was specific for RSV, a competitive inhibition assay was carried out. Plates were coated with RSV Ag overnight at a 5μg/ml. Anti-peptide sera at a dilution of 1/50 (final 1/25) were mixed with half-log dilutions of the corresponding peptide starting at 2mg/ml, and added to the wells of RSV-coated ELISA. Plates were incubated for 3 hours. The anti-peptide sera were against peptide 24, 36, 41, and 48.

RSV inhibitions assay using anti-peptide 24 sera:

The presence of peptide 24 free in solution did not affect the RSV binding activity in sera from mice immunized with peptide 24 (mice1, and 2) to the RSV Ag in the ELISA (Figure 6.11), suggesting that the binding of the anti-peptide serum to RSV antigen was not related to the anti-peptide reactivity.

RSV inhibitions assay using anti- peptide 36 sera:

The RSV binding activity in sera from mice immunized with peptide 36 (1, 2, and 4) was not effected by the presence of the peptide free in solution (Figure 6.12). Again suggesting that the binding of the anti-peptide serum to RSV antigen was not related to the anti-peptide reactivity..

RSV inhibitions assay using anti- peptide 41 sera

The presence of peptide 41 free in solution inhibited the binding of anti-peptide antibody to the RSV Ag in the ELISA; a decrease of 0.2 OD reading binding was observed with sera from animal 4, and a decrease of 0.1 OD reading in the sera from animal 1. The inhibition observed was concentration-dependent in sera from both mice(Figure6.13).

RSV inhibitions assay using anti-peptide 48 sera

The binding of anti-peptide 48 serum to RSV was not affected by the presence of peptide 48 in free solution, which again suggest that the binding of the serum to RSV antigen was not related to the peptide binding reactivity (Figure 6.14).

These results suggest that of the peptides tested, only peptide 41 represents an epitope expressed on the RSV antigen to which the anti-peptide antibodies bound.

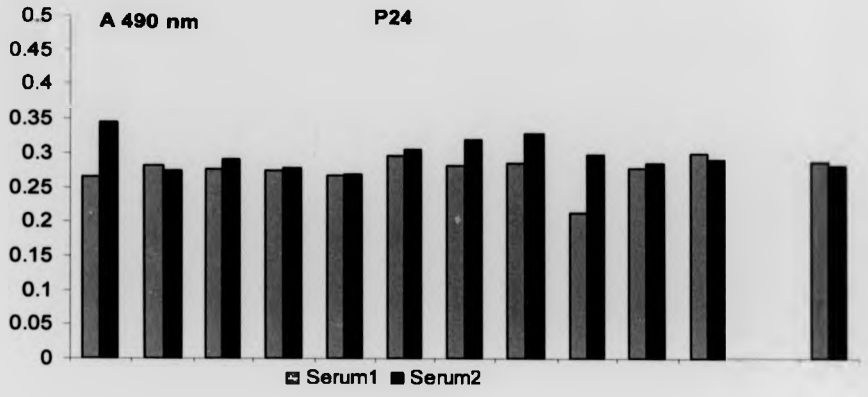


Figure 6.11

Competitive inhibition ELISA using peptide 24 and mouse anti-peptide sera against RSV Ag. The OD shown is the maen of 2 OD readings.

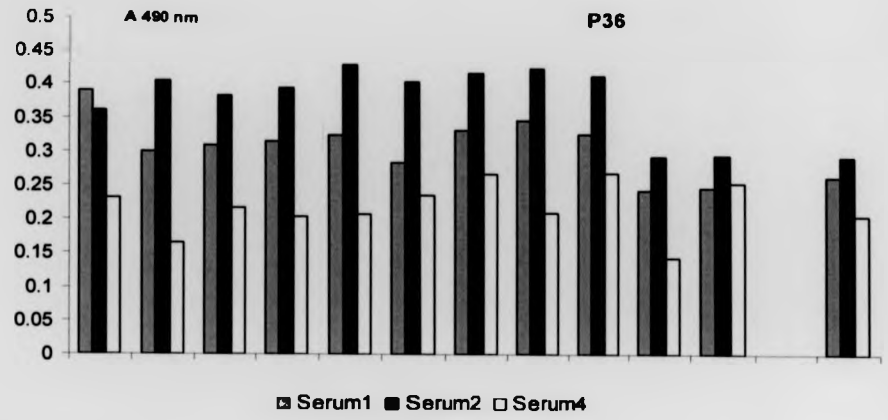


Figure 6.12

Competitive inhibition ELISA using peptide 36 and mouse anti-peptide sera against RSV Ag. The OD shown is the maen of 2 OD readings.

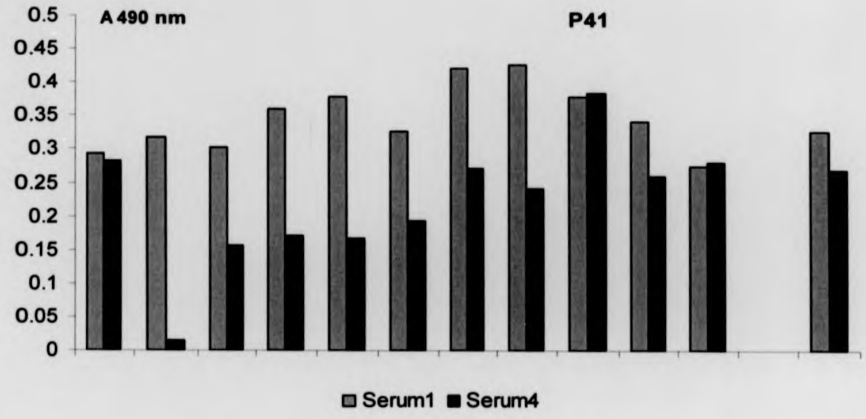


Figure 6.13

Competitive inhibition ELISA using peptide 41 and mouse anti-peptide sera against RSV Ag. The OD shown is the maen of 2 OD readings.

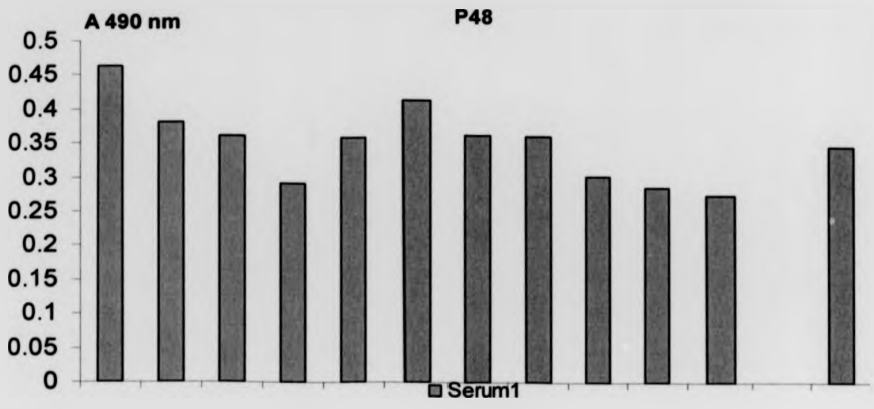


Figure 6.14

Competitive inhibition ELISA using peptide 48 and mouse anti-peptide sera against RSV Ag.The OD shown is the maen of 2 OD readings.

6.2.5 Antibody response following immunization with mimotopes

As described in Chapter 5, a human monoclonal antibody (RF2) that recognizes a conformational epitope on the fusion protein of HRSV was used to screen a solid phase combinatorial peptide library to identify mimotopes of this conformational epitope. Three potential sequences were identified:

RH1, 2, and 3.The immunogenicity of these mimotopes was tested individually in groups of four of female BALB/c mice. It was also hoped to be able to use the mimotopes in which the sequences had been modified by amino acid substitutions to give higher affinity binding by RF2. However lack of time and problems in the Biological Service Unit of the school seriously interfered with the planned experiments. BALB/c mice were sub-cutaneously immunized with 50μg of peptide in (CFA), and boosted three weeks later by the same route with the same dose of the same peptide in IFA. Sera from individual animals were collected prior to the boost and then 20 days after the boost. The ability of each peptide to induce an antibody response was assessed by direct peptide ELISA. Sera from the animals from each group were obtained just before the start of the experiment and used as a negative control for reactivity against both RSV Ag and the tested peptide in every assay. Two positive controls were included: (1) serum from mice intranasally immunized and challenged with RSV and (2) the RF2 Mab.

Since the mimotopes were only 8 amino acids long, simply attaching them to the ELISA plate (NUNC) would possibly induce a number of conformational changes that would influence antibody binding. Therefore, two other approaches were used to present the mimotope on the ELISA solid phase in addition to the direct coating of peptides (RH1, RH2, and RH3):

1. peptides were coupled to KLH as a carrier protein (KRH1,KRH2, and KRH3 - see Chapter 2 for details); and

2. Peptides were biotinylated and bound to streptavidin coated plates (bRH1, bRH2, and bRH3-see Chapter 2 for details).

6.2.5.1 Antibody binding to mimotopes coated directly on the ELISA plate:

RH1

Sera from all four RH-1 immunized animals showed antibody binding at a dilution of 1/10 that was greater than the negative controls and all the four sera had titres between 2.2 and 2.5 respectively (Figure 6.15).

RH2

Sera from all the four RH2 immunized animals showed antibody binding at a dilution of 1/10 that was 3 times the cut off with titres between 2.2 and 2.5 (Figure 6.16).

RH3

Sera from all four immunized mice (1, 2, 3, and 4) showed antibody binding at a dilution of 1/10 that was greater than the negative controls at a titre of 2.2 (Figure 6.17). It should be noted that although immunization with the mimotopes induced anti-mimotope antibodies, none of these reacted to RSV antigen (data not shown).

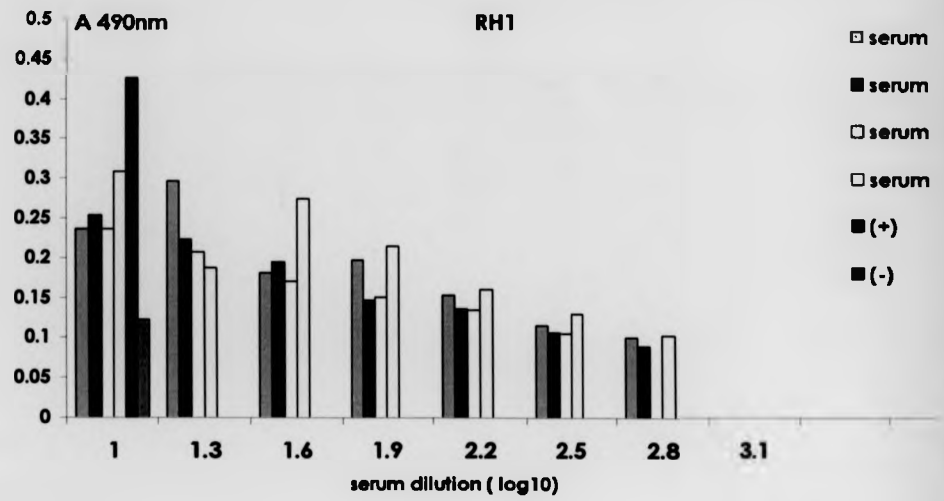


Figure.6.15

Antibody binding to mimotope RH1 coated directly on the ELISA plate. The OD shown is the maen of 2 OD readings.

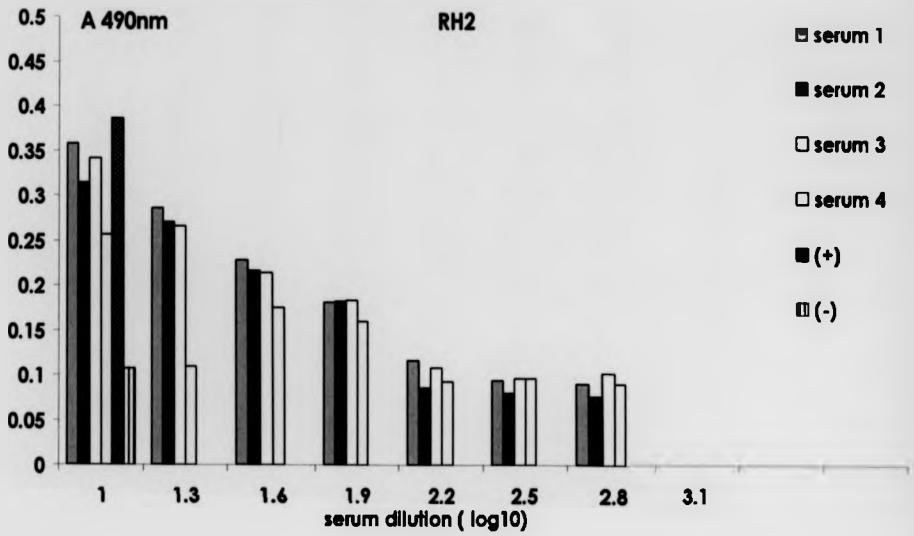


Figure 6.16

Antibody binding to mimotope RH2 coated directly on the ELISA plate. The OD shown is the maen of 2 OD readings.

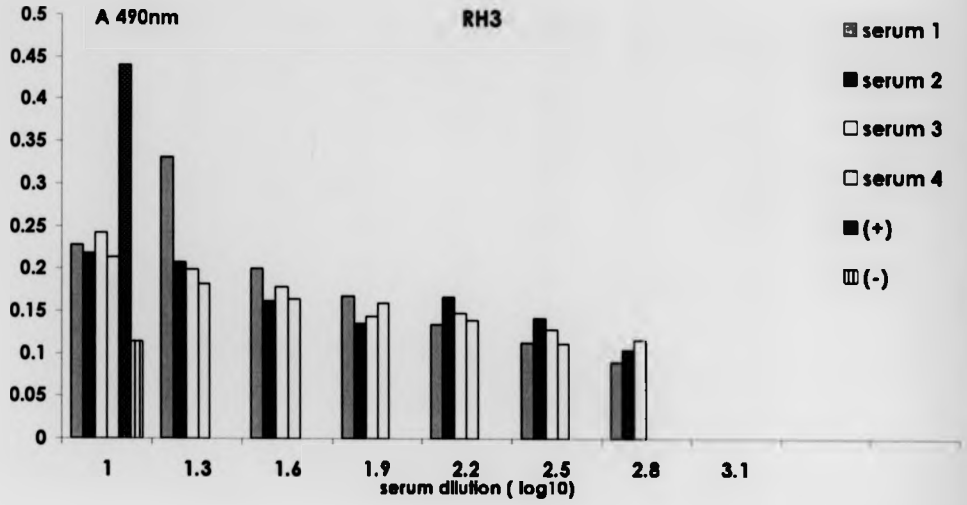


Figure 6.17

Antibody binding to mimotope RH3 coated directly on the ELISA plate. The OD shown is the maen of 2 OD readings.

6.2.5.5.2. Antibody binding response to mimotopes coupled to KLH on the ELISA plate.

KRH1

Sera from the three of the four RH1 immunized animals (1, 2, and 3) showed antibody binding that was 3 and 4 times greater than the negative controls with titres between 2.2 and 2.8(Figure 6.18).

KRH2

Sera from all RH2 immunized animals showed antibody binding at a dilution of 1/10 that was 3 times the cut off at 1/10 and with a titre of 2.2 (Figure 6.19).

KRH3

Sera from the four RH3 immunized animals (1, 2, 3 and 4) showed antibody binding at a dilution of 1/10 that was 3 times greater than the negative controls, with titres between 1.9 and 2.2 (Figure 6.20).

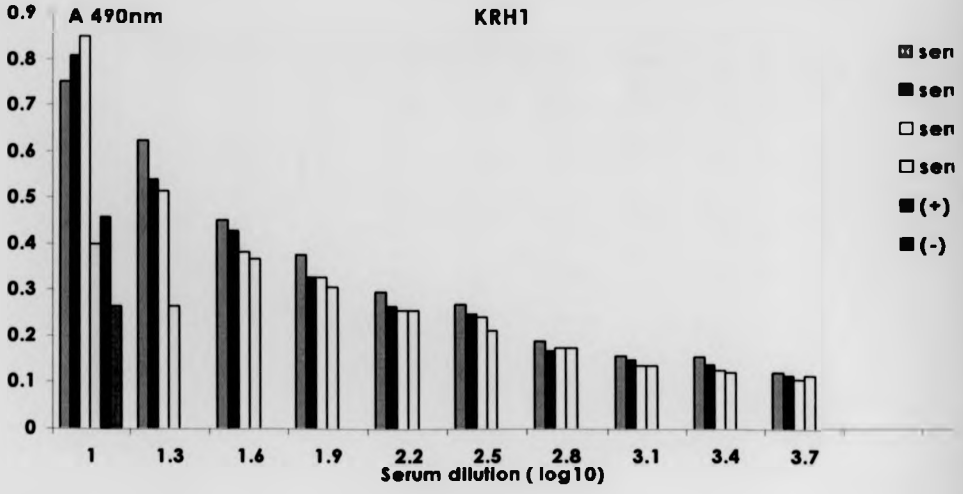


Figure.6.18

Antibody binding to mimotope RH1, coupled to KLH on the ELISA plate. The OD shown is the maen of 2 OD readings.

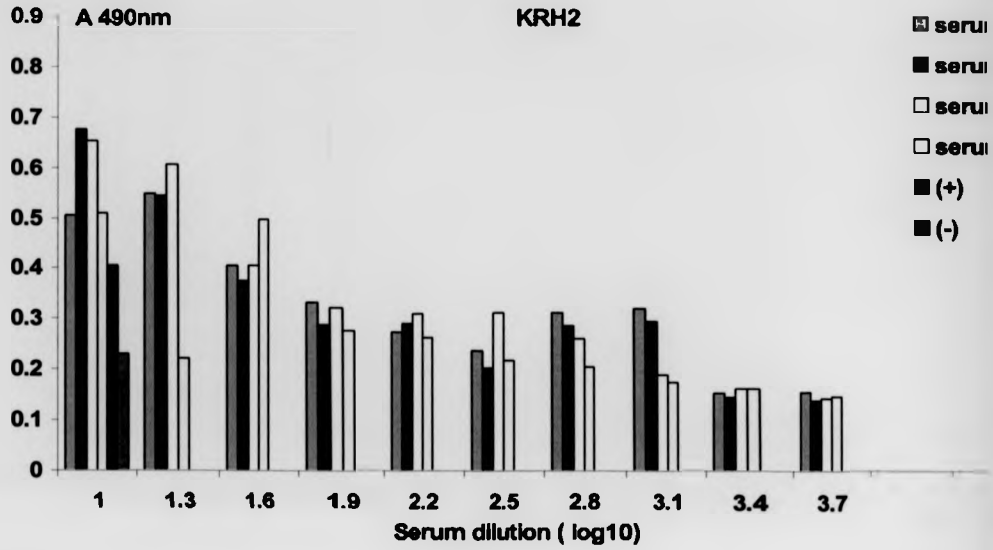


Figure.6.19

Antibody binding to mimotope RH2, coupled to KLH on the ELISA plate. The OD shown is the maen of 2 OD readings.

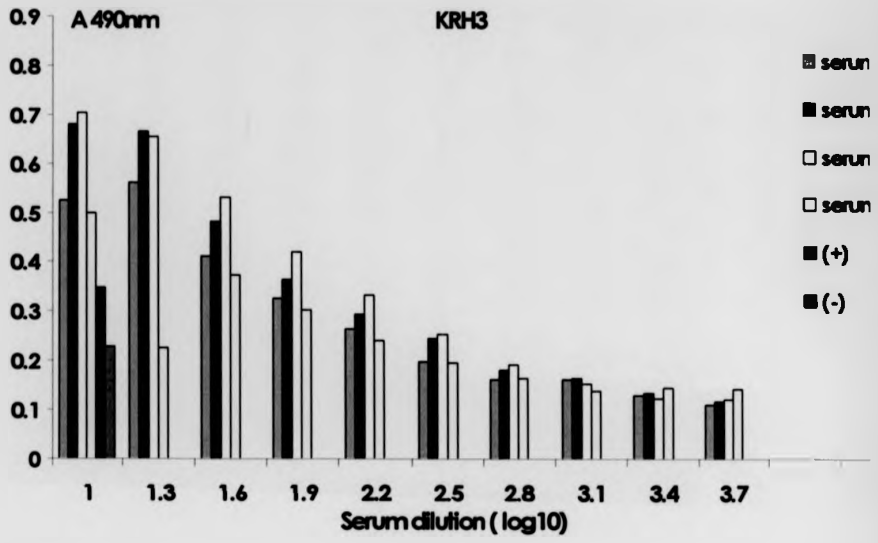


Figure.6.20

Antibody binding response to mimotope RH3, coupled to KLH on the ELISA plate. The OD shown is the maen of 2 OD readings.

6.2.5.3 Antibody binding to the biotinylated mimotopes coupled to Streptavidin - coated plates

bRH1

Sera from the four RH1 immunized mice showed antibody binding at a dilution of 1/10 that was 3 times the cut off. Three of the sera (mouse 1, 2, and 3) had high binding at a dilution of 1/20. The titre for all the immunized animals was 1.6 (Figure 6.21).

bRH2

Sera from all the four RH2 immunized animals (1,2, 3and 4) showed high level of anti-body binding at a dilution 1/10 that was 3, and 4 times greater than that of the negative controls, and four sera had titres between 1.9 and 2.2 (Figure 6.22).

bRH3

No detectable levels of anti-mimotope antibody were detected in any of the sera.

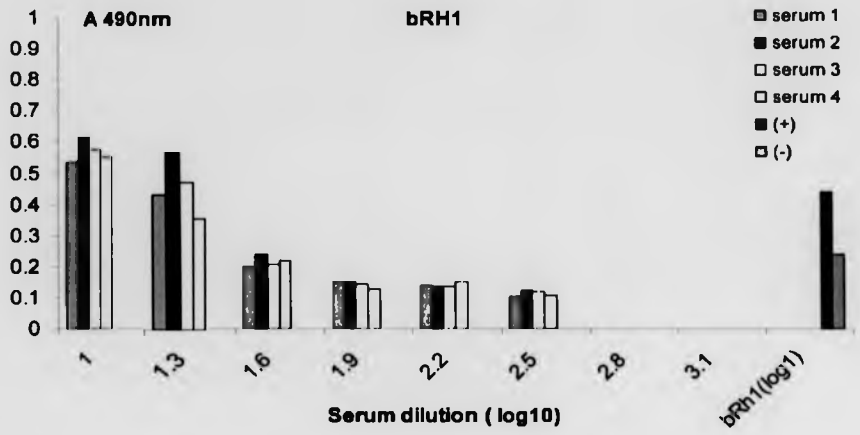


Figure 6.21

Antibody binding to the RH1 biotinylated mimotope coupled to Streptavidin coated plates. The OD shown is the maen of 2 OD readings.

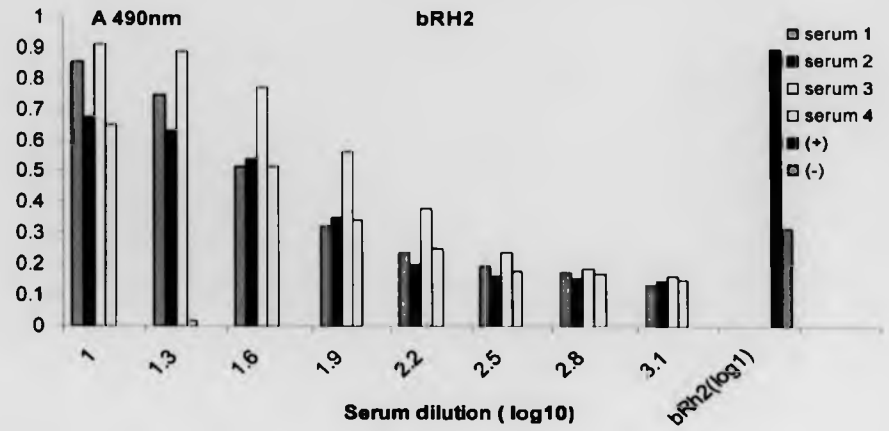


Figure.6.22

Antibody binding to the RH2 biotinylated mimotope coupled to Streptavidin coated plates. The OD shown is the maen of 2 OD readings.

6.2.6 The reactivity of the control sera to the mimotopes

Both the negative and the positive control varied in their response to the three mimotopes in their different form of presentation on the plates.

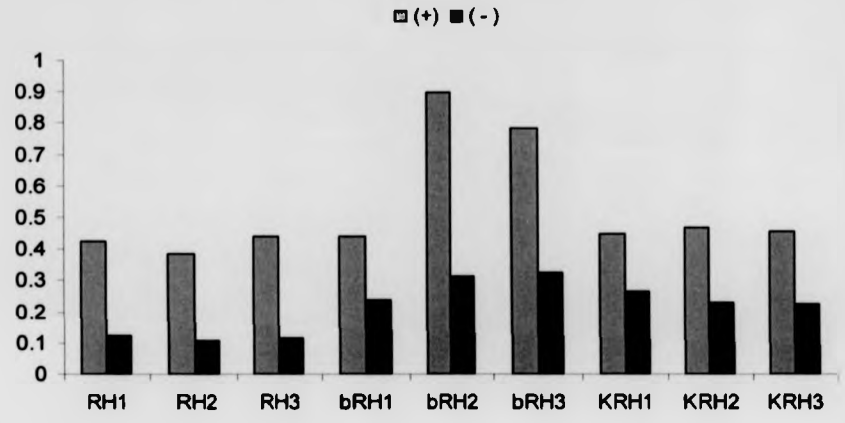


Figure.6.23

The reactivity of the control sera to the different mimotopes. The OD shown is the maen of 2 OD readings.

6.2.7 Discussion

Results from this study are summarized in Table 6.1: only 2 of the linear peptides (8 and 11) induced anti-peptide antibodies in the immunized mice. Sera from all the mice immunized with peptide 8 had anti-peptide antibodies, whereas only 2 of the animals immunized with peptide 11 showed anti-peptide antibody. None of the sera reacted with RSV antigen in an ELISA. When the competition inhibition ELISA was preformed with the sera from mice immunized with peptide 8, peptide 8 did not inhibit the binding of the anti-peptide antibody to P8 on the plate. Similarly, in the competition inhibition ELISA preformed with peptide 11, the binding only slightly inhibited with the peptide in free solution. The main purpose of raising anti-peptide antibodies is to obtain a reagent capable of reacting with the original protein; the success of an immunization procedure depends on the cross-reactive properties of the anti-serum and not solely on the titre of antibodies reactive with homologous peptide immunogen. Although none of the sera from mice immunized with peptide 8 or 11 reacted with RSV Ag on the plate, this could be due to the way the RSV Ag was presented on the solid phase. These results do not totally rule out the possibility that peptide 8 and peptide 11 could represent real epitopes on RSV.

Sera from mice immunized with peptide 24, 36, 41, and 48 did not produce detectable anti-peptide antibodies either following priming or boosting in ELISA. Interestingly, some of these sera reacted with RSV Ag on the plate. This could be a result of few factors;

1) The peptides could have assumed an inappropriate conformation on the ELISA plate and therefore the antibody could not recognize the shape of the epitope that induced it in the first place. 2) The lack of anti-peptide antibody could be also due to the lack of T-cell help even though there was a source of T-help presented in by co-

immunizing with peptide TTB. 3) The nature of the adjuvant used as well as the route of immunization plays an important role in the induction of a protective anti-peptide antibody response (Obeid, Stanley et al. 1996) and thus these factors need to be studied.

In the case of sera from peptide 24 immunized mice, it is possible that the sera recognized a linear epitope on the RSV Ag, it should be noted that peptide 24 has been identified as a human B-cell epitope (see Table 1.3). When a competitive inhibition ELISA was carried out using the sera from the mice immunized with peptide 24, 36, 41, and 48, the presence of these peptides in solution did not inhibit the binding of the sera to RSV Ag in the ELISA. Therefore, this suggests that these peptides do not mimic the structure in RSV to which the RSV binding activity was detected. Meanwhile, peptide 41 in free solution inhibited the RSV binding of reactivity in the ELISA which suggests that the peptide may represent an important epitope on the protein of RSV.

Although peptides 4 and 14 were recognized by both the adult and children's sera from Bissau, the hope that they would represent potential immunogen was not supported by the data obtained.

peptides	Antibody response		
	Anti-peptide		Anti-RSV
	+Ve Response	Titre	+Ve Response
4	0/4	_	0/4
8	4/4	1.6-2.2	0/4
11	3/4	2.5-2.8	2/4
14	0/4	_	0/4
19	0/4	_	0/4
20	0/4	_	0/4
24	0/4	_	2/4
32	0/4	_	0/4
36	0/4	_	3/4
41	0/4	_	1/4
48	0/4	_	1/4

Table 6.1

Antibody responses following immunization groups of 4 BALB/c mice with the panel of peptides recognized by human sera.

The immunogenicity of the mimotopes was tested in the mice and the results are summarized in Table 6.2. Although immunization with the mimotopes induced anti-mimotope antibodies, none of these antibodies cross- reacted with RSV. The anti-mimotope antibody titre was quite low and this could be because in this initial immunization, no source of T-cell helper was provided. In addition the mimotopes are short and therefore unlikely to be weak immunogens. It was also noted that biotinylating the mimotopes resulted in the detection of a lower antibody response. Furthermore, the biotinylated peptides were not recognized by the anti-RH3 sera. The controls used in all the ELISAs gave different levels of reactivity towards the mimotopes presented in the various forms which highlights the difficulty in interpreting these data.

The failure of the mimotopes to induce anti-RSV antibodies could mean: 1) that RSV Ag on the plate does not present the epitopes in the right conformation for the antibody to react with them; 2) or that mimotopes are not true mimics of the native epitopes on the F protein which implies that they are not likely to have any potential as vaccine candidates.

Mimotopes in ELISA	Immunizing peptides					
	RH1		RH2		RH3	
	+Ve Response	Titre	+Ve Response	Titre	+Ve Response	Titre
Directly on the plate	* 4/4	2.2-2.5	4/4	2.2-2.5	4/4	2.2
KLH Bound	4/4	2.2-2.8	4/4	2.2	4/4	1.9-2.2
Biotinylated	4/4	1.0	4/4	1.9-2.2	0/4	-

Table.6.2

Antibody responses in sera from mice immunized with mimotopes in ELISA.

The mimotopes were coupled to the ELISA plates either directly, bound to KLH or biotinylated.

*** number of mice > negative control/ the number of mice immunized.**

Blank
In
Original

CHAPTER 7

CONCLUDING REMARKS

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis and pneumonia in infants and young children worldwide and as yet, there is no effective vaccine against RSV infection. The development of an epitope-based synthetic peptide vaccine that was able to induce virus-neutralizing antibodies against RSV would seem to be a suitable approach to this problem. However, this approach requires the identification of epitopes capable of inducing the appropriate antibody (B-cell epitopes) and cellular (T-cell) immune responses. However, it seems that B-cell epitopes capable of inducing virus-neutralizing, protective antibody responses are likely to be conformational in nature and therefore, the identification of mimics (mimotopes) of these epitopes along with the linear epitopes (Steward 2001) would be valuable.

The aim of this work was to identify both linear, and conformational epitopes from the Fusion protein of HRSV to be used in an epitope based vaccine. Furthermore, it was proposed to try and gain a better understanding of repeated infections with RSV by analyzing the antibody response to the Fusion protein in sera from different stages of exposure to the virus. In this regard, no clear conclusions could be drawn from the results of the study presented in Chapter 4.This could be due to several factors that may have influenced the analysis of the data:

i) The negative controls we had were the so-called pre-primary infection samples which are from children who did not have clinical RSV before 18 months of age- but they may well have had sub-clinical infection which could explain the presence of anti-RSV antibody reactivity.

ii) The negative or positive reactivity of some sera with RSV Ag was not always correlated with the presence of antibodies to peptides, this caused confusion in interpreting the data.

iii) The presence of maternally-derived antibodies could have also interfered with the analysis of the data. Maternal antibody has been shown in previous studies to interfere with how babies respond to RSV.

Analysis of the binding the adult sera to the mimotopes showed that mimotopes

M1, M2, RH1 and RH2 could represent epitopes that may have importance in generating a protective antibody response. However, when the reactivity of RF2 with the detected mimotopes was tested in ELISA, no binding was detectable. This is possibly due to the conformation they adopt on binding to the solid phase in the ELISA plates. In order to both improve the binding of RF2 to the identified mimotopes by presenting them in a more appropriate conformation to RF2, they were synthesized in the SPOTs system.

Although the results from experiments describing the substitutions of amino acids in the sequences of the detected mimotopes in Chapter 5 showed that amino acid substitution in the mimotopes could enhance their potential as vaccine candidates, there were difficulties in assessing the data. Furthermore, the fact that the binding results with certain peptides were not consistent was also a problem. These inconsistencies could have arisen from the relative insensitivity of the assay. The affinity of binding between RH1, RH2, and RH3 with RF2 needs to measure. This could highlight which of the mimotopes could be of potential use as a vaccine. Data from the immunization study suggested that peptide 41 may represent an important epitope on the protein of RSV. However, it is also possible that peptides 8 and peptide 11 could represent important epitopes on RSV.

Although peptides 4 and 14 were recognized by both the adult and children sera from Bissau, data in Chapter 6 showed that they were unlikely to represent potential immuonogens.

The design of a successful peptide vaccine against RSV will require further detailed knowledge of the mechanisms of protective immunity involving B-cell and T-cell epitopes, as well as parameters that affect peptide immunogenicity, epitope sequence and structure. Also the need for carrier or adjuvant, changing the route of immunization and method of delivery could be considered in detail in future studies. In addition, an ideal peptide vaccine that can be used universally for all diverse high risk groups to RSV infection will not be easy.

Statistical Analysis:

- To estimate the population mean from the same mean. The mean plus or minus 2 standard errors would be calculated for all samples. This would represent that the population mean was 95 times out of 100 of lying within the two standard errors.
- Data from Chapter 3:

To determine the peptide which is going to be mostly reactive among the adults sera , the *Kruskal-Wallis* test will be used as shown in the table below to find out which of the 8 peptides has a significantly different proportion of individuals with OD readings that are equal or higher than the cut-off.

	Peptide							
	Pep1	**Pep2**	**Pep3**	**Pep4**	**Pep5**	**Pep6**	**Pep7**	**Pep8**
Number of individuals	A	B	C	D	E	F	G	H

with OD reading ≥ cut-off								
Number of individuals with OD reading < cut-off	I	J	K	L	M	N	O	P
TOTAL	9	9	9	9	9	9	9	9

- Data from Chapter 4:

Peptides with a frequency of more than 40% among the children sera will be further analyzed. In children sera with pre and first-post infection samples *the Student T-test* will be applied to compare the differences between the mean ODs for all children in the pre-infection samples against the first-post infection samples.

In children sera with pre, first-post and second-post infection samples *Kruskal Wallis* test will be applied for each peptide individually to compare the means of all ODs observed for the peptide reactivity among the sera from the three sample groups. This will allow me to find out which peptides had significantly different mean ODs in pre, first-post and second-post infection samples.

	Pre - infection samples	**First-post infection samples**	**Second - post infection samples**
Peptide type	Mean of all ODs	Means of all ODs	Means of all ODs
Pep1	Means of all ODs	Means of all ODs	Means of all ODs
Pep8	Means of all ODs	Means of all ODs	Means of all ODs

- Data from Chapter 6:

To test the effect of inhibiting sera reactivity to the inducing peptide, data from immunised animals (using Peptide 8 and 11) will be arranged as shown below.

The *McNemar* test will be applied to find out if inhibition causes a significant decrease in the proportion of animals that produced ODs above the cut-off.

	Before inhibition	**After inhibition**
Number of animals with ODs ≥cut off	X1	Y1
Number of animals with ODs <cut off	X2	Y2

The *McNemar* test will also be applied on data from mice immunised with peptide 24, 36, 41, 48 individually to test the change in the reactivity of the sera against the inducing peptides when the sera was inhibited by RSV Ag. Data will also be arranged as shown above.

The *Kruskal-Wallis* test will be applied to test the difference between the proportions of mice with ODs above and below the cutoff when immunized with each mimotope individually using the three different methods of peptide coatings on the plates. The data will be arranged as below:

	Peptide on plate	**Peptide/biotin**	**Peptide/KLH**
Number of mice with ODs ≥cut off	X1	Y1	Z1
Number of mice with ODs <cut off	X2	Y2	Z2

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APPENDIX

Sample	Appendix 1 -PEPTIDE NUMBER																	
	1	2	3	5	7	9	10	12	13	15	16	17	19	20	21	22	23	25
A	0.137	0	0.203	0.159	0	0.396	0.367	0.413	0.249	0.348	0.337	0.336	0.342	0.388	0.368	0.32	0.242	0.298
B	0	0	0	0.118	0.112	0.379	0.395	0.346	0.201	0.354	0.274	0.398	0.421	0.302	0.321	0.345	0.232	0.289
C	0.187	0.146	0.262	0.255	0.22	0.377	0.327	0.378	0.293	0.297	0.359	0.365	0.354	0.278	0.354	0.283	0.239	0.202
D	0.254	0	0.282	0.166	0.227	0.432	0.49	0.386	0.381	0.416	0.375	0.4	0.4	0.377	0.444	0.227	0.28	0.246
E	0.562	0.546	0.52	0.523	0.46	0.476	0.456	0.488	0.337	0.457	0.391	0.589	0.529	0.587	0.517	0.447	0.394	0.401
F	0.308	0.179	0.232	0.348	0.335	0.348	0.33	0.282	0.268	0.338	0.226	0.241	0.197	0.285	0.133	0.211	0.208	0.219
G	0.37	0.232	0.273	0.553	0.591	0.385	0.442	0.265	0.307	0.256	0.242	0.243	0.2	0.247	0.156	0.192	0.225	0.224
H	0.495	0.24	0.245	0.409	0.435	0.352	0.406	0.323	0.327	0.2	0.286	0.246	0.237	0.331	0.176	0.249	0.266	0.269
I	0.308	0.154	0.37	0.335	0.355	0.376	0.381	0.288	0.289	0.284	0.227	0.244	0.181	0.2	0.223	0.194	0.185	0.221

Sample	Appendix2-PEPTIDE NUMBER																	
	26	27	28	29	30	31	32	33	34	35	37	38	39	40	42	43	44	45
A	0.306	0.12	0.679	0.243	0.213	0.215	0.267	0.366	0.255	0.208	0.653	0.253	0.179	0.159	0.139	0.154	0.196	0.203
B	0.324	0.227	0.52	0.328	0.261	0.262	0.326	0.287	0.193	0.312	0.73	0.216	0.185	0.18	0.253	0.165	0.145	0.198
C	0.287	0.324	0.617	0.3	0.198	0.273	0.235	0.258	0.344	0.205	0.806	0.306	0.276	0.227	0.298	0.175	0.132	0.246
D	0.215	0.213	0.814	0.293	0.216	0.228	0.257	0.308	0.36	0.377	0.934	0.24	0.303	0.154	0.258	0.19	0.144	0.199
E	0.407	0.364	0.876	0.383	0.379	0.313	0.274	0.387	0.397	0.332	1.407	0.452	0.388	0.249	0.325	0.245	0.256	0.324
F	0.234	0.252	0.274	0.229	0.23	0.165	0.313	0.178	0.128	0.475	0.67	0.207	0.406	0.321	0.184	0.138	0.184	0.204
G	0.214	0.227	0.275	0.305	0.227	0.168	0.276	0.195	0.14	0.402	0.373	0.176	0.379	0.258	0.159	0.138	0.187	0.227
H	0.233	0.197	0.389	0.211	0.254	0.276	0.345	0.268	0.18	0.333	0.8	0.221	0.485	0.321	0.214	0.159	0.241	0.307
I	0.262	0.178	0.225	0.221	0.221	0.18	0.188	0.227	0.187	0.378	0.665	0.198	0.4	0.246	0.207	0.139	0.208	0.234

Sample	Appendix-3-PEPTIDE NUMBER									
	46	47	48	49	50	51	52	53	54	55
A	0.187	0.384	0.456	0.176	0.341	0.44	0.25	0.34	0.167	0.187
B	0.176	0.329	0.246	0.184	0.4	0.385	0.21	0.287	0.214	0.154
C	0.196	0.276	0.386	0.202	0.378	0.354	0.23	0.307	0.202	0.2
D	0.3	0.297	0.332	0.227	0.285	0.227	0.263	0.296	0.187	0.197
E	0.341	0.437	0.56	0.388	0.546	0.478	0.245	0.407	0.253	0.231
F	0.206	0.338	0.335	0.308	0.293	0.44	0.2	0.346	0.176	0.238
G	0.178	0.224	0.297	0.223	0.345	0.316	0.209	0.27	0.184	0.137
H	0.298	0.198	0.332	0.334	0.31	0.326	0.238	0.387	0.202	0.212
I	0.273	0.336	0.198	0.208	0.307	0.209	0.269	0.328	0.227	0.191

Appedix 4

Sequences producing significant alignments with peptide 4. Score/Identity =53/100%, 44/92%, 41/84%, 31/81%, 30/69%, 29/57-60%

Sequences producing significant alignments:	Score (bits)	E Value
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	53	6e07
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	53	6e07
gi|1912295|gb|AAC57027.1| Fusion protein (F)	53	6e07
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	53	6e07
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	53	6e07
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	53	6e07
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	53	6e07
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	53	6e07
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	53	6e07
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	53	6e07
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	53	6e07
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	53	6e07
gi|227299|prf||1701388A fusion glycoprotein	53	6e07
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	53	6e07
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	53	6e07
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	53	6e07
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	53	6e07
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	53	6e07
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	53	6e07
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	53	6e07
gi|226438|prf||1512372A fusion protein	53	6e07
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	53	6e07
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	53	6e07
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	53	6e07
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	53	6e07
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	53	6e07
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	53	6e07
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	53	6e07
gi|480767|pir||S37254 cell fusion protein - human respirato...	53	6e07
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	53	6e07
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	53	6e07
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	53	6e07
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	53	6e07
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	53	6e07
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	44	3e04
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	41	0.004
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	41	0.004
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	41	0.004
gi|281366|pir||JQ1583 cell fusion protein precursor - bovin...	41	0.004
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	41	0.004
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	41	0.004
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	41	0.004
gi|17939990|gb|AAL49399.1| fusion protein [Bovine respirato...	41	0.004
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	41	0.004
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	31	3.3
gi|24372027|ref|NP_716069.1| aconitate hydratase 2 [Shewane...	30	4.5
gi|113785|sp|P19584|AMYB_THETU Beta-amylase, thermophilic p...	30	6.0
gi|16759155|ref|NP_454772.1| aconitate hydratase 2 (citrate...	29	11
gi|15921053|ref|NP_376722.1| 572aa long hypothetical granul...	29	11
gi|15799802|ref|NP_285814.1| aconitate hydrase B [Escherich...	29	11
gi|26246058|ref|NP_752097.1| Aconitate hydratase 2 [Escheri...	29	11

Appendix 5

Sequences producing significant alignments (peptide 8). Score/Identity= 52/100%, 48/93%, 42/80%, 41/80%, 38/73%, 36/73%, 30/76%, 28/45-76%, 26-60-100%

Sequences producing significant alignments:	Score (bits)	E Value
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	52	1e06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	48	2e05
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	48	2e05
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	48	2e05
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	48	2e05
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	48	2e05
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	48	2e05
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	48	2e05
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	45	2e04
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	45	2e04
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	45	2e04
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	45	2e04
gi|227299|prf||1701388A fusion glycoprotein	45	2e04
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	45	2e04
gi|226438|prf||1512372A fusion protein	45	2e04
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	45	2e04
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	45	2e04
gi|480767|pir||S37254 cell fusion protein - human respirato...	45	2e04
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	45	2e04
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	45	2e04
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	42	0.001
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	42	0.001
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	42	0.001
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	42	0.002
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	41	0.002
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	41	0.002
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	41	0.004
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	41	0.004
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	41	0.004
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	41	0.004
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	41	0.004
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	41	0.004
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	41	0.004
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	38	0.031
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	36	0.099
gi|49238350|emb|CAF27574.1| hypothetical protein [Bartonell...	30	6.1
gi|21955241|ref|NP_523865.2| CG16778-PB [Drosophila melanog...	28	20
gi|33865290|ref|NP_896849.1| probable aminopeptidase N [Syn...	26	86
gi|15603581|ref|NP_246655.1| Lig [Pasteurella multocida Pm7...	26	86
gi|45185037|ref|NP_982754.1| ABL193Cp [Eremothecium gossypi...	26	86
gi|47214801|emb|CAF89628.1| unnamed protein product [Tetrao...	26	86
gi|45198639|ref|NP_985668.1| AFR121Wp [Eremothecium gossypi...	26	86
gi|28829374|gb|AAL88727.2| similar to Homo sapiens (Human)....	26	86
gi|22971986|ref|ZP_00018894.1| hypothetical protein [Chloro...	26	86
gi|46579935|ref|YP_010743.1| conserved hypothetical protein...	26	115
gi|49124153|ref|XP_412584.1| hypothetical protein AN8447.2 ...	26	115
gi|12846941|dbj|BAB27371.1| unnamed protein product [Mus mu...	26	115
gi|39579193|emb|CAE57000.1| Hypothetical protein CBG24867 [...	26	115
gi|39592595|emb|CAE63672.1| Hypothetical protein CBG08174 [...	26	115
gi|22028422|gb|AAH34901.1| 2510040D07Rik protein [Mus muscu...	26	115
gi|83856|pir||JQ1469 hypothetical 10.1K protein (INF56 regi...	26	115
gi|23593529|ref|XP_135092.2| RIKEN cDNA 2510040D07 [Mus mus...	26	115
gi|22959017|ref|ZP_00006676.1| COG4176: ABC-type proline/gl...	26	115
gi|24475539|dbj|BAC22716.1| vitellogenin [Crassostrea gigas]	26	115
gi|26349055|dbj|BAC38167.1| unnamed protein product [Mus mu...	26	115
gi|416|9024|tpg|DAA04062.1| TPA: HDC13868 [Drosophila melan...	26	115

Appenix 6

Sequences producing significant alignments (peptide11). Score/Identity=49/100%, 46/100%, 43/92%, 39/85%, 31/81%,

27/66-81%, 26/72-88%

Sequences producing significant alignments:	Score (bits)	E Value
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	49	1e05
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	49	1e05
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	49	1e05
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	49	1e05
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	49	1e05
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	46	9e05
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	46	9e05
gi|226438|prf||1512372A fusion protein	46	9e05
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	46	9e05
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	46	9e05
gi|480767|pir||S37254 cell fusion protein - human respirato...	46	9e05
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	46	9e05
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	46	9e05
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	46	9e05
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	43	9e04
gi|1912295|gb|AAC57027.1| Fusion protein (F)	43	9e04
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	43	9e04
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	43	9e04
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	43	9e04
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	43	9e04
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	43	9e04
gi|227299|prf||1701388A fusion glycoprotein	43	9e04
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	43	9e04
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	43	9e04
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	43	9e04
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	43	9e04
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	43	9e04
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	39	0.013
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	39	0.013
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	39	0.013
gi|22748321|gb|AAN05323.1| Hypothetical protein [Oryza sati...	31	3.4
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	31	3.4
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	31	3.4
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	31	3.4
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	31	3.4
gi|19112634|ref|NP_595842.1| telomerase reverse transcripta...	27	48
gi|7493461|pir||T03838 telomerase catalytic chain - fission...	27	48
gi|49134466|ref|XP_413229.1| hypothetical protein AN9092.2 ...	27	48
gi|42760033|emb|CAE01390.1| tuber borchii white collar-1 [T...	27	64
gi|23481167|gb|EAA17525.1| AT hook motif, putative [Plasmod...	27	64
gi|23482650|gb|EAA18573.1| hypothetical protein [Plasmodium...	27	64
gi|23957740|ref|NP_705854.1| hypothetical protein [Plasmodi...	27	64
gi|22208753|emb|CAA21447.2| SPCC1442.13c [Schizosaccharomyc...	27	64
gi|19075827|ref|NP_588327.1| hypothetical protein [Schizosa...	27	64
gi|15223158|ref|NP_172303.1| DNA-directed DNA polymerase ep...	26	86
gi|3721933|dbj|BAA33760.1| orotidine-5'-phosphate decarboxy...	26	115
gi|118356|sp|P07817|PYRF_ASPNG Orotidine 5'-phosphate decar...	26	115

Appendix 7

Sequences producing significant alignments(peptide 14). Score/Identity = 48/100%, 46/93-100%, 32/100%, 31/83%, 28/100%.

Sequences producing significant alignments:	Score (bits)	E Value
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	49	1e05
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	49	1e05
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	49	1e05
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	49	1e05
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	49	1e05
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	49	1e05
gi|281366|pir||JQ1583 cell fusion protein precursor - bovin...	49	1e05
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	49	1e05

gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	49	1e05
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	49	1e05
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	49	1e05
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	49	1e05
gi|17939990|gb|AAL49399.1| fusion protein [Bovine respirato...	49	1e05
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	49	1e05
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	46	6e05
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	46	6e05
gi|1912295|gb|AAC57027.1| Fusion protein (F)	46	6e05
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	46	6e05
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	46	6e05
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	46	6e05
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	46	6e05
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	46	6e05
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	46	6e05
gi|227299|prf||1701388A fusion glycoprotein	46	6e05
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	46	6e05
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	46	6e05
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	46	6e05
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	46	6e05
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	46	6e05
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	46	6e05
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	46	6e05
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	46	6e05
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	46	6e05
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	46	6e05
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	46	6e05
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	46	6e05
gi|480767|pir||S37254 cell fusion protein - human respirato...	46	6e05
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	46	6e05
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	46	1e04
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	46	1e04
gi|226438|prf||1512372A fusion protein	46	1e04
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	46	1e04
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	46	1e04
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	46	1e04
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	46	1e04
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	46	1e04
gi|12084115|pdb|1G2C|A Chain A, Human Respiratory Syncytial...	32	1.9
gi|46133522|ref|ZP_00203193.1| COG0846: NAD-dependent prote...	31	3.4
gi|48868061|ref|ZP_00321452.1| COG0846: NAD-dependent prote...	31	3.4
gi|2137577|pir||I55466 N-methyl-D-aspartate receptor subuni...	28	26

Appendix 8

Sequences producing significant alignments(peptide 18). Score/Identity = 52/100%, 48/93%, 46/86-92%.

Sequences producing significant alignments:	**Score (bits)**	**E Value**
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	52	1e06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	52	1e06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	52	1e06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	52	1e06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	52	1e06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	52	1e06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	52	1e06
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	52	1e06
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	52	1e06
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	52	1e06
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	52	1e06
gi|227299|prf||1701388A fusion glycoprotein	52	1e06
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	52	1e06
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	52	1e06
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	52	1e06
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	52	1e06
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	52	1e06
gi|12084115|pdb|1G2C|A Chain A, Human Respiratory Syncytial...	52	1e06
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	52	1e06
gi|226438|prf||1512372A fusion protein	52	1e06
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	52	1e06

gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	52	1e06
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	52	1e06
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	52	1e06
gi|480767|pir||S37254 cell fusion protein - human respirato...	52	1e06
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	52	1e06
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	52	1e06
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	52	1e06
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	52	1e06
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	52	1e06
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	48	3e05
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	46	9e05
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	46	9e05
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	46	1e04
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	46	1e04
gi|10765409|gb|AAG23033.1| fusion protein [Bovine respirato...	46	1e04
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	46	1e04
gi|10765431|gb|AAG23044.1| fusion protein [Bovine respirato...	46	1e04
gi|10765425|gb|AAG23041.1| fusion protein [Bovine respirato...	46	1e04
gi|10765417|gb|AAG23037.1| fusion protein [Bovine respirato...	46	1e04
gi|10765433|gb|AAG23045.1| fusion protein [Bovine respirato...	46	1e04
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	46	1e04
gi|10765419|gb|AAG23038.1| fusion protein [Bovine respirato...	46	1e04
gi|10765427|gb|AAG23042.1| fusion protein [Bovine respirato...	46	1e04
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	46	1e04
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	46	1e04
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	46	1e04
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	46	1e04
gi|17939990|gb|AAL49399.1| fusion protein [Bovine respirato...	46	1e04
gi|10765421|gb|AAG23039.1| fusion protein [Bovine respirato...	46	1e04
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	46	1e04
gi|10765389|gb|AAG23023.1| fusion protein [Bovine respirato...	46	1e04
gi|10765391|gb|AAG23024.1| fusion protein [Bovine respirato...	46	1e04

Appendix 9

Sequences producing significant alignments (peptide 19). Score/Identity = 27/60%, 29/100, 51/100, 47/93, 46/93%,

44/86%, 43/86%, 39/85%, 29/100%, 27/86%, 27/60%.

Sequences producing significant alignments:	Score (bits)	E Value
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	51	3e06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	51	3e06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	51	3e06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	51	3e06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	51	3e06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	51	3e06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	51	3e06
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	51	3e06
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	51	3e06
gi|227299|prf||1701388A fusion glycoprotein	51	3e06
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	51	3e06
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	51	3e06
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	51	3e06
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	51	3e06
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	51	3e06
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	51	3e06
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	51	3e06
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	51	3e06
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	51	3e06
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	51	3e06
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	47	5e05
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	47	5e05
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	47	5e05
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	47	5e05
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	47	5e05
gi|480767|pir||S37254 cell fusion protein - human respirato...	47	5e05
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	47	5e05

gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	47	5e05
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	47	5e05
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	46	6e05
gi|226438|prf||1512372A fusion protein	44	3e04
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	43	9e04
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	43	9e04
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	43	9e04
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	39	0.009
gi|12084115|pdb|1G2C|A Chain A, Human Respiratory Syncytial...	29	15
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	27	36
gi|19682989|gb|AAL92614.1| hypothetical protein [Dictyostel...	27	36
gi|10765409|gb|AAG23033.1| fusion protein [Bovine respirato...	27	36
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	27	36
gi|38201765|ref|NP_938132.1| SefH [Serratia entomophila] >g...	27	36
gi|10765425|gb|AAG23041.1| fusion protein [Bovine respirato...	27	36
gi|10765417|gb|AAG23037.1| fusion protein [Bovine respirato...	27	36
gi|10765433|gb|AAG23045.1| fusion protein [Bovine respirato...	27	36
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	27	36
gi|10765419|gb|AAG23038.1| fusion protein [Bovine respirato...	27	36
gi|10765427|gb|AAG23042.1| fusion protein [Bovine respirato...	27	36
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	27	36
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	27	36
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	27	36
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	27	36
gi|10765389|gb|AAG23023.1| fusion protein [Bovine respirato...	27	36
gi|10765391|gb|AAG23024.1| fusion protein [Bovine respirato...	27	36
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	27	48

Appendix 10

Sequences producing significant alignments (peptide 20). Score/Identity = 52/100%, 49/93%, 48/93%. 46/92%, 45/86%, 44/86%.

Sequences producing significant alignments:	**Score (bits)**	**E Value**
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	52	1e-06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	52	1e-06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	52	1e-06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	52	1e-06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	52	1e-06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	52	1e-06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	52	1e-06
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	52	1e-06
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	52	1e-06
gi|227299|prf||1701388A fusion glycoprotein	52	1e-06
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	52	1e-06
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	52	1e-06
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	52	1e-06
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	52	1e-06
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	52	1e-06
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	52	1e-06
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	52	1e-06
gi|10765409|gb|AAG23033.1| fusion protein [Bovine respirato...	49	1e-05
gi|10765425|gb|AAG23041.1| fusion protein [Bovine respirato...	49	1e-05
gi|10765417|gb|AAG23037.1| fusion protein [Bovine respirato...	49	1e-05
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	49	1e-05
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	49	1e-05
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	49	1e-05
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	49	1e-05
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	49	1e-05
gi|226438|prf||1512372A fusion protein	49	1e-05
gi|10765419|gb|AAG23038.1| fusion protein [Bovine respirato...	49	1e-05
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	49	1e-05
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	49	1e-05
gi|10765423|gb|AAG23040.1| fusion protein [Bovine respirato...	49	1e-05
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	49	1e-05
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	49	1e-05
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	49	1e-05
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	49	1e-05

gi|480767|pir||S37254 cell fusion protein - human respirato...	49	1e-05
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	49	1e-05
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	49	1e-05
gi|10765421|gb|AAG23039.1| fusion protein [Bovine respirato...	49	1e-05
gi|10765389|gb|AAG23023.1| fusion protein [Bovine respirato...	49	1e-05
gi|10765391|gb|AAG23024.1| fusion protein [Bovine respirato...	49	1e-05
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	49	1e-05
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	48	2e-05
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	48	2e-05
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	48	2e-05
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	48	2e-05
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	46	1e-04
gi|281366|pir||JQ1583 cell fusion protein precursor - bovin...	45	2e-04
gi|17939990|gb|AAL49399.1| fusion protein [Bovine respirato...	45	2e-04
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	44	3e-04
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	44	3e-04
gi|10765427|gb|AAG23042.1| fusion protein [Bovine respirato...	44	3e-04
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	44	3e-04
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	44	4e-04
gi|10765431|gb|AAG23044.1| fusion protein [Bovine respirato...	44	4e-04

Appendix 11

Sequences producing significant alignments (peptide 24). Score/Identity =52/100%.

Sequences producing significant alignments:	Score (bits)	E Value
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	52	2e-06
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	52	2e-06
gi|10765409|gb|AAG23033.1| fusion protein [Bovine respirato...	52	2e-06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	52	2e-06
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	52	2e-06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	52	2e-06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	52	2e-06
gi|10765431|gb|AAG23044.1| fusion protein [Bovine respirato...	52	2e-06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	52	2e-06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	52	2e-06
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	52	2e-06
gi|10765425|gb|AAG23041.1| fusion protein [Bovine respirato...	52	2e-06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	52	2e-06
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	52	2e-06
gi|10765417|gb|AAG23037.1| fusion protein [Bovine respirato...	52	2e-06
gi|10765433|gb|AAG23045.1| fusion protein [Bovine respirato...	52	2e-06
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	52	2e-06
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	52	2e-06
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	52	2e-06
gi|6653464|gb|AAF22722.1| fusion protein [Bovine respirator...	52	2e-06
gi|227299|prf||1701388A fusion glycoprotein	52	2e-06
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	52	2e-06
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	52	2e-06
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	52	2e-06
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	52	2e-06
gi|281366|pir||JQ1583 cell fusion protein precursor - bovin...	52	2e-06
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	52	2e-06
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	52	2e-06
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	52	2e-06
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	52	2e-06
gi|226438|prf||1512372A fusion protein	52	2e-06
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	52	2e-06
gi|10765419|gb|AAG23038.1| fusion protein [Bovine respirato...	52	2e-06
gi|10765427|gb|AAG23042.1| fusion protein [Bovine respirato...	52	2e-06
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	52	2e-06
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	52	2e-06
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	52	2e-06
gi|10765423|gb|AAG23040.1| fusion protein [Bovine respirato...	52	2e-06
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	52	2e-06
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	52	2e-06
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	52	2e-06
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	52	2e-06

gi|38230490|gb|AAR14266.1|	F [Human Respiratory syncytial v...	52	2e-06
gi|19550336|gb|AAL91342.1|	fusion protein [Ovine respirator...	52	2e-06
gi|961611|gb|AAB38518.1|	fusion protein [Human respiratory ...	52	2e-06
gi|480767|pir||S37254	cell fusion protein - human respirato...	52	2e-06
gi|138251|sp|P03420|VGLF_HRSVA	Fusion glycoprotein precurso...	52	2e-06
gi|17939990|gb|AAL49399.1|	fusion protein [Bovine respirato...	52	2e-06
gi|37674750|gb|AAQ97029.1|	fusion protein precursor [Human ...	52	2e-06
gi|138252|sp|P12568|VGLF_HRSVL	Fusion glycoprotein precurso...	52	2e-06
gi|21689579|gb|AAM68154.1|	fusion glycoprotein [Human respi...	52	2e-06
gi|10765421|gb|AAG23039.1|	fusion protein [Bovine respirato...	52	2e-06
gi|10765389|gb|AAG23023.1|	fusion protein [Bovine respirato...	52	2e-06
gi|10765391|gb|AAG23024.1|	fusion protein [Bovine respirato...	52	2e-06
gi|37674744|gb|AAQ97026.1|	fusion protein precursor [Human ...	52	2e-06

Appendix 12

Sequences producing significant alignments (peptide 32). Score/Identity =56/100%.

Sequences producing significant alignments:		Score (bits)	E Value
gi|138248|sp|P23728|VGLF_BRSVR	Fusion glycoprotein precurso...	56	1e-07
gi|267344|sp|P29791|VGLF_BRSVA	Fusion glycoprotein precurso...	56	1e-07
gi|21689587|gb|AAM68160.1|	fusion glycoprotein [Human respi...	56	1e-07
gi|10765409|gb|AAG23033.1|	fusion protein [Bovine respirato...	56	1e-07
gi|21263086|gb|AAM44851.1|	fusion glycoprotein [Human respi...	56	1e-07
gi|3451386|emb|CAA76980.1|	F protein [Bovine respiratory sy...	56	1e-07
gi|1912295|gb|AAC57027.1|	Fusion protein (F)	56	1e-07
gi|94063|pir||B28929	cell fusion glycoprotein - human respi...	56	1e-07
gi|10765431|gb|AAG23044.1|	fusion protein [Bovine respirato...	56	1e-07
gi|961615|gb|AAB38520.1|	fusion protein [Human respiratory ...	56	1e-07
gi|1353201|sp|P11209|VGLF_HRSVR	Fusion glycoprotein precurs...	56	1e-07
gi|29290043|gb|AAO72325.1|	fusion protein [Human respirator...	56	1e-07
gi|10765425|gb|AAG23041.1|	fusion protein [Bovine respirato...	56	1e-07
gi|21689583|gb|AAM68157.1|	fusion glycoprotein [Human respi...	56	1e-07
gi|9629375|ref|NP_044596.1|	Fusion protein (F) [respiratory...	56	1e-07
gi|10765417|gb|AAG23037.1|	fusion protein [Bovine respirato...	56	1e-07
gi|10765433|gb|AAG23045.1|	fusion protein [Bovine respirato...	56	1e-07
gi|37674746|gb|AAQ97027.1|	fusion protein precursor [Human ...	56	1e-07
gi|74932|pir||VGNZR2	cell fusion glycoprotein precursor - h...	56	1e-07
gi|37674754|gb|AAQ97031.1|	fusion protein precursor [Human ...	56	1e-07
gi|6653464|gb|AAF22722.1|	fusion protein [Bovine respirator...	56	1e-07
gi|227299|prf||1701388A	fusion glycoprotein	56	1e-07
gi|3172522|gb|AAC18601.1|	fusion protein [Human respiratory...	56	1e-07
gi|138250|sp|P13843|VGLF_HRSV1	Fusion glycoprotein precurso...	56	1e-07
gi|281366|pir||JQ1583	cell fusion protein precursor - bovin...	56	1e-07
gi|9629206|ref|NP_056863.1|	fusion protein [Human respirato...	56	1e-07
gi|1695263|gb|AAC55970.1|	fusion glycoprotein precursor [Hu...	56	1e-07
gi|29290041|gb|AAO72324.1|	fusion protein [Human respirator...	56	1e-07
gi|226438|prf||1512372A	fusion protein	56	1e-07
gi|2582041|gb|AAB82446.1|	fusion protein [Human respiratory...	56	1e-07
gi|10765419|gb|AAG23038.1|	fusion protein [Bovine respirato...	56	1e-07
gi|10765427|gb|AAG23042.1|	fusion protein [Bovine respirato...	56	1e-07
gi|138247|sp|P22167|VGLF_BRSVC	Fusion glycoprotein precurso...	56	1e-07
gi|425678|gb|AAB28458.1|	fusion protein F2 subunit [bovine ...	56	1e-07
gi|37674752|gb|AAQ97030.1|	fusion protein precursor [Human ...	56	1e-07
gi|10765423|gb|AAG23040.1|	fusion protein [Bovine respirato...	56	1e-07
gi|37674748|gb|AAQ97028.1|	fusion protein precursor [Human ...	56	1e-07
gi|9631275|ref|NP_048055.1|	fusion glycoprotein precursor; ...	56	1e-07
gi|29290039|gb|AAO72323.1|	fusion protein [Human respirator...	56	1e-07
gi|961613|gb|AAB38519.1|	fusion protein [Human respiratory ...	56	1e-07
gi|38230490|gb|AAR14266.1|	F [Human Respiratory syncytial v...	56	1e-07
gi|19550336|gb|AAL91342.1|	fusion protein [Ovine respirator...	56	1e-07
gi|480767|pir||S37254	cell fusion protein - human respirato...	56	1e-07
gi|138251|sp|P03420|VGLF_HRSVA	Fusion glycoprotein precurso...	56	1e-07
gi|17939990|gb|AAL49399.1|	fusion protein [Bovine respirato...	56	1e-07
gi|37674750|gb|AAQ97029.1|	fusion protein precursor [Human ...	56	1e-07
gi|138252|sp|P12568|VGLF_HRSVL	Fusion glycoprotein precurso...	56	1e-07
gi|21689579|gb|AAM68154.1|	fusion glycoprotein [Human respi...	56	1e-07
gi|10765421|gb|AAG23039.1|	fusion protein [Bovine respirato...	56	1e-07
gi|17940002|gb|AAL49410.1|	fusion protein [Bovine respirato...	56	1e-07

gi|10765389|gb|AAG23023.1|	fusion protein [Bovine respirato...	56	1e-07
gi|10765391|gb|AAG23024.1|	fusion protein [Bovine respirato...	56	1e-07
gi|37674744|gb|AAQ97026.1|	fusion protein precursor [Human ...	56	1e-07

Appendix 13

Sequences producing significant alignments (peptide 36). Score/Identity = 39/73%,41/80%, 46/86%, 49/93%, 51/100%.

Sequences producing significant alignments:		Score (bits)	E Value
gi|94063|pir||B28929	cell fusion glycoprotein - human respi...	51	3e-06
gi|138251|sp|P03420|VGLF_HRSVA	Fusion glycoprotein precurso...	51	3e-06
gi|21263086|gb|AAM44851.1|	fusion glycoprotein [Human respi...	49	1e-05
gi|37674746|gb|AAQ97027.1|	fusion protein precursor [Human ...	49	1e-05
gi|37674754|gb|AAQ97031.1|	fusion protein precursor [Human ...	49	1e-05
gi|3172522|gb|AAC18601.1|	fusion protein [Human respiratory...	49	1e-05
gi|61211|emb|CAA26143.1|	unnamed protein product [Human res...	49	1e-05
gi|1695263|gb|AAC55970.1|	fusion glycoprotein precursor [Hu...	49	1e-05
gi|961607|gb|AAB38516.1|	fusion protein [Human respiratory ...	49	1e-05
gi|226438|prf||1512372A	fusion protein	49	1e-05
gi|37674752|gb|AAQ97030.1|	fusion protein precursor [Human ...	49	1e-05
gi|37674748|gb|AAQ97028.1|	fusion protein precursor [Human ...	49	1e-05
gi|480767|pir||S37254	cell fusion protein - human respirato...	49	1e-05
gi|37674750|gb|AAQ97029.1|	fusion protein precursor [Human ...	49	1e-05
gi|138252|sp|P12568|VGLF_HRSVL	Fusion glycoprotein precurso...	49	1e-05
gi|37674744|gb|AAQ97026.1|	fusion protein precursor [Human ...	49	1e-05
gi|21689587|gb|AAM68160.1|	fusion glycoprotein [Human respi...	46	6e-05
gi|1912295|gb|AAC57027.1|	Fusion protein (F)	46	6e-05
gi|961615|gb|AAB38520.1|	fusion protein [Human respiratory ...	46	6e-05
gi|1353201|sp|P11209|VGLF_HRSVR	Fusion glycoprotein precurs...	46	6e-05
gi|29290043|gb|AAO72325.1|	fusion protein [Human respirator...	46	6e-05
gi|21689583|gb|AAM68157.1|	fusion glycoprotein [Human respi...	46	6e-05
gi|9629375|ref|NP_044596.1|	Fusion protein (F) [respiratory...	46	6e-05
gi|74932|pir||VGNZR2	cell fusion glycoprotein precursor - h...	46	6e-05
gi|227299|prf||1701388A	fusion glycoprotein	46	6e-05
gi|961609|gb|AAB38517.1|	fusion protein [Human respiratory ...	46	6e-05
gi|29290041|gb|AAO72324.1|	fusion protein [Human respirator...	46	6e-05
gi|29290039|gb|AAO72323.1|	fusion protein [Human respirator...	46	6e-05
gi|961613|gb|AAB38519.1|	fusion protein [Human respiratory ...	46	6e-05
gi|961611|gb|AAB38518.1|	fusion protein [Human respiratory ...	46	6e-05
gi|21689579|gb|AAM68154.1|	fusion glycoprotein [Human respi...	46	6e-05
gi|138250|sp|P13843|VGLF_HRSV1	Fusion glycoprotein precurso...	41	0.003
gi|9629206|ref|NP_056863.1|	fusion protein [Human respirato...	41	0.003
gi|2582041|gb|AAB82446.1|	fusion protein [Human respiratory...	41	0.003
gi|38230490|gb|AAR14266.1|	F [Human Respiratory syncytial v...	41	0.003
gi|138248|sp|P23728|VGLF_BRSVR	Fusion glycoprotein precurso...	39	0.009
gi|267344|sp|P29791|VGLF_BRSVA	Fusion glycoprotein precurso...	39	0.009
gi|10765409|gb|AAG23033.1|	fusion protein [Bovine respirato...	39	0.009
gi|3451386|emb|CAA76980.1|	F protein [Bovine respiratory sy...	39	0.009
gi|10765431|gb|AAG23044.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765425|gb|AAG23041.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765417|gb|AAG23037.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765433|gb|AAG23045.1|	fusion protein [Bovine respirato...	39	0.009
gi|6653464|gb|AAF22722.1|	fusion protein [Bovine respirator...	39	0.009
gi|281366|pir||JQ1583	cell fusion protein precursor - bovin...	39	0.009
gi|10765419|gb|AAG23038.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765427|gb|AAG23042.1|	fusion protein [Bovine respirato...	39	0.009
gi|138247|sp|P22167|VGLF_BRSVC	Fusion glycoprotein precurso...	39	0.009
gi|425678|gb|AAB28458.1|	fusion protein F2 subunit [bovine ...	39	0.009
gi|10765423|gb|AAG23040.1|	fusion protein [Bovine respirato...	39	0.009
gi|9631275|ref|NP_048055.1|	fusion glycoprotein precursor; ...	39	0.009
gi|19550336|gb|AAL91342.1|	fusion protein [Ovine respirator...	39	0.009
gi|17939990|gb|AAL49399.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765421|gb|AAG23039.1|	fusion protein [Bovine respirato...	39	0.009
gi|17940002|gb|AAL49410.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765389|gb|AAG23023.1|	fusion protein [Bovine respirato...	39	0.009
gi|10765391|gb|AAG23024.1|	fusion protein [Bovine respirato...	39	0.009

Appendix 14

Sequences producing significant alignments (peptide 41). Score/Identity = 28/80%, 48/100%, 50-51/100%.

Sequences producing significant alignments:	Score (bits)	E Value
gi|138248|sp|P23728|VGLF_BRSVR Fusion glycoprotein precurso...	50	5e-06
gi|267344|sp|P29791|VGLF_BRSVA Fusion glycoprotein precurso...	50	5e-06
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	50	5e-06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	50	5e-06
gi|3451386|emb|CAA76980.1| F protein [Bovine respiratory sy...	50	5e-06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	50	5e-06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	50	5e-06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	50	5e-06
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	50	5e-06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	50	5e-06
gi|9629375|ref|NP_044596.1| Fusion protein (F) [respiratory...	50	5e-06
gi|37674746|gb|AAQ97027.1| fusion protein precursor [Human ...	50	5e-06
gi|37674754|gb|AAQ97031.1| fusion protein precursor [Human ...	50	5e-06
gi|227299|prf||1701388A fusion glycoprotein	50	5e-06
gi|3172522|gb|AAC18601.1| fusion protein [Human respiratory...	50	5e-06
gi|61211|emb|CAA26143.1| unnamed protein product [Human res...	50	5e-06
gi|961609|gb|AAB38517.1| fusion protein [Human respiratory ...	50	5e-06
gi|138250|sp|P13843|VGLF_HRSV1 Fusion glycoprotein precurso...	50	5e-06
gi|281366|pir||JQ1583 cell fusion protein precursor - bovin...	50	5e-06
gi|9629206|ref|NP_056863.1| fusion protein [Human respirato...	50	5e-06
gi|1695263|gb|AAC55970.1| fusion glycoprotein precursor [Hu...	50	5e-06
gi|961607|gb|AAB38516.1| fusion protein [Human respiratory ...	50	5e-06
gi|29290041|gb|AAO72324.1| fusion protein [Human respirator...	50	5e-06
gi|226438|prf||1512372A fusion protein	50	5e-06
gi|2582041|gb|AAB82446.1| fusion protein [Human respiratory...	50	5e-06
gi|138247|sp|P22167|VGLF_BRSVC Fusion glycoprotein precurso...	50	5e-06
gi|37674752|gb|AAQ97030.1| fusion protein precursor [Human ...	50	5e-06
gi|37674748|gb|AAQ97028.1| fusion protein precursor [Human ...	50	5e-06
gi|9631275|ref|NP_048055.1| fusion glycoprotein precursor; ...	50	5e-06
gi|29290039|gb|AAO72323.1| fusion protein [Human respirator...	50	5e-06
gi|961613|gb|AAB38519.1| fusion protein [Human respiratory ...	50	5e-06
gi|38230490|gb|AAR14266.1| F [Human Respiratory syncytial v...	50	5e-06
gi|19550336|gb|AAL91342.1| fusion protein [Ovine respirator...	50	5e-06
gi|961611|gb|AAB38518.1| fusion protein [Human respiratory ...	50	5e-06
gi|480767|pir||S37254 cell fusion protein - human respirato...	50	5e-06
gi|138251|sp|P03420|VGLF_HRSVA Fusion glycoprotein precurso...	50	5e-06
gi|17939990|gb|AAL49399.1| fusion protein [Bovine respirato...	50	5e-06
gi|37674750|gb|AAQ97029.1| fusion protein precursor [Human ...	50	5e-06
gi|138252|sp|P12568|VGLF_HRSVL Fusion glycoprotein precurso...	50	5e-06
gi|21689579|gb|AAM68154.1| fusion glycoprotein [Human respi...	50	5e-06
gi|17940002|gb|AAL49410.1| fusion protein [Bovine respirato...	50	5e-06
gi|37674744|gb|AAQ97026.1| fusion protein precursor [Human ...	50	5e-06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	48	3e-05
gi|74932|pir||VGNZR2 cell fusion glycoprotein precursor - h...	48	3e-05
gi|425678|gb|AAB28458.1| fusion protein F2 subunit [bovine ...	48	3e-05
gi|23485704|gb|EAA20535.1| hypothetical protein [Plasmodium...	28	26
gi|11128019|ref|NP_061820.1| cytochrome c [Homo sapiens] >g...	28	26
gi|46250408|gb|AAH68464.1| Cytochrome c [Homo sapiens]	28	26
gi|65436|pir||CCCZ cytochrome c - chimpanzee (tentative seq...	28	26
gi|15929398|gb|AAH15130.1| CYCS protein [Homo sapiens]	28	26

Appendix 15

Sequences producing significant alignments (peptide 48). Score/Identity = 51/100%.44/86%, 32/78%, 30/80%, 28/88-100%

Sequences producing significant alignments:	Score (bits)	E Value
gi|21689587|gb|AAM68160.1| fusion glycoprotein [Human respi...	51	3e-06
gi|21263086|gb|AAM44851.1| fusion glycoprotein [Human respi...	51	3e-06
gi|1912295|gb|AAC57027.1| Fusion protein (F)	51	3e-06
gi|94063|pir||B28929 cell fusion glycoprotein - human respi...	51	3e-06
gi|961615|gb|AAB38520.1| fusion protein [Human respiratory ...	51	3e-06
gi|1353201|sp|P11209|VGLF_HRSVR Fusion glycoprotein precurs...	51	3e-06
gi|29290043|gb|AAO72325.1| fusion protein [Human respirator...	51	3e-06
gi|21689583|gb|AAM68157.1| fusion glycoprotein [Human respi...	51	3e-06

gi|9629375|ref|NP_044596.1|	Fusion protein (F) [respiratory...	51	3e-06
gi|37674746|gb|AAQ97027.1|	fusion protein precursor [Human ...	51	3e-06
gi|74932|pir||VGNZR2	cell fusion glycoprotein precursor - h...	51	3e-06
gi|37674754|gb|AAQ97031.1|	fusion protein precursor [Human ...	51	3e-06
gi|227299|prf||1701388A	fusion glycoprotein	51	3e-06
gi|3172522|gb|AAC18601.1|	fusion protein [Human respiratory...	51	3e-06
gi|61211|emb|CAA26143.1|	unnamed protein product [Human res...	51	3e-06
gi|961609|gb|AAB38517.1|	fusion protein [Human respiratory ...	51	3e-06
gi|138250|sp|P13843|VGLF_HRSV1	Fusion glycoprotein precurso...	51	3e-06
gi|9629206|ref|NP_056863.1|	fusion protein [Human respirato...	51	3e-06
gi|1695263|gb|AAC55970.1|	fusion glycoprotein precursor [Hu...	51	3e-06
gi|961607|gb|AAB38516.1|	fusion protein [Human respiratory ...	51	3e-06
gi|29290041|gb|AAO72324.1|	fusion protein [Human respirator...	51	3e-06
gi|226438|prf||1512372A	fusion protein	51	3e-06
gi|2582041|gb|AAB82446.1|	fusion protein [Human respiratory...	51	3e-06
gi|12084116|pdb|1G2C|B	Chain B, Human Respiratory Syncytial...	51	3e-06
gi|37674752|gb|AAQ97030.1|	fusion protein precursor [Human ...	51	3e-06
gi|37674748|gb|AAQ97028.1|	fusion protein precursor [Human ...	51	3e-06
gi|29290039|gb|AAO72323.1|	fusion protein [Human respirator...	51	3e-06
gi|961613|gb|AAB38519.1|	fusion protein [Human respiratory ...	51	3e-06
gi|38230490|gb|AAR14266.1|	F [Human Respiratory syncytial v...	51	3e-06
gi|961611|gb|AAB38518.1|	fusion protein [Human respiratory ...	51	3e-06
gi|480767|pir||S37254	cell fusion protein - human respirato...	51	3e-06
gi|138251|sp|P03420|VGLF_HRSVA	Fusion glycoprotein precurso...	51	3e-06
gi|37674750|gb|AAQ97029.1|	fusion protein precursor [Human ...	51	3e-06
gi|138252|sp|P12568|VGLF_HRSVL	Fusion glycoprotein precurso...	51	3e-06
gi|21689579|gb|AAM68154.1|	fusion glycoprotein [Human respi...	51	3e-06
gi|37674744|gb|AAQ97026.1|	fusion protein precursor [Human ...	51	3e-06
gi|138248|sp|P23728|VGLF_BRSVR	Fusion glycoprotein precurso...	44	4e-04
gi|267344|sp|P29791|VGLF_BRSVA	Fusion glycoprotein precurso...	44	4e-04
gi|3451386|emb|CAA76980.1|	F protein [Bovine respiratory sy...	44	4e-04
gi|281366|pir||JQ1583	cell fusion protein precursor - bovin...	44	4e-04
gi|138247|sp|P22167|VGLF_BRSVC	Fusion glycoprotein precurso...	44	4e-04
gi|425678|gb|AAB28458.1|	fusion protein F2 subunit [bovine ...	44	4e-04
gi|9631275|ref|NP_048055.1|	fusion glycoprotein precursor; ...	44	4e-04
gi|19550336|gb|AAL91342.1|	fusion protein [Ovine respirator...	44	4e-04
gi|17939990|gb|AAL49399.1|	fusion protein [Bovine respirato...	44	4e-04
gi|17940002|gb|AAL49410.1|	fusion protein [Bovine respirato...	44	4e-04
gi|45525184|ref|ZP_00176429.1|	hypothetical protein Cwat361...	32	1.4
gi|46119850|ref|ZP_00177482.2|	COG1900: Uncharacterized con...	30	4.5
gi|47222570|emb|CAG02935.1|	unnamed protein product [Tetrao...	28	27
gi|23114746|ref|ZP_00100032.1|	COG1502: Phosphatidylserine/...	28	27
gi|48838151|ref|ZP_00295099.1|	hypothetical protein Meth020...	28	27