Analysis of the molecular basis of the immune response to *Streptococcus pneumoniae* capsular polysaccharide

Dr Helen E Baxendale BSc (Hons) MB BS MRCP (UK)

This thesis is presented to the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine

July 2001

Immunobiology Unit
Institute of Child Health
University College London
London WC1N 1EH
ABSTRACT

Streptococcus pneumoniae is a major cause of infection world wide, particularly in the very young and the elderly. Serotype specific anti-capsular antibodies are known to protect against infection. Studies of human antibodies specific for bacterial derived capsular polysaccharide antigens reveal these antibodies to be oligoclonal and of limited diversity. Relatively little is known of the molecular basis of the human immune response to pneumococcal polysaccharide. With new generation pneumococcal conjugate vaccines now becoming available a better understanding of the human immune response to Streptococcus pneumoniae is of increasing importance.

This thesis describes an analysis of the immune response to two types of pneumococcal vaccine (plain polysaccharide and conjugate) in healthy adult volunteers. Using heterohybridoma technology to produce antigen specific monoclonal antibodies, the diversity of the immune response to pneumococcal polysaccharides was analysed and the molecular characteristics of the antibodies were correlated with in vitro functional activity. A high proportion of the hybridomas produced were isotype switched and highly mutated, inconsistent with their being derived from a primary immune response. Identical genes were used by a number of individuals to generate antibodies to a variety of serotypes and a common replacement mutation was identified in the CDR2 of two clones derived from different individuals and of different sero-specificity. Both ranked highly in the functional assays. Two of the hybridomas generated were isotype switch variants of the same clone and demonstrated marked differences in antibody avidity and opsonophagocytic activity.

These data suggest that the antibody repertoire induced by pneumococcal vaccination in adults may be restricted in V gene use with common V genes and somatic mutations demonstrated to a variety of serotypes and shared between individuals. Mutation analysis demonstrates that the antibody repertoire of adult vaccinees may be dictated not by vaccine formulation but by immune history of each individual in whom priming for memory has already occurred.
ACKNOWLEDGEMENTS

There have been many people who have provided support throughout this period of research. I would particularly like to thank David Goldblatt who, having provided invaluable support in getting this project underway gave me the freedom to run with it. He retained a clarity of vision throughout and as I delved into the minutiae at risk of getting lost, he retained the broad perspective.

Freda Stevenson, Myfanwy Spellerburg and Zadie Davis taught me the laboratory skills essential to get the project off the ground. They and their colleagues at the Molecular Immunology Unit, Tenovus Laboratories, Southampton, were both constructive in trouble shooting and sociable making my time in Southampton most enjoyable.

Robin Callard and Mac Turner provided useful advice during the inevitable lows that come with the job and Robin’s support in providing an exclusive mycoplasma free facility was one of the most important contributing factors making this project a success.

Harry White has been both a pleasure to know and to work with. With extensive knowledge and experience in the field of molecular biology his advice and practical contributions have enabled this work to move into new and exciting areas.

This work has been funded through a Clinical Training Fellowship from the Wellcome Trust.
DEDICATION

This thesis is dedicated to my father. He took me into a virology lab when I was barely 4. We looked down microscopes and he explained what we saw in a way I could understand. As children we were always encouraged to ask questions and ignorance was never misread as stupidity. This encouraged my persistent interest in exploring the wherefore and the why. Life is far richer and more exciting as a result.
1. GENERAL INTRODUCTION

1.1 General Introduction ...................................................... 2
1.2 *Streptococcus pneumoniae* .................................................. 8
1.3 Antibody ................................................................................. 18
1.4 Development of the humoral immune response to *S. pneumoniae* ...................................................... 33
1.5 Aims of this study ................................................................. 46

2. MATERIALS AND METHODS

2.1 General reagents .................................................................. 49
2.2 Plastics/Glassware ............................................................... 51
2.3 Vaccines ................................................................................. 51
2.4 Cell lines ............................................................................... 51
2.5 Software ................................................................................ 52
<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1</strong></td>
<td>9</td>
</tr>
<tr>
<td>Representation of the capsular structure of <em>S.pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.2</strong></td>
<td>17</td>
</tr>
<tr>
<td>Representation of antibody (Ab) mediated opsonophagocytosis of pneumococcus</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.3</strong></td>
<td>19</td>
</tr>
<tr>
<td>Immunoglobulin (Ig)</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.4</strong></td>
<td>24</td>
</tr>
<tr>
<td>Schematic representation of the Ig $V_H$ domain</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.5</strong></td>
<td>27</td>
</tr>
<tr>
<td>The sequence of genetic rearrangement events to produce functional immunoglobulin from germline genes</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.6</strong></td>
<td>29</td>
</tr>
<tr>
<td>Representation of the immunoglobulin variable region and the antigen binding site</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.7</strong></td>
<td>32</td>
</tr>
<tr>
<td>Surface representation of the heavy atoms of 2 Fv fragments fitting the canonical conformations 1-1-2-1-1 and 1-4-3-1-1 (left and right respectively)</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.8</strong></td>
<td>36</td>
</tr>
<tr>
<td>Representation of the T cell independent response to pneumococcal plain polysaccharide vaccines</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.9</strong></td>
<td>39</td>
</tr>
<tr>
<td>The incidence of invasive Haemophilus influenzae type b (Hib) disease in England and Wales between 1989 and 1996</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1.10</td>
<td>40</td>
</tr>
<tr>
<td>1.11</td>
<td>45</td>
</tr>
<tr>
<td>3.1a</td>
<td>79</td>
</tr>
<tr>
<td>3.1b</td>
<td>79</td>
</tr>
<tr>
<td>3.1c</td>
<td>80</td>
</tr>
<tr>
<td>3.2</td>
<td>82</td>
</tr>
<tr>
<td>3.3</td>
<td>88</td>
</tr>
<tr>
<td>3.4</td>
<td>90</td>
</tr>
<tr>
<td>4.1</td>
<td>104</td>
</tr>
</tbody>
</table>

**Legend:**
- **Figure 1.10**
  Representation of the T cell dependent response to pneumococcal conjugate vaccines.

- **Figure 1.11**
  The dynamics of the T-dependent antibody response following primary and secondary antigen exposure.

- **Figure 3.1a**
  Vaccination schedule and post vaccination bleeds.

- **Figure 3.1b**
  Heterohybridoma production.

- **Figure 3.1c**
  Screening heterohybridoma supernatants by ELISA for pneumococcal specific antibody production, subcloning and generation of antibody stocks.

- **Figure 3.2**
  Hoechst stain of mycoplasma contaminated OURI cell line.

- **Figure 3.3**
  Sero-specificity of the 13 serotype specific monoclonal antibodies as determined by competitive inhibition ELISA.

- **Figure 3.4**
  Immunocytochemistry. Cytospin of AbA5 hybridoma cells stained with peroxidase labelled antihuman IgG.

- **Figure 4.1**
  Summary of methods used to sequence the V region DNA from hybridomas.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 4.2</strong></td>
<td>105</td>
</tr>
<tr>
<td>Agarose gels demonstrating $V_h$ gene Pcr products from a panel of pneumococcal hybridomas</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.3</strong></td>
<td>106</td>
</tr>
<tr>
<td>Agarose gels demonstrating $V_l$ gene Pcr products from a panel of pneumococcal hybridomas</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.4</strong></td>
<td>107</td>
</tr>
<tr>
<td>Isotype specific restriction Pcr of whole blood cDNA</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.5</strong></td>
<td>110</td>
</tr>
<tr>
<td>$V_h$ gene amino acid alignments for the 15 pneumococcal specific Mabs</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.6</strong></td>
<td>111</td>
</tr>
<tr>
<td>$V_l$ gene amino acid alignments for the 15 pneumococcal specific Mabs</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.7</strong></td>
<td>112</td>
</tr>
<tr>
<td>Nucleotide sequence alignments of the light chain of Db8C11 and Db7D4 aligned to the $V_{\text{ll}}$-A17 germline gene</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.8</strong></td>
<td>113</td>
</tr>
<tr>
<td>Nucleotide sequence alignments of the heavy chain of Db8C11 and Db7D4 aligned to the $V_{\text{h}}$3-07 germline gene</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.9</strong></td>
<td>117</td>
</tr>
<tr>
<td>Restriction Pcr profile of six Mabs</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.10</strong></td>
<td>120</td>
</tr>
<tr>
<td>Isotype specific restriction Pcr profile of D7 peripheral blood $V_{\text{h}}$3 genes</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 4.11</strong></td>
<td>121</td>
</tr>
<tr>
<td>Isotype specific restriction Pcr profile of D7 peripheral blood $V_{\text{h}}$1 genes</td>
<td></td>
</tr>
<tr>
<td>LIST OF FIGURES cont.</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Figure 5.1</strong></td>
<td>140</td>
</tr>
<tr>
<td>Outline of procedure to evaluate opsonophagocytosis of pneumococci by serum and Mabs +/- complement</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.2</strong></td>
<td>141</td>
</tr>
<tr>
<td>Avidity of binding of Mab Db3G9, D0 and D28 post vaccination serum to PncPS serotypes 6B as determined by ammonium thiocyanate elution ELISA</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.3</strong></td>
<td>144</td>
</tr>
<tr>
<td>Histogram plots showing percentage opsonophagocytosis of FITC labelled pneumococci by peripheral blood PMNs after pre-incubation with three pneumococcal Mabs</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.4</strong></td>
<td>145</td>
</tr>
<tr>
<td>Opsonophagocytosis of pneumococci by IgG Mabs and post vaccination sera</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.5</strong></td>
<td>146</td>
</tr>
<tr>
<td>Opsonophagocytosis of pneumococci by three IgA and one IgM Mabs and post vaccination sera</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.6</strong></td>
<td>147</td>
</tr>
<tr>
<td>Opsonophagocytosis of four different pneumococcal serotypes by polyreactive IgM Mabs</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.7</strong></td>
<td>150</td>
</tr>
<tr>
<td>Correlation of Avidity Index (Al) and opsonophagocytic activity of IgG Mabs at a concentration of 0.5μg/ml</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 5.8</strong></td>
<td>150</td>
</tr>
<tr>
<td>Correlation of Avidity Index (Al) and opsonophagocytic activity of IgA Mabs at a concentration of 0.5μg/ml</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1 20
Characteristics of the human antibody isotype and subclasses

Table 1.2 30
Distribution of human Vh genes within the 3 clans

Table 1.3 32
Clan allocation and H1-H2 canonical structure classification for human Ig Vh gene families

Table 2.1 65
Primer sequences for PCR

Table 3.1 74
Frequency, absolute numbers and isotype of circulating AbSC specific for different pneumococcal serotypes and CWPS from a pool of eight subjects on day 7 post-vaccination with 23V-PS

Table 3.2 83
Results of mycoplasma DNA hybridisation test for batch 1 OURI cells

Table 3.3 83
Results of mycoplasma DNA hybridisation test for batch 2 OURI cells

Table 3.4 85
Serum antibody concentration (μg/ml) of IgM, IgA and IgG to 5 pneumococcal serotypes pre- and day 28 post-pneumococcal vaccination

Table 3.5 86
Serotype and Isotype distribution of hybridoma clones
LIST OF TABLES cont.

Table 3.6 89
Immunohistochemistry of selected hybridomas to evaluate % of cells producing human Mab

Table 3.7 92
Antibody concentration in fresh supernatants and supernatant concentrates from serotype specific IgG producing hybridomas as determined by ELISA

Table 4.1 109b
Molecular profiles of 15 Human Monoclonal Antibodies (Mab) from 5 subjects

Table 4.2 115
Canonical Structure classification of Mabs. N/A = not available ND = not determined *Unassigned

Table 5.1 142
Avidity indices (AI) measured by modified ELISA from 7 pre and post vaccination serum samples for serotype specific IgG, 5 IgG, 3 IgA and 1 IgM Mab

Table 5.2 148
Opsonophagocytic indices (OI) for Mabs
ABBREVIATIONS

Ab  antibody
Abs  absorbance
AbSC  antibody secreting cell
AFC  antibody forming cell
Ag  antigen
AI  avidity index
APC  antigen presenting cell
BcR  B cell receptor
BSA  bovine serum albumin
BSA-PBS-T  phosphate buffered saline / 0.5% tween 20 / 1% bovine serum albumin
C  complement
CbpA  choline binding protein A
cDNA  complementary DNA
CDR  cluster of differentiation region
Ch  heavy chain constant region
Cl  light chain constant region
CL  confidence limits
CR  complement receptor
CRM 197  mutant diphtheria toxin cross reactive material 197
CRP  C reactive protein
CWPS  cell wall polysaccharide
D  diversity
D7  day 7 post vaccination
D28  day 28 post vaccination
DAB  3,3-diaminobenzidine
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
dsDNA  double stranded deoxyribonucleic acid
EBV  Epstein Barr virus
EBVTB  EBV transformed B cells
ELISA  enzyme linked immunosorbent assay
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MØ</td>
<td>macrophage</td>
</tr>
<tr>
<td>Mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Men C</td>
<td>meningococcus serotype C</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>M-MuLV</td>
<td>monkey maloney leukaemia virus</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cell</td>
</tr>
<tr>
<td>M-OMP</td>
<td>meningococcal outer membrane protein</td>
</tr>
<tr>
<td>MWt</td>
<td>molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NOC</td>
<td>non opsonin control</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OI</td>
<td>opsonophagocytic index</td>
</tr>
<tr>
<td>OURI</td>
<td>ouabain resistant</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>P</td>
<td>palindromic nucleotide</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate buffered saline / 0.5% Tween 20</td>
</tr>
<tr>
<td>PC</td>
<td>phosphorylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>Pnc</td>
<td>pneumococcus</td>
</tr>
<tr>
<td>PPS</td>
<td>pneumococcal capsular polysaccharide</td>
</tr>
<tr>
<td>PRP</td>
<td>polyribose phosphate</td>
</tr>
<tr>
<td>PS</td>
<td>polysaccharide</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>R₉</td>
<td>RPMI 1640 no additives</td>
</tr>
<tr>
<td>R₁₀</td>
<td>RPMI 1640 with additives and 10% foetal calf serum</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
</tr>
<tr>
<td>R/S</td>
<td>replacement substitution</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S</td>
<td>switch region</td>
</tr>
<tr>
<td>slg</td>
<td>surface immunoglobulin</td>
</tr>
<tr>
<td>slgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosis</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T dependent</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotide transferase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>$T_h$</td>
<td>T helper lymphocyte</td>
</tr>
<tr>
<td>$T_{h1}$</td>
<td>T helper lymphocyte type 1</td>
</tr>
<tr>
<td>$T_{h2}$</td>
<td>T helper lymphocyte type 2</td>
</tr>
<tr>
<td>TI</td>
<td>T independent</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>V</td>
<td>variable</td>
</tr>
<tr>
<td>$V_L$</td>
<td>light chain variable region</td>
</tr>
<tr>
<td>$V_H$</td>
<td>heavy chain variable</td>
</tr>
<tr>
<td>7V-CRM$_{197}$</td>
<td>Pneumococcal 7 valent polysaccharide conjugate vaccine</td>
</tr>
<tr>
<td>23V-PS</td>
<td>Pneumococcal 23 valent plain polysaccharide vaccine</td>
</tr>
</tbody>
</table>
DISCLAIMER

The author performed all of the experimental work described in this thesis with the exception of the following:

i. Mrs Z. Davis (Molecular Immunology Unit, Tenovus Laboratories, Southampton General Hospital, Southampton UK) performed the heavy chain sequencing reaction for 6 of the 15 hybridomas from PCR amplified Vh genes provided by the author.

ii. Dr H. White (Molecular Immunology and Immunobiology Unit, ICH, London UK) performed the restriction digest Pcr on cDNA produced from hybridomas and whole blood lymphocytes provided by the author.

iii. Dr J. Reinholdt (Dept Microbiology, Aarhus University, Aarhus, Denmark) determined the IgA subclasses of the 4 IgA antibodies.
CHAPTER ONE
INTRODUCTION

1.1 General Introduction ................................................................. 2
1.2 Streptococcus pneumoniae ............................................................. 8
  1.2.1 Structure .............................................................................. 10
  1.2.2 Pathogenesis ....................................................................... 13
  1.2.3 Host defence mechanisms .................................................... 15
1.3 Antibody ....................................................................................... 18
  1.3.1 Antibody structure and function .......................................... 18
  1.3.2 Immunoglobulin Isotypes and the mechanism of class switching .... 20
  1.3.3 Immunoglobulin variable region genes and the generation of antibody diversity ................................................................. 23
  1.3.4 Evolution of V genes and their canonical structures ................. 30
1.4 Development of the humoral immune response to S.pneumoniae.. 33
  1.4.1 Primary response ................................................................. 34
  1.4.2 Secondary Immune response and generation of memory .......... 44
1.5 Aims of this study ........................................................................ 46
1.1 General Introduction

*Streptococcus pneumoniae* (pneumococcus, Pnc) are pathogenic bacteria which are a leading cause of serious disease worldwide. They are one of the leading causes of pneumonia, septicaemia and meningitis in man. In addition they cause a number of other less severe but more common diseases such as otitis media and pharyngitis with considerable morbidity. Infection can occur in any age group although it is the very young and the old who are particularly susceptible to severe disease. Other high risk groups include patients with sickle cell disease, nephrotic syndrome and primary and secondary immunodeficiencies including HIV disease.

Over 90 different pneumococcal serotypes have been identified and the dominant disease-causing serotypes vary with age, race and geographical location (Smart *et al.*, 1987; Sniadack *et al.*, 1995; Scott *et al.*, 1996). In addition, they may be different for invasive and non-invasive disease. Antibiotic resistant strains of this bacteria are emerging and have spread globally (Crook & Spratt, 1998; Barry, 1999; Tomasz, 1999) with some European strains demonstrating up to 40% resistance to penicillin and 10% resistance to macrolide antibiotics.

*Streptococcus pneumoniae* remains an important pathogen, accounting for up to 5 million deaths per annum worldwide. It affects all age groups although infants and the elderly are particularly susceptible to infection.

Serotype specific anticapsular antibody protects against invasive pneumococcal disease and the first study demonstrating successful immunisation against invasive pneumococcal disease was reported in the Lancet in 1914 (Wright *et al.*, 1914). In this investigation South African miners were vaccinated with heat killed whole pneumococci. The basis for the protection provided by whole bacteria was not known until 1930 when it was found that protective antibodies induced by natural disease were specific for the pneumococcal polysaccharide capsule (Francis & Tillett, 1930).
By 1940 a hexavalent pneumococcal polysaccharide vaccine was being used in US military recruits. With the advent of antibiotics, further research and development slowed considerably in anticipation of the successful treatment and cure of bacterial infection with these drugs. In the 1960s, however it was reported that despite the use of antibiotics the morbidity and mortality associated with pneumococcal disease remained high and by the 1970s a number of pneumococcal strains resistant to antibiotics had been isolated. By 1977 a 14 valent vaccine had been developed and licensed in the USA. This was quickly followed by a 23 valent polysaccharide vaccine (licensed in 1983) which is still in use today.

The plain polysaccharide vaccine has an efficacy of 60-80% for protection from invasive pneumococcal disease although immunity wanes after about 5 years with no anamnestic response on subsequent antigen challenge. The vaccine is not immunogenic in infancy and protection in the elderly is unreliable. Consequently it fails to protect the two groups within the population that bear the major burden of disease.

The explanation for this is that polysaccharides are poor immunogens and unlike the response to protein antigens, the immune response to polysaccharides is described as T-independent with the features of delayed ontogeny (ie. poor response in children under 2 yrs of age), no affinity maturation and the absence of immunological memory.

Polysaccharides were recognised to be poor immunogens relative to peptides or proteins in the early 1900s and the effect of conjugating polysaccharides to protein to improve the immunogenicity of the polysaccharide was first described in 1929 when a number of polysaccharides were conjugated to albumin and studied in a rabbit model (Goebel, 1929). The effect of conjugation was to convert the immune response to the polysaccharide from a T independent response to a T dependent response and immunogenicity was improved. The additional benefits of conjugate vaccines: immunogenicity in infancy, affinity maturation and the development of immunological
memory were only later appreciated (Beuvery et al, 1983; Adderson et al, 1998; Kayhty et al, 1989; Anderson et al, 1987).

The first human polysaccharide conjugate vaccine to be licensed was for *Haemophilus influenzae* type b, the leading cause of bacterial meningitis in infancy. Since the introduction of this vaccine into the UK infant immunisation schedule in 1992, invasive Hib disease has been almost eliminated in the target population (Fritzell & Plotkin, 1992; Booy et al, 1994; Mulholland et al, 1997; Heath, 1998; Bower et al, 1998; Dagan et al, 1999). Meningococcus type C (Men C) conjugate vaccine was subsequently developed and licensed for use in 1999. It has also been introduced into the infant immunisation schedule and a nation-wide program of catch up vaccination for all children under the age of 18 was started in November 1999. A recent report has demonstrated a dramatic decline in the incidence of invasive Men C disease in the vaccinated groups (Ramsay et al, 2001). More recently polyvalent pneumococcal conjugate vaccines have been developed. They have been shown to be immunogenic in all age groups and prime for memory in both young adults and infants (Steinhoff et al, 1994; Kayhty et al, 1995; Anderson et al, 1996; O'Brien et al, 1996; Dagan et al, 1997; Eskola, 2000). In addition, the conjugate vaccines are known to be immunogenic in over 50% of adult non-responders to the plain polysaccharide vaccine (Zielen et al, 2000).

Following the successful completion of the first two efficacy studies of a heptavalent pneumococcal mutant diphtheria toxin conjugate vaccine (7V-CRM197) (Black et al, 2000; Eskola J. et al, 2001), the first pneumococcal conjugate vaccine was licensed for use in the USA (Prevanir™ Wyeth Lederle) in February 2000. This was followed one year later with approval for use in Europe. Both studies demonstrated clinical efficacy of the vaccine which was able to reduce the incidence of invasive pneumococcal disease and otitis media (OM) in the vaccinated groups. In the first study the vaccine
demonstrated 94% efficacy in preventing invasive disease by vaccine serotypes. In addition, a reduction in the incidence of all causes of OM of 7% was demonstrated with a 67% reduction in OM caused by vaccine serotypes. The second study assessed vaccine efficacy in reducing the incidence of OM only and produced similar results. A 57% reduction in episodes of OM caused by vaccine serotypes was demonstrated with a 34% reduction in all serotype pneumococcal OM and a 6% reduction in all causes of OM.

Although the clinical efficacy of these vaccines has been clearly demonstrated, the risk of replacement disease caused by non-vaccine related serotypes is an ongoing concern. Replacement disease was not demonstrated with long term follow-up following Hib immunisation however there are many more pathogenic pneumococcal serotypes so that the risk of replacement pneumococcal disease is considered to be higher. The large scale double blind control studies described above demonstrated that the incidence of all type invasive disease was reduced by pneumococcal vaccination with no evidence of replacement disease (Eskola J, 1999; Black et al, 2000) however, this may change as more of the population is immunised. Despite the success of the 2 conjugate vaccine studies described for protection in infancy, it is unclear whether they offer advantage over the simple polysaccharide vaccine in protection of other high risk groups such as the elderly and those with HIV. Serum studies suggest that pneumococcal conjugate vaccines will confer no improvement in immunogenicity over plain polysaccharide vaccines as a single dose in the elderly (Powers et al, 1996), but may be more effective in protecting those with HIV infection (Kroon et al, 2000). Clinical efficacy studies are awaited for both these groups.

Serotype coverage is limited due to the sheer bulk of antigen involved in polysaccharide vaccines. The 7-valent vaccine described comprises the dominant
serotypes of the Western world and does not include 2 of the major disease causing serotypes in developing countries where the burden of disease is greatest (Types 1 and 5). This emphasises the importance of finding non-serotype restricted vaccine antigens. A number of non-serotype specific pneumococcal surface proteins are currently under investigation as potential vaccine candidates (Briles et al, 1997; Paton et al, 1997; Srivastava et al, 2000) to facilitate wider vaccine coverage.

The immune response to pneumococcal vaccines has been shown to vary widely between individuals and different subpopulations. The molecular basis for the variability in antibody response between individuals is poorly understood. It is recognised that the diversity of antibody to polysaccharide antigens may be limited at a genetic level (Shackelford et al, 1988; Adderson et al, 1991; Adderson et al, 1993; Park et al, 1996; Sun et al, 1999; Kowal et al, 1999) and the immune repertoire has also been shown to vary with the age of the individual (Lucas et al, 1993; Schroeder-HW et al, 1995). It has been suggested that this may in part explain the high susceptibility of the young and the old to invasive pneumococcal disease. A number of functional and genetic studies comparing the antibody response to pneumococcal polysaccharide vaccines in young, mature and aged population groups have been described.

Conjugate and plain polysaccharide pneumococcal vaccines are known to be less immunogenic in the elderly (Shelly et al, 1997, Rubins et al. 1999). Antibody titres are lower overall and in vitro tests of antibody function (avidity and opsonophagocytic activity) have demonstrated reduced functional activity in this age group (Romero-steiner et al. 1999). However studies looking at the immunoglobulin genetic diversity in the aged have not been described.

The ontogeny of the immune response to polysaccharide antigens has been studied by a number of groups. Phosphorylcholine (PC, a component of the cell wall of many
encapsulated bacteria) has been used as a hapten to study the immune response to polysaccharides. Chen et al, 1992a looked at the ontogeny of antibody diversity in response to PC in non-immunised mice. They discovered that 2 types of anti-PC antibodies were produced in new-born mice. One group (group 1) reacted with PC and bacteria containing PC (environmental antigens) whereas the other group (group 2) reacted only with PC-protein conjugates (non-environmental antigens). Group 2 antibodies dominated early in the new-born period and was gradually replaced from day 7 onwards with group 1 antibodies. The V genes known to be dominant in the adult group 1 response to PC (T15 idiotype) were not dominant in the new-born mice and the avidity of the new-born group 1 antibodies was lower than that of the adults. In contrast, the characteristics of the group 2 antibodies which were found at low titre in the adult mice were similar in both groups. This study raises the question: How would neonatal or infant immunisation with a PC-protein conjugate vaccine effect the V gene repertoire of the adult and could this have functional consequences?

Lucas et al, 1993 demonstrated a similar distinction in V gene use between adults an infants in response to a single dose of Hib conjugate vaccine. They found that the pattern of light chain V gene use was markedly different in infants and adults and although the functional consequences of this were not described for the study group, other authors have demonstrated that the V gene selected may have functional consequences (Chung et al, 1995, Lucas & Granoff, 1995).

In addition to the effect of age on immune repertoire, a number of other factors may also be important. It is recognised that both conjugate and simple pneumococcal polysaccharide vaccines are less immunogenic in HIV infected individuals than in healthy adult controls as evaluated by antibody titre (Ahmed et al, 1996; Kroon et al, 2000). To investigate the molecular basis for this, Chang et al, 2000 looked at the effect of infection with human immunodeficiency virus (HIV) on V gene repertoire in
response to pneumococcal vaccination. They found that the immune repertoire of the infected group was markedly different from that of matched non-infected individuals and suggest that aberrant expression of particular V genes may result in a poor vaccine response.

It has been proposed that the individual and sub-population differences in immunoglobulin V gene use in response to bacterial polysaccharides may explain, in part, the increased susceptibility to invasive disease of certain population groups. Diversity of the immune response may also be influenced by the nature of the immunising antigen. A number of Hib studies have demonstrated that different vaccine formulations induce differing antibody V gene repertoires (Granoff et al, 1993; Steinhoff et al, 1994) and as V gene use has been shown to correlate with antibody functional activity this may have important consequences for clinical efficacy of the vaccine. Although there are a number of studies looking at the diversity of the immune response to polysaccharides there is very limited data available describing the diversity of the immune response to pneumococcus. Without this information, the effect of different vaccine formulations or the age at vaccination on antibody repertoire can not be described.

The objective of this study was to determine the genetic characteristics of the immune response to pneumococcal polysaccharide and conjugate vaccines in adult man. The genetic data was then correlated with \textit{in vitro} assays of antibody functional activity to determine whether dominant use of specific genes or gene groups was associated with superior antibody function.

\subsection{1.2 \textit{Streptococcus pneumoniae}}

\textit{Streptococcus pneumoniae} (Pnc) is a gram positive facultatively anaerobic encapsulated bacteria. It grows in pairs or chains and is seen on blood agar as round colonies of 0.5 -1.5mm diameter. The cell wall structure is detailed in Figure 1.1.
Figure 1.1 Representation of the capsular structure of *S. pneumoniae*. The polysaccharide capsule forms a thick coat around the bacterial cell wall into which peptidoglycans, pneumococcal surface proteins such as PpsA, teichoic acids and lipoteichoic acids are anchored. A phospholipid bi-layer forms the cell membrane. Within the cytoplasm a number of lytic enzymes including autolysin, pneumolysin, neuraminidase and hyaluronidase are produced.
Chapter 1  Introduction

Three major layers are seen: the polysaccharide capsule, the cell wall and the plasma membrane. The cell wall consists of a peptidoglycan framework into which teichoic acids, lipoteichoic acids, proteins and capsular polysaccharides (PS) are anchored. The PS capsule varies in thickness depending on the growth phase of the bacteria and is at its thickest when the bacterium is in an exponential phase of growth. The exposure of cell wall constituents, including surface proteins is dependent on capsular thickness.

1.2.1 Structure

Capsular polysaccharide

Antigenic variation in the capsule accounts for the 90 different capsular serotypes identified to date. The classification is dependent on the Quellung reaction in which the bacteria are incubated with a panel of rabbit antisera of known serospecificity. Serotype is confirmed by the demonstration of a halo around the bacterial colonies of homologous serotype (Merrill et al, 1973; Wasilauskas & Hampton, 1984; Mundy et al, 1998). American and Danish classification systems have been used. The American system numbers serotypes sequentially in order of discovery, the Danish system numbers serotypes into serogroups according to antigenic and structural characteristics. Antibody production is generally serotype specific (AlonsoDeVelasco et al, 1995) although some cross reactivity between serotypes but within serogroups has been demonstrated (Nahm et al, 1997). 46 serogroups have been classified within which the serotypes are designated by a capital letter. Some groups contain many serotypes (e.g. 23 A-F) and others only one (eg.14). The Danish classification is more generally accepted internationally and has been used for this study.

The polysaccharide capsule is the major virulence factor for pneumococcal disease. This is due to the ability of the encapsulated pneumococcus to resist phagocytosis in the absence of type specific PS antibody. The capsule is hydrophobic and does not activate complement well in the absence of type specific antibody. It masks other antigens in the cell wall which when exposed are immunogenic and may activate...
complement directly (Holzer et al., 1984). Within a serotype, the quantity of PS is associated with invasiveness. Phenotypes with a high level of encapsulation are opaque and demonstrate enhanced invasiveness (Kim & Weiser, 1998; Jakobsen et al., 1999), less well encapsulated (transparent) phenotypes were less invasive but better at nasal colonisation (Weiser et al., 1994; Cundell et al., 1995b).

**Cell wall polysaccharide and proteins**

A number of antigens are present in the cell wall below the PS capsule. These are also immunogenic and, unlike the PS capsule, are potent inflammatory mediators. They may play a role in colonisation of the nasal mucosa and invasion of the bacterium through host epithelia. Antibody to these antigens, although alone not protective against invasive disease, may complement the effect of PS capsular antibody.

Cell wall polysaccharide (CWPS) (a complex of teichoic acid containing phosphorylcholine (PC) associated with peptidoglycan) and Forssmann antigen (a lipoteichoic acid containing PC covalently attached to the cell membrane) are the two most potent inflammatory components (Briles & Tomasz, 1973). The PC moiety binds the acute phase reactant C reactive protein (CRP) and CWPS when exposed activates complement directly via the alternative pathway (Winkelstein, 1981; Holzer et al., 1984). CWPS is immunogenic and antibodies (non-serotype specific) to CWPS develop early in ontogeny (Gray et al., 1980; Gray et al., 1983; Goldblatt et al., 1992) and are found in the serum of most adults (Briles et al., 1987). However, although anti-CWPS antibodies are protective in mice models of invasive pneumococcal disease (Yother et al., 1982; Briles et al., 1984) there is no evidence that they are protective in man (Chudwin et al., 1985; Musher et al., 1990; Goldblatt et al., 1992). It has been suggested that whilst not able to reduce the incidence of invasive disease, anti-CWPS
Chapter 1  Introduction

Abs may modify disease severity by neutralising the potent inflammatory activity of CWPS (Holzer et al, 1984).

A number of protein antigens have been investigated for immunogenicity in the search for vaccine target antigens. Pneumococcal surface proteins are found anchored to the bacterial cell wall via a choline-teichoic acid/lipoteichoic acid interaction. Although a number of protein antigens have been described, attention has been largely focused on pneumococcal surface protein A (PspA), Autolysin (LytA) and Choline binding protein A (CbpA or SpsA or PspC) (Yother et al, 1982; Briles et al, 1997; Hvalbye et al, 1999). These proteins all appear to be more important as virulence determinants than as immunogens. PspA, a cell-surface protein of variable structure is present on all strains of pneumococci. The functional role of this protein is unclear but it may prevent complement activation and thus inhibit opsonophagocytosis. It is immunogenic in mice and man and has been shown to elicit non-serotype specific protective antibody responses in mice in the absence of capsular polysaccharide (Langermann et al, 1994; Wu et al, 1997; Oggunyi et al, 2000; Briles et al, 2000), protecting against nasal colonisation, pneumonia and bacteraemia. Human studies have also demonstrated that immunisation with PspA can elicit non-serotype specific antibody responses (Nabors et al, 2000) and efficacy studies are now awaited.

LytA is associated with virulence. It can break down the bacterial cell wall releasing the inflammatory components described above (Hvalbye et al, 1999). CbpA binds secretory IgA (sIgA) and facilitates bacterial adherence to human epithelial and endothelial cells.

A number of surface binding proteins have also recently been discovered as important virulence determinants. These include neuraminidase, hyaluronidase, pyruvate oxidase and IgA protease and may inhibit activation of the alternative pathway of complement by preventing C3bBb formation (Tu et al, 1999). Of these enzymes only
the IgA protease, pneumolysin has been assessed as a potential vaccine target antigen. Pneumolysin is an intracellular cytolytic toxin with protease activity specific for human IgA1 and is released in vivo during the autolysis of pneumococci. Pneumolysin is immunogenic and nasopharyngeal IgA antibodies to pneumolysin are produced during pneumococcal colonisation (Virolainen et al., 1995). However mucosal antibody levels do not correlate with serum levels and the role of pneumolysin antibodies in defence against invasive disease in man is unclear.

1.2.2 Pathogenesis

Colonisation

Pneumococcus is generally first encountered in man via the nasal epithelium. Nearly all healthy individuals have been colonised by pneumococcus at some stage of life with the level of colonisation varying depending on age and population (Gwaltney-JM et al., 1975; Gray et al., 1980; Musher et al., 1986; Smith et al., 1993; Greenwood, 1999; Lopez et al., 1999). Highest levels of colonisation are found in the young (pre-school) and where overcrowding is prominent.

The first line of defence against pneumococcal colonisation and invasion is an intact mucosal epithelium. In addition to being a physical barrier, the production of mucus and antibacterial peptides and the ciliation of the epithelium facilitates expulsion of invading bacteria. Pneumococcus adheres to nasal epithelial cells via specific receptors (Andersson et al., 1988) and produces pneumolysin which cleaves IgA into Fc and Fab portions. This renders IgA non-functional thus facilitating nasal colonisation (Kilian et al., 1988) (Paton, 1993).

Studies from Denmark demonstrate that 2-35% of healthy adults are nasal carriers of pneumococcus at any one time (Heilmann, 1990). Colonisation is generally asymptomatic and can persist for several weeks to years. In a small percentage of
cases, invasion of the middle ear, sinuses, bronchial tree or the blood stream may occur. This too is most commonly asymptomatic although in a minority of cases invasive disease ensues. Colonisation is known to be higher in children with middle ear and respiratory tract infections than in age matched controls (Herva et al, 1980; Faden et al, 1990).

Whilst the mechanism for transition from carrier state to disease is not well understood, it is clear that high carriage rates are associated with higher incidence of invasive disease and that recently acquired serotypes are more commonly pathogenic (Gray et al, 1980; Tuomanen et al, 1995; Kellner et al, 1998). However, the correlation between carriage and disease is not linear and, although the serotypes which are prevalent in nasopharyngeal carriage studies are often those causing invasive disease, the rank order of frequency may be different and some disease causing serotypes have never been found in carriage studies (Gray et al, 1980; Hansman & Morris, 1988; Smith et al, 1993; Takala et al, 1996; Kellner et al, 1998).

Nasal colonisation is associated with the development of specific antibody. Recent studies in mice have demonstrated that nasal immunisation with heat killed pneumococci induces local IgA and serum IgG production and protects mice against invasive disease (Hvalbye et al, 1999). The potential for mucosal immunisation in man has not yet been thoroughly investigated. Whilst antibodies induced post systemic immunisation with conjugate vaccines have been shown to reduce carriage of vaccine serotypes (Dagan et al, 1996); (Mbelle et al, 1999) and antibodies to PC and serotype specific PS have been shown to develop with colonisation in man (Svinhufvud et al, 1993), it is unclear whether antibodies induced though mucosal immunisation of man limit colonisation or are protective against disease.

Invasion
Chapter 1  Introduction

The mechanism of invasion of pneumococci through the nasal epithelium is under investigation. Specific glycoconjugate receptors on the respiratory epithelial surface have been identified and are thought to interact with pneumococcal surface proteins and PC to facilitate adhesion (Andersson *et al*, 1988; Cundell *et al*, 1995a). It has been demonstrated that pneumococcal adhesiveness to nasal epithelium is increased with adenoviral co-infection (Hakansson *et al*, 1994) and it is proposed that the mechanism for this may involve viral mediated upregulation of the pneumococcal glycoconjugate receptors. This may explain the well recognised association of viral infection predisposing to invasive bacterial disease.

The thickness of the Pnc polysaccharide capsule of the invading bacteria is important in determining the bacteria-endothelial interaction. Pneumococci with lower levels of encapsulation (transparent phenotype) demonstrate enhanced adherence to epithelial cells and more stable colonisation than the highly encapsulated strains (opaque phenotype) (Weiser *et al*, 1994; Cundell *et al*, 1995b; Weiser *et al*, 1996).

1.2.3 Host defence mechanisms

Innate immunity

Once bacteria have penetrated the epithelium, they are met by both innate and acquired arms of the immune system. The innate mechanism of lectinophagocytosis is important in providing protection from invasion by many bacteria that, although prevalent, are not pathogenic in healthy individuals. Lectins such as surfactant proteins A and D, CRP and mannose binding lectin (MBL) facilitate direct recognition of the invading bacteria by combining with various sugar groups on the bacteria and subsequently interact with special receptors on polymorphonuclear lymphocytes (PMN). Ingestion (phagocytosis) and killing of the bacteria by phagocytes ensues (Ofek & Sharon, 1988; Ofek, 1989; Ofek *et al*, 1995). Lectinophagocytosis has been proposed to operate in defence against pneumococcal disease (Beuth *et al*, 1987;
proposed to operate in defence against pneumococcal disease (Beuth et al, 1987; Hartshorn et al, 1998), however the evidence for this is inconsistent and it has been suggested that it may be useful only for a limited number of serotypes (Silvennoinen & Koskela, 1986; AlonsoDeVelasco et al, 1995; Alonso-De et al, 1995).

The role of complement in innate immunity to Pnc is also unclear. It has been shown to mediate opsonophagocytosis and bacterial clearance of some serotypes however others are resistant to complement fixation (Winkelstein et al, 1981; Johnston et al, 1981). In addition to serotype, the level of encapsulation may also be important. The polysaccharide capsule is known to be a poor activator of the alternative arm of the complement pathway and C3b can be degraded on the capsular surface into less opsonically active components (Angel et al, 1994). CRP when bound to PC residues in the CWPS can fix C3b, however CWPS is buried in the capsule in exponentially growing bacteria and thus may only be useful to facilitate killing of the transparent phenotype (Kim 1999).

**Humoral immunity**

Antibody is the antigen specific product of mature B cells and the production of antibody in the response to pneumococcal infection forms the basis of protective immunity to pneumococcal disease. In the presence of type specific anticapsular antibody, opsonisation, complement fixation, phagocytosis and intracellular killing of the bacteria occurs by PMNs and macrophages (MØ) (Musher et al, 1986). The multiple repeating epitopes of the polysaccharide capsule of the pneumococcus permits multimeric binding of antibody to the bacterial surface via the Fab (fragment antigen binding) region and multimeric binding of antibody to the PMN Fc Receptor (FcR) via the Fc (fragment crystallisable) region (Figure 1.2).
Figure 1.2 Representation of antibody (Ab) mediated opsonophagocytosis of the pneumococcus (Pnc). The pneumococcal polysaccharide capsule is recognised by serotype specific antibody and becomes coated (opsonised). The Fc regions of the opsonising antibody bind to the Fc receptors (FcR) of polymorphonuclear leukocytes (PMN). Phagocytosis with intracellular bacteriolysis ensues.
Multimeric binding increases the avidity of what is a low affinity interaction at a monomer level such that antibody coated (opsonised) bacteria can be phagocytosed. FcR cross-linking at the PMN surface results in PMN activation with subsequent phagocytosis and intracellular bacteriolysis through lysosomal degranulation. Bacterial clearance ultimately occurs in the liver and spleen and it is well recognised that post splenectomy individuals have an increased risk of invasive pneumococcal disease (Kingston & MacKenzie, 1979).

1.3 Antibody

Pnc serotype specific antibody protects against invasive Pnc disease. The structure, function and diversity of antibody will now be described.

1.3.1 Antibody structure and function

A single antibody molecule comprises four chains (Porter 1973), two identical heavy and two identical light chains. The light chains are made up of one variable (\(V_J\)) and one constant region domain (\(C_J\)) and the heavy chain is made up one variable region domain (\(V_{\text{H}}\)) and three or four constant region domains (\(C_{\text{H}}\)). The four chains connect through both non-covalent interactions and disulphide bonds to form a Y shaped molecule of 3 equal sized segments loosely joined by a flexible hinge region (Figure 1.3). There are five main forms of heavy chain constant region (isotypes). Each is associated with a different spectrum of effector responses.

Antibody has two main functions: recognition of specific antigen and recruitment of effector cells to remove the antigen expressing pathogen. These functions are organised through separate regions of the antibody molecule. Proteolytic enzymes have been used to dissect the IgG antibody molecule and determine which parts of the molecule are responsible for different functions (Porter et al 1973). Pepsin cleaves the IgG in the hinge region to generate a bivalent F(\(ab\')_2\) fragment and a dimer of the \(C_{\text{H}}3\)
domains called the pFc' fragment. Papain cleaves the IgG into three fragments (Figure 1.3), two identical Fab fragments and an Fc fragment. The Fab fragments contain the antigen binding sites and consist of the complete light chains paired with \( V_{h1} \) and \( C_{h1} \) of the heavy chains. The Fc fragment is responsible for effector cell recruitment and complement activation and is made up of the paired \( C_{h2} \) and \( C_{h3} \) domains.

Figure 1.3 Immunoglobulin (Ig). Ig consists of two light chains and two heavy chains each with constant and variable region domains. The light chain is of kappa or lambda type and consists of one variable (\( V_l \)) and one constant (\( C_l \)) domain. The heavy chain is of \( \mu, \alpha, \gamma, \varepsilon, \) or \( \delta \) type and consists of one variable (\( V_{h} \)) and 3 or 4 constant region domains (\( C_{h1-4} \)) depending on isotype. Non-covalent and disulphide bonds link the four chains and between the constant domains \( C1 \) and \( C2 \), is a hinge region which facilitates flexibility of antibody binding. The antigen binding site is formed at the V region end by \( V_{h} \) and \( V_{l} \). Within these V regions are three regions of hypervariability which play an important role in antigen binding specificity and affinity.
Truncated Fabs comprising only the V region of the heavy chain linked by synthetic peptide to the V region of the light chain have been developed and are termed single chain Fv (Winter and Milstein 1991). It is this part of the antibody molecule which forms the antigen binding site and determines antigen binding specificity.

1.3.2 Immunoglobulin Isotypes and the mechanism of class switching

The heavy chain C region determines the structure of the antibody molecule. Since the speciation of mammals, five main immunoglobulin classes (isotype) have arisen each with distinct and specialised functional activity (Janeway et al 1967, Spiegelberg 1974 & 1989). The isotypes show a high degree of homology in sequence with the major differences residing in the hinge region. This is particularly marked in the IgG3 subclass which has an elongated hinge which may confer functional advantage.

Any Vh region may associate with any of the isotypes through the process of class switching. The five isotypes are IgM, IgD, IgG, IgA and IgE. IgG is further subdivided into four subclasses: IgG1, IgG2, IgG3 and IgG4. IgA is subdivided into two subclasses called IgA1 and IgA2. The characteristics of the different isotypes are shown in Table 1.1.

<table>
<thead>
<tr>
<th>Isotype</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
<th>IgM</th>
<th>IgA1</th>
<th>IgA2</th>
<th>IgE</th>
<th>IgD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWt (kDa)</td>
<td>146</td>
<td>146</td>
<td>165</td>
<td>146</td>
<td>970</td>
<td>160</td>
<td>160</td>
<td>184</td>
<td>188</td>
</tr>
<tr>
<td>Serum level : mean adult (mg/ml)</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>0.5</td>
<td>1.5</td>
<td>3.0</td>
<td>0.5</td>
<td>0.03</td>
<td>5x10^-5</td>
</tr>
<tr>
<td>Half-life in serum (days)</td>
<td>21</td>
<td>20</td>
<td>7</td>
<td>21</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Complement activation (classical pathway)</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcR binding</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Reactivity with Staph. Protein A</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.1 Characteristics of the human immunoglobulins.
IgG, IgA and IgD have three $\gamma$ domains and IgM and IgE have four $\gamma$ domains. All isotypes within blood except IgM have a molecular weight (MWt) of 146-190 kDa. IgM is a pentamer of four chain subunits and has a MWt of ~900kDa. The serum levels differ widely. IgG1 has the highest serum concentration and the half-life in serum ranges from 2-21 days with IgG being the most long lived. Functional activity is also diverse. IgG and IgM both activate complement efficiently and IgG1, IgG3 and IgA can bind to phagocytes via Fc receptors.

Both IgM and IgA can form polymers. IgM exists mainly as a pentamer although hexamers have occasionally been found in the serum. IgA is found mainly as a dimer in secretions although in plasma it is in monomeric form. Polymerisation of Ig is thought to be important in the binding of repetitive epitopes such as polysaccharides. The strength of binding of individual Ag binding sites (affinity) to polysaccharides is often poor however multiple epitope binding increases the overall strength of the interaction considerably such that the functional affinity (avidity) of antigen binding is high.

While all the progeny of a given B cell express the same V region genes, the C-region genes expressed may change as the B cell matures and proliferates and thus isotypic variants of the same clone may be seen (Radbruch et al 1980). Every B cell initially expresses IgM and IgD. During maturation of the B cell, IgD expression is lost and, following encounter with antigen, IgM secretion begins. As the response matures, the irreversible process of isotype switching to IgG, IgA or IgE isotypes may occur. This process is largely cytokine driven and requires the costimulation of T cell derived signals (Wu et al, 1999).

Murine studies using mouse B cells, bacterial lipopolysaccharide and cytokines in vitro have shown that different cytokines preferentially induce switching to specific isotypes, inhibiting production of others (Callard & Turner, 1990). IL-4 preferentially induces
IgG1 and IgE production, IL-5 augments IgA production, IFN-γ induces IgG3 and IgG2a production and TGF-β induces IgA and IgG2b production. These cytokines appear to work by inducing transcription of heavy chain switch regions (Goodman et al., 1993).

IgM is the first isotype to be produced. Early IgM is generally of low affinity as it is produced before the process of somatic hypermutation has commenced (Klein et al. 1983). However the polymeric nature of IgM (it has 10 Ag binding sites) may result in a high overall binding affinity (avidity). IgM activates complement efficiently and is usually confined to the blood. IgM is also produced in the secondary immune response after somatic hypermutation but is not the dominant isotype and overall IgM accounts for only 10% of circulating antibody. Other isotypes are smaller and are able to diffuse out of the blood stream to other tissues. IgA may be dimeric but IgG and IgE are always monomeric and the affinity of the individual Ag binding sites of these isotypes is important for overall function.

Different isotypes are directed to different parts of the body as determined by their distinctive effector activities. The role of IgA in systemic immunity is unclear and its major function is as a neutralising antibody preventing pathogen adherence and invasion through the mucosal barrier (Mestecky and McGhee 1987). Thus IgA dominates in secretions from mucosal epithelium of the gastrointestinal and respiratory tract. IgG is more effective in the control of systemic infection. It fixes complement and is an effective opsonin. IgG is found equally distributed between the intravascular and extravascular spaces.

IgE, which is involved in anti-parasitic immunity, is found at very low levels in the blood and locates mainly on mast cells just below the skin and mucosa (Metzger 1991). Ag-IgE binding triggers mast cell degranulation with release of histamine and other mediators which induce coughing, sneezing and vomiting, reactions which facilitate
expulsion of the invading pathogen. The function of IgD is unclear (Vladutiu 2000, Preud'homme et al. 2000 review). It is found in the serum at very low concentrations but is expressed at high concentrations on the B cell surface. It may play a regulatory role in the humoral response and the maintenance of B cell memory.

1.3.3 Immunoglobulin variable region genes and the generation of antibody diversity

The antibody repertoire within an individual consists of at least $10^{11}$ different antigen specificities. This diversity is achieved through a number of mechanisms all of which produce amino acid sequence variability within the immunoglobulin variable region domains.

The constant and variable region encoding genes are separate in all cells except lymphocytes committed to the B cell lineage. Early in B cell ontogeny, DNA rearrangement occurs which brings the separate regions together. The heavy chain variable domain is encoded by three genes, a V gene, a diversity gene (D gene) and a J gene (Hozumi et al. 1976) (Figure 1.4).

There are multiple copies of all the gene segments in germ line DNA (Pascual & Capra, 1991). The exact number of functional gene segments varies between individuals by insertion or deletion during meiotic recombination and through mutation which may transform a functional gene into a non-functional pseudogene (Cook & Tomlinson, 1995). The functional genes are organised in three clusters: κ and λ light chain genes and heavy chain genes. They are all on separate chromosomes and are organised differently between species. In man, the κ genes are on chromosome 2. A cluster of Vκ genes (n~40) is followed by a cluster of Jκ genes (n~5) and a single Cκ gene. The λ genes are on chromosome 22 with a cluster of Vλ genes (n~30) followed by pairs of Jλ (n~4) and Cλ genes. The heavy chain genes are mainly found
on chromosome 14 and are clustered in a similar way to the \( \kappa \) genes (\( V_\kappa n = 51, D_\kappa n = 27, J_\kappa n = 6 \)) (Cook & Tomlinson, 1995). A number of non-functional \( V_\kappa \) genes have also been located on chromosomes 15 and 16 (Tomlinson et al, 1994).

Figure 1.4 Schematic representation of the Ig \( V_\kappa \) domain. The \( V_\kappa \) region is made up of \( V, D \) and \( J \) genes joined to a \( C_\kappa \) region. There are 51 functional \( V \) genes, 27 \( D \) genes and 6 \( J \) genes identified to date. Within the \( V \) region are three areas of hypervariability: CDR1, 2 & 3. The first two lie within the \( V \) gene and the third arises at the VDJ junction.

Somatic Recombination

A complex of cleavage and repair enzymes orchestrated the somatic recombination of \( V(D)J \) genes to produce functional antibody. The initiating enzymes are specialised for immunoglobulin gene rearrangement and are derived from the recombination activating genes RAG-1 and RAG-2 (Oettinger et al 1990). Heavy chain gene rearrangement occurs first with DJ combination, a \( V \) gene then rearranges to complete the segment (Figure 1.5).
In both light and heavy chains, the J genes are separated from the C region genes (C genes) by non-coding DNA that is spliced out after transcription. VDJ gene rearrangement is guided by flanking sequences adjacent to the point of recombination (Tonegawa 1983). The sequences consist of a heptamer : 5’CACAGTG3’ contiguous with the coding sequence and followed by a spacer of between 12-23 base pairs. This is followed by a nonamer : 5’ACAAAAACC3’. The spacer sequence differs but is conserved in length and corresponds with 1-2 turns of the DNA double helix. This brings the heptamer/nonamer sequences to the same side of the helix to bind the proteins catalysing recombination. The heptamer-spacer-nonamer arrangement is known as the recombination signal sequence (RSS). The length of the spacer segment determines which genes can recombine and prevents direct V-J joining in the heavy chain. It has been suggested that D-D joining can occur (Meek et al, 1989; Mansikka & Toivanen, 1991; Bertoni et al, 1997; Numasaki et al, 1998) and does so in ~5% of human antibody. This would add to repertoire diversity and may be the most common reason for extra long CDR3 loops in the heavy chain of some antibodies. However, Corbett et al (1997) refute the possibility of D-D joining and suggest that conventional VDJ joining alone can account for the variability of the CDR3.

Following successful rearrangement, the μ heavy chain associates with a surrogate or pseudo light chain (V_{μεβλ}) and this complex is expressed on the B cell surface (Nemazee, 2000). The light chain VJ genes then rearrange (Tsubata et al 1992). Successful joining of the rearranged heavy and light chains results in expression of complete IgM on the immature B cell surface. This, in association with alpha and beta protein chains is known as the B cell receptor (BcR) (Venkitaraman 1991). Not all V genes are used with the same frequency and not all V_{μr}-V_{λ} combinations pair successfully.
The multiple V(D)J gene combinations available and the pairing of different heavy and light chain combinations may give rise to $\sim 2.5 \times 10^8$ different antibody molecules. Further diversity is achieved during the recombination process where imprecise joining of V(D)J segments and N and P addition occurs (Berek and Milstein 1987, Golding et al 1987). N nucleotides (non template encoded) are added by the enzyme: terminal deoxynucleotide transferase (TdT) to single stranded DNA after cleavage and before recombination. Up to 20 nucleotides may be added in this way. Repair enzymes will trim unmatched ends and synthesise complementary bases to produce double stranded DNA. N addition was originally thought to be more common in heavy chains as TdT is expressed for only a brief time in murine B cell development, during heavy chain gene rearrangement. However more recent data from man demonstrates high levels of N addition in both the kappa and lambda light chains suggesting that TdT activity continues throughout the whole process of gene rearrangement (Bridges-SL, 1998). P nucleotides (palindromic) make up palindromic sequences which are also added to the ends of the gene segments during gene rearrangement. Repair enzymes add the complementary nucleotides to produce double stranded palindromic sequences at the V(D)J joins.

Palindromic sequences are found in most light chain rearrangements and are produced after N addition in the heavy chains. Nucleotides may also be deleted at the junctions by exonucleases: This accounts for the very short CDR3 in some V domains and explains why D gene assignment may be impossible in some cases. This nucleotide addition and deletion is random and may disrupt the reading frame of the coding sequence beyond the joint: a frameshift mutation. This normally leads to a non-functional rearrangement.
Chapter 1

Introduction

Through these mechanisms the CDR3 becomes the most hypervariable region of the V domain and is thought to be largely responsible for differences in fine specificity of antigen binding (Jahn et al., 1995; Xu & Davis, 2000).

Figure 1.5 The sequence of genetic rearrangement events to produce functional immunoglobulin from germline genes.

**Somatic Mutation**

It is proposed that the specificity of antigen binding is determined by the sequence and size of the CDRs which form the complementary surface to which antigen binds (Jones et al., 1986). The only mechanism through which immunoglobulin specificity
may be altered once functional heavy and light chains have been generated is somatic hypermutation of the rearranged V regions (Benner and Milstein 1966, Tonegawa 1983). Point mutations may be introduced throughout the V-regions of rearranged heavy and light chain genes. However, the variability is not distributed evenly (Wu and Kabat 1970). There are three hypervariable regions found roughly between residues 28-35, 49-59 and 92-103 in both heavy and light chains which lie within the CDRs and are more susceptible to mutation. The HV regions lie between four framework regions, designated FR1, FR2, FR3 & FR4, which are relatively constant. The framework regions form β pleated sheets which constitute the structural framework of the variable domain and the hypervariable regions form the loops at the edge of the sheet. When the V_{H} and the V_{L} domains come together the HV loops or CDRs from each domain come together to form a single hypervariable antigen binding site at the tip of the Fab (Figure 1.6).

Mutational hot spots consisting of four to five nucleotides have also been found in both HV and framework regions (Lara et al, 1995). Silent mutations which preserve amino acid sequence are unselected and found scattered throughout the sequence. Replacement mutations which occur within a CDR and are associated with improved antigen binding specificity and affinity may be positively selected (Kocks & Rajewsky, 1988; Bye et al, 1992; Davies & Riechmann, 1996; England et al, 1999). Expansion of B cells expressing the mutated antibody then occurs, a process known as affinity maturation (Steiner et al 1967, Siskind et al 1969, Berek and Milstein 1987, Nossal 1992).
Figure 1.6 Representation of the immunoglobulin variable region and the antigen binding site. The top picture represents a side on view and the bottom picture represents a view from above. The framework regions are shown in grey and the hypervariable regions/CDRs are shown in colour. Reproduced from the IMGT, the international ImMunoGeneTics database http://imgt.cnusc.fr:8104.
1.3.4 Evolution of V genes and their canonical structures

Sequence analysis of immunoglobulin variable region genes have demonstrated that V genes are grouped into families, canonical (structural) classes and clans on the basis of amino acid sequence and structural homology which reflects the early events of gene duplication in the evolution of the locus (Sims & Taussig, 1990; Schroeder-HW et al, 1990; Vargas et al, 1997). Members of V gene families show over 80% sequence homology at the nucleotide level (Kodaira et al, 1986; Lee et al, 1987; Berman et al, 1988; Willems-van et al, 1989; Tomlinson et al, 1992) and V gene restriction or the use of dominant V genes in response to specific antigens has been described. In man there are seven Vh andVk families and eight Vl families.

The seven Vh families are further sub-grouped into three clans (Kirkham et al, 1992) (Table 1.2) which share similar clustering with other animal groups including non-mammalian species suggesting common ancestral origin (Schroeder et al, 1990).

<table>
<thead>
<tr>
<th>Clan</th>
<th>V gene families</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vh1, Vh5, Vh7</td>
</tr>
<tr>
<td>2</td>
<td>Vh2, Vh4, Vh6</td>
</tr>
<tr>
<td>3</td>
<td>Vh3</td>
</tr>
</tbody>
</table>

Table 1.2 Distribution of human VH genes within the three clans.

Canonical class is determined by the HV loop length and the location of conserved residues at key positions within both the hypervariable and the framework regions (Tomlinson et al, 1995; Vargaz-Medrazo et al, 1995a&b; Almagro et al, 1996). Although ~300 possible canonical conformations are possible, analysis of antibodies of known atomic structure has revealed that over 85% of antibodies of known
sequence use one of a small number of main chain conformations or canonical structures for L1, L2, L3 and H1 and H2 regions (Chothia & Lesk, 1987; Chothia et al, 1989; Vargas et al, 1995) which are conserved between species.

The canonical structure defines the recognition properties of the antibody (Vargas-Madrazo E, 1995; Almagro et al, 1997; Vargas et al, 1997) and it is thought that H1, H2 and L1 are most important for antigen binding. Antibodies raised against native proteins use a different canonical structure to that used by smaller antigens such as peptides, haptens and polysaccharides and it has been suggested that the nature of the epitope binding in these 2 groups is different. Antibodies to proteins are considered to bind to surface epitopes that are discontinuous with the primary structure of the protein (Figure 1.7). In contrast, antibodies to smaller antigens usually bind in a more continuous fashion to the HV loops formed by the cleft between V\textsubscript{H} and V\textsubscript{L} (Lara et al, 1996).

V gene family identity is based on nucleic acid sequence homology although there may be marked structural diversity of antibodies within the same V gene family. V gene canonical classification has demonstrated that each V gene family uses a minimum of two different types of canonical structure (Table 1.3). V\textsubscript{H}1 is most homogeneous with the two canonical groups used by this family having a similar conformation. V\textsubscript{H}3 makes the largest contribution to structural diversity with members in each of 4 very different canonical groups. V\textsubscript{H}2, 4 and 6 (Clan 2 genes) also contribute significantly to structural diversity and use three quite distinct canonical conformations.
Chapter 1

Introduction

Figure 1.7 Surface representation of the heavy atoms of two Fv fragments fitting the canonical conformations 1-1-2-1-1 and 1-4-3-1-1 (left and right respectively). The structure depicted are derived from antibodies specific for lysozyme and phosphocholine respectively.

Table 1.3 Clan allocation and H1-H2 canonical structure classification for human Ig \( V_h \) gene families (Vargaz Madrazo et al 1997).

<table>
<thead>
<tr>
<th>Vh family</th>
<th>Clan</th>
<th>Canonical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vh1</td>
<td>1</td>
<td>1-2, 1-3</td>
</tr>
<tr>
<td>Vh2</td>
<td>2</td>
<td>1-1, 2-1, 3-1</td>
</tr>
<tr>
<td>Vh3</td>
<td>3</td>
<td>1-1, 1-3, 1-4, 1-6</td>
</tr>
<tr>
<td>Vh4</td>
<td>2</td>
<td>1-1, 2-1, 3-1</td>
</tr>
<tr>
<td>Vh5</td>
<td>1</td>
<td>1-2, 1-3</td>
</tr>
<tr>
<td>Vh6</td>
<td>2</td>
<td>1-1, 2-1, 3-1</td>
</tr>
<tr>
<td>Vh7</td>
<td>1</td>
<td>1-2, 1-3</td>
</tr>
</tbody>
</table>

These analyses suggest that antibody recognition of antigen occurs in two stages. Initially antibody with a canonical structure complementary to the specific antigen type
binds. Selective expansion of B cells then occurs using V genes with a shape or chemical composition which produces a tighter fit. Further selection then follows through the process of somatic hypermutation in which the fine specificity and affinity of antigen binding is improved although the overall conformation is unaltered.

1.4 Development of the humoral immune response to *S.pneumoniae*

A number of studies analysing the humoral response to a variety of antigens including bacterial polysaccharides, proteins and autoantigens have demonstrated that the mature antibody response is oligoclonal with the preferential expression of specific V genes and light chains. In addition, identical somatic mutations have been demonstrated between individuals producing antibody to a common antigen. It is not known, however, whether the diversity of the immune response to wild type infection is similar to that induced by vaccination with the most immunogenic antigens expressed by the pathogen. This issue has particular relevance with regard to vaccine antigens inducing a T cell dependent (TD) immune response with the induction of memory. Multiple vaccinations in infancy may have a profound effect on the immune repertoire of the vaccinee. This repertoire is likely to be affected by a large range of factors such as age, race, immune history, number of vaccine antigens included in the schedule and the formulation of the vaccines used.

In most of the studies analysing the ontogeny of the antibody response to pneumococcus, pneumococcal polysaccharide and conjugate vaccines have been used as the immunising antigen. Little data is available in which the response to infection with the encapsulated bacteria is described. In the light of new data documenting the presence of pre-vaccination memory to bacterial polysaccharide in an adult vaccinee (Hougs *et al*, 1999), it is unclear whether the data produced from
vaccine studies can be used as a surrogate to understand the mechanisms involved in the ontogeny of immunity to the live bacterium. Using data generated from vaccine studies in mouse and man, the current understanding of the ontogeny of the immune response to pneumococcal polysaccharide and conjugate vaccines is discussed.

1.4.1 Primary response

Antibody is the antigen specific product of mature B cells. B cells do not produce antibody until they have been stimulated by specific antigen which they recognise through their surface immunoglobulin: the B cell receptor (BcR). There are two main types of B cell response: T dependent (TD) and T independent (TI), each with quite distinct characteristics. Most antigens induce TD responses although the immune response to plain polysaccharides such as pneumococcal capsular polysaccharide is characterised by being TI.

1.4.1.1 T-independent response

The classification of TI originates from murine studies in which nude, athymic mice have been shown to produce antibody in response to Pnc PS vaccination (Jutila et al, 1975; Fairchild & Braley, 1983). TI antigens may be further subdivided into type 1 (TI-1) and type 2 (TI-2) antigens which have distinct characteristics (Mosier et al 1982). TI-1 antigens include some polysaccharides, polypeptides and polynucleotides. They are immunogenic in neonates and induce polyclonal B cell activation which is totally independent from the regulatory activity of T cells (Mosier et al. 1977). TI-2 antigens are multideterminate high molecular weight polymers such as pneumococcal capsular polysaccharides (Sela et al.1972; Mosier et al. 1977). They are not immunogenic in infancy and immunogenicity only develops once the child is between two - four years of age (Smith et al 1973, Peltola et al 1977). TI-2 antigens are not polyclonal activators and the multiple repeating oligosaccharide units of the polysaccharide bind
to the mature B cell via a specific interaction with the BcR. Multimeric BcR binding induces BcR cross-linkage and subsequent lymphocyte activation with clonal expansion and antibody production (Figure 1.8) (Mond et al. 1995).

Additional co-stimulation may be provided by co-ligation of the CD19/CD21 complex through complement fragments such as C3d which bind to polysaccharide and antigen-antibody complexes (Fearon and Carroll 2000).

Although TI-2 antigens may induce antibody in the absence of T cells (Jutila et al., 1975) animals with no T cells at all have greatly diminished antibody responses to these antigens and it is clear that when present, T cells are involved in regulating the humoral response (Schuler et al., 1984; Heilmann et al., 1988; Baker, 1990; Mond et al., 1995). The mechanism underlying T cell regulation of the TI-2 response is unknown.

The T cell receptor is specific for protein or peptide antigens and does not classically bind polysaccharide antigens. It has been suggested that regulation may be mediated through a population of non-thymus derived peripheral T cells exists which may recognise polysaccharide antigen (Baker, 1992; Poccia et al., 1998).

Other features of the TI immune response include delayed isotype switch, little affinity maturation and the absence of immunological memory with little or no boosting of antibody titre on subsequent antigen challenge (Braley-Mullen 1978). These features can largely be explained by the lack of cognate T-B-Ag interaction in which T cell derived cytokines such as IL-4, IL-5, IFN-γ and TGF-β are known to induce B cell differentiation into plasma and memory cells, isotype switch and V gene somatic hypermutation (Wu et al. 1999, Guttormsen et al. 1999) (see below).
Chapter 1 Introduction

Figure 1.8 Representation of the T cell independent response to pneumococcal plain polysaccharide vaccines. The branched multiple repeating oligosaccharide units of the pneumococcal polysaccharide capsule permit cross linking of the B cell receptor (BcR) which consists of surface immunoglobulin (Ig) in a complex with an unknown number of invariant proteins Igα and Igβ. When Ig is ligated by antigen, the invariant proteins are required to signal to the B cell to mature and divide producing a population of antibody secreting plasma cells. Additional co-stimulatory signals may be produced by the B cell co-receptor complex which consists of CD21 (the complement receptor 2 or CR2) and CD19. CD21 is a receptor for complement fragments such as C3d which are known to bind to antigen antibody complexes. Co-ligation of the B cell receptor (BCR) with its CD19 and CD21 amplifies the signalling through the BCR considerably.

The delayed ontogeny of the response to polysaccharide antigens is thought to be due to immaturity of the infant B cell population (Barrett, 1985). Whilst the exact
mechanism for the poor responsiveness of infants to TI-2 antigens is not known, infant B cells differ from adult B cells in a number of ways. It is recognised that immature B cells are inactivated by multiple repeating epitopes which characterise polysaccharide antigens. Neonates also demonstrate low expression of the complement receptor CR2 in their splenic marginal zone lymphocytes (Timens et al, 1989). The recognition of C3d by CR2 is believed to provide an important stimulation signal in the absence of T cell involvement (Griffioen et al, 1992).

The expression of CD5 may also be relevant. IgM+ B cells are divided into CD5+ and CD5- populations which have both phenotypic and functional differences (Kearney, 1993; Hardy and Hayakawa, 1994). In adults, 5% of peripheral B cells express CD5, and 95% are CD5 negative. The CD5- B cell subset has been shown to be the dominant subset of AbSC to pneumococcus (Barrett et al, 1992) and this subpopulation is rare in infancy, increasing in proportion with age in a temporal course similar to the acquisition of responsiveness to PS antigens.

Most studies analysing the dynamics of the immune response have looked at the response to TD antigens and the dynamics of the B cell response to TI antigens is less well defined. Murine studies looking at the TI-2 response to a synthetic antigen: 4-hydroxy-3-nitrophenyl acetyl Ficoll have demonstrated that within 24 hours of intraperitoneal immunisation, antigen specific B cells can be found distributed throughout the splenic tissue (de-Garcia et al, 1999). Selective accumulation in the outer T zone then occurs. The exponential growth that leads to plasma cell production occurs only in the extra follicular regions with a proliferation peak by day five post vaccination. However persistent presence of proliferating antibody secreting cells have been found in the spleen at least three months post vaccination. It is not known whether the persisting Ag specific B cells are derived from an ongoing recruitment of virgin B cells or sustained self renewal of the early responders however the phenotype
of these antigen specific cells remains the same and a memory phenotype does not develop.

1.4.1.2 T-dependent response

The immune response to the new pneumococcal conjugate vaccines has the features of a TD response which contribute to the superior efficacy of polysaccharide-conjugate vaccines compared with simple polysaccharide vaccines. These features include immunogenicity in infancy with effective antibody production possible from birth, early isotype switch, affinity maturation and the generation of memory (Shinefield et al 1999, Balck et al 2000). The clinical significance of such antigen modification has been demonstrated by the success of the Haemophilus type b (Hib) infant immunisation schedule in which Hib polysaccharide-peptide conjugate vaccines have virtually eliminated invasive Hib disease in infancy (Booy et al 1994, Heath et al 1998)(Figure 1.9).

To achieve a TD response, T and B cells must interact with the same antigen (cognate recognition) (Guttormsen et al 1999) although the epitope recognised by the two cells may be different (linked recognition). As has been discussed, T cells do not recognise polysaccharide, but when polysaccharide is conjugated to a peptide, linked recognition can occur with the T cell recognising the carrier peptide which is linked to the polysaccharide. Through linked recognition T dependency is conferred on the humoral response to the polysaccharide. A detailed description of the Ag-T-B cell interaction is shown in Figure 1.10.
Figure 1.9 Graphical representation of the incidence of invasive Haemophilus influenzae type b (Hib) disease in England and Wales between 1989 and 1996. Incidence in infancy is represented by the dotted line and all age incidence is represented by the continuous line. Seasonal fluctuations were seen prior to the introduction of the Hib conjugate vaccine in October 1992 after which there was a sharp decline which has persisted.
Figure 1.10. Representation of the T cell dependent response to pneumococcal conjugate vaccines. The polysaccharide moiety of the vaccine antigen is recognised by serotype specific antibody on the surface of the B cell, the B cell receptor (BcR). The polysaccharide conjugate is internalised and digested. The peptide moiety is then expressed on the B cell surface in association with MHC class 2 (MHC II). It is recognised by peptide specific T cells and T-B binding through the TcR, CD40L-CD40 and CD28-B7 occurs. This stimulates T cell production of cytokines such as IL-4 & 5, IFN γ, and TGF β which are received by the B cell. They provide the stimulation for the B cells to divide and differentiate into plasma cells which produce antibody or into memory B cells which move to the bone marrow.
Chapter 1  Introduction

The B cell recognises the polysaccharide moiety of the conjugate via the BcR. The polysaccharide conjugate is internalised and degraded and the B cell then acts as an antigen presenting cell for the conjugate peptide presenting it to the T cell receptor (TcR) of CD4+ T helper cells (Th) in association with MHC class 2 (MHC II) (Gurroms et al 1999). Although the two cells recognise different antigens, the antigens are linked which confers specificity on the interaction.

A number of other T-B receptor-ligand interactions including CD40-CD40 ligand (CD40L) and B7-CD28 are also required to produce a TD humoral response (Wu et al., 1999; Gurroms et al., 1999). The direct cellular interactions that result lead to the production of T cell cytokines such as IL-4 and IL-5 which drive B cell proliferation and development.

B cells can also present antigen non-specifically if it has been internalised via the Fc receptor. However B cells binding a specific antigen via the BcR are much more efficient at presenting degradation products of that antigen than those binding antigen non-specifically (Moller et al 1991, Zimmerman et al 1999, Alfonso et al 1999). The mechanisms of antigen presentation and B cell stimulation, proliferation and the development of a memory compartment has been more intensely studied for TD antigens. Murine studies have shown that in a T-dependent antibody response (TD) antigen is captured and processed by professional antigen presenting cells which migrate from the site of antigen exposure to T cell areas of lymphoid tissue where they then trap antigen specific recirculating T and B cells to stimulate a humoral immune response (Banchereau et al 2000). Direct T-B interaction through cognate or linked recognition and subsequent Th cytokine production drives a new primary focus of B cell expansion in the lymphoid tissue.

By five days post antigen challenge, the B cell progeny then migrate from the T cell rich areas of lymphoid tissue. They move either to extrafollicular foci such as the
medullary cords of lymph nodes or the red pulp of spleen where they become short lived antibody secreting plasma cells (AbSC) (van Rooijen et al 1986, Jacob et al 1991) or to the B cell areas where they form primary follicles which develop into germinal centres (Liu et al 1991, MacLennan 1994, Tarlinton et al 2000). The AbSC of the early foci first secrete IgM but subsequently switch to other isotypes (Nossal and Reidel, 1989, Jacob et al 1991). The antibody produced by these AbSCs is of low affinity antibody (Lalor et al. 1992) and is encoded by V gene segments that have not been altered by somatic hypermutation (Jacob et al 1993, McHeyzer-Williams et al 1993). Despite the low affinity of the early IgM, the pentameric structure of this antibody can bind multivalent antigens such as pneumococcal polysaccharide polyvalently producing high functional affinity (avidity). In addition, IgM is a potent activator of complement (Zhong et al 1999). The combination of early production, polymeric binding and complement activation suggests IgM may have an important role in protection against invasive pneumococcal disease. IgM is the first isotype to be produced but with time, the IgG and IgA isotypes will dominate the response. Early antibody may also help trap antigen on the surface of follicular dendritic cells in the germinal centres (GC) (Tew et al 1997) to facilitate antigen presentation.

T cells comigrate with B cells to form the primary follicles and they produce the cytokines IL-5 and IL-6, which induce further B cell differentiation (Takatsu, 1997). Germinal centers (GCs) develop from the primary follicles with the first week and usually persist for a further three to four weeks. Within the GCs V gene somatic hypermutation occurs. There are about 360 base pairs in heavy and light chain variable genes. While the centroblast is dividing, mutations accumulate in the variable genes at a rate of about one per $10^3$ divisions per base pair (Allen et al, 1987). This results in every second cell acquiring a mutation in the V region with each division. Selection then occurs on the basis of affinity of binding to antigen presented by FDCs.
Chapter 1  Introduction

Higher affinity binders are preferentially expanded and further differentiation subsequently occurs (Weiss et al 1992; Jacob et al 1993). These processes result in the rapid enrichment of clonal B cells expressing surface immunoglobulin with increased affinity for the immunizing antigen (Nossal 1992, MacLennan 1994, Kelsoe 1996).

Selected B cells then move to T cell rich areas of the light zone of the spleen where they exchange signals to induce further proliferation of antigen specific T and B cells and differentiation of B cells into antibody secreting plasma cells and memory B cells. After two weeks of rapid proliferation, the numbers of GC Ag specific B cells decline (Jacob et al 1991, McHeyzer-Williams et al 1993, Smith et al 1994, Liu et al 1996) and a stable, recirculating memory B-cell pool is established which remains in the spleen for many weeks after immunisation (Smith et al 1994). Memory cells also circulate moving from the germinal centre to the marginal zones of the spleen, the subcapsular sinus of lymph nodes and the gut and mucosal associated lymphoid tissue. This population includes switch recombined IgG, IgA and a significant number of IgM+ IgD-expressing cells (Klein et al 1997). Germinal centers then involute although they may persist for over 3 months (Ridderstat et al 1998).

The B cells which differentiate along the plasma cell lineage leave the germinal centres as plasmablasts (= pre-plasma cells) and migrate to a number of sites for final differentiation and to produce antibody locally. Those originating from lymph node or spleen migrate to bone marrow, those from Peyers patches migrate to the lamina propria of the gut and those developing in mesenteric lymph nodes migrate to epithelial surfaces (Liu et al 1995). The majority of antibody production occurs in the bone marrow and AbSCs producing high affinity antibody have been detected in bone marrow shortly after immunization (Zachau et al 1989, Smith et al 1996 & 1997). These cells live for up to a month although some live much longer and with age the majority
of IgG production occurs in the bone marrow (Benner et al 1982).

B cells are continually circulating in the blood through the secondary lymphoid organs (spleen, lymph nodes and mucosa associated lymphoid tissue (MALT)). Most B cells survive in the circulation for three to four days before dying by apoptosis and a fairly constant level of circulating B cell numbers is maintained by daily replenishment of 5-10% of the circulating B cell pool from the maturing population in the bone marrow (Tough & Sprent, 1995).

Antigen can remain attached to FDCs in the lymphoid follicles for prolonged periods of time which facilitates long lived antibody production (Mandel et al 1980). Studies of non-replicating antigens show that germinal centres are present for only three to four weeks after the extrafollicular antigen supply has been exhausted, however a few proliferating B cells may remain in the follicles for months and these may be the precursors of bone marrow and mucosal antigen specific B cells for years (Gray et al 1986 & 1996). This is particularly relevant for polysaccharide antigens which take a long time to degrade. It may account to the long lived immunity to simple polysaccharide antigens in mature vaccinees despite the absence of the induction of memory by such antigens.

### 1.4.2 Secondary immune response and generation of memory

A T dependent immune response produces immunological memory in which a population of antigen specific clonally expanded mature lymphocytes exist primed to respond to antigen challenge more rapidly and more effectively than a naïve population. There is a 10-100 fold increase in the frequency of antigen specific B cells after priming. Antibody production starts earlier, reaches a higher titre and is classically dominated by IgG with small amounts of IgM and some IgA (Figure 1.11) (Neuberger et al 2000).
The antibody is of high affinity and may demonstrate high levels of somatic mutation. Memory persists for the lifetime of the individual (MacLennan et al. 2000) although the mechanism by which it is maintained is unclear. Two hypotheses exist. One suggests that memory is sustained by a population of long lived lymphocytes which persist in a resting state ready to be reactivated on re-encounter with antigen.

**Primary and Secondary Antibody Responses**

![Graph showing antibody response](image)

*Figure 1.11* The dynamics of the T-dependent antibody response following primary and secondary antigen exposure. In the primary response, serum antibody is measurable from day 5 onwards. IgM dominates although IgG achieves a higher final concentration. On secondary or subsequent exposure, there is more rapid antibody production and far higher IgG concentrations are achieved which last for longer.

The second suggests that there is continual restimulation of primed lymphocytes by chronically retained antigen, cross-reactive antigens or cytokines produced to new antigens which provide by-stander stimulation to memory cells in the milieu.

Animal studies using protein antigens have shown that antigen specific memory B cells appear a few days post antigen challenge and peak in the spleen and bone.
marrow within a month post secondary exposure (Driver et al 2001). This is slower than T cells which peak by day five post-challenge and may reflect the need for specific T-B interaction and B cell proliferation and selection.

Memory B cells express CD27 (Klein et al 1998), CD148 (Tangye et al 1998) and demonstrate higher levels of MHC class II expression than naïve cells (Sproul et al 2000). This facilitates more efficient antigen uptake, presentation and T-B interaction such that primed cells are preferentially activated and can respond to lower doses of antigen than naïve B cells.

1.5 Aims of this study

There clearly remain a plethora of unanswered questions regarding the molecular basis of the immune response to polysaccharide antigens and the effect primarily of age on the nature of this response. In this study, the molecular profile of the adult immune response to two of the pneumococcal vaccines described above (23V-PS and 7V-CRM$_{197}$) was assessed.

- Human heterohybridomas secreting pneumococcal specific human monoclonal antibody (Mab) were generated from adult vaccinees.
- Mab serotype and isotype were determined and in vitro functional activity was assessed using avidity and opsonophagocytic assays.
- Heterohybridomas were sequenced to look at immunoglobulin V gene use, CDR3 characteristics and somatic mutation.
- The genetic data was compared with the functional data to determine whether specific V gene use and the presence of common mutations correlated with superior functional activity.
Although the technology used provided only a limited impression of repertoire it was demonstrated how this technology could be applied on a larger scale to population groups to study the immune repertoire and the generation of memory to specific antigens and the factors that may influence this.

In addition, the use of heterohybridomas generated a number of serotype specific and polyreactive human anti-pneumococcal Mabs of a range of isotypes which have been used by a number of collaborative groups to investigate the role of antibody in protection against mucosal colonisation with pneumococcus and to look for peptide mimics of pneumococcal polysaccharides in the search for surrogate pneumococcal antigens to be included in a new generation of pneumococcal vaccines.
## CHAPTER TWO

MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 General Reagents</td>
<td>49</td>
</tr>
<tr>
<td>2.2 Plastics/Glassware</td>
<td>51</td>
</tr>
<tr>
<td>2.3 Vaccines</td>
<td>51</td>
</tr>
<tr>
<td>2.4 Cell Lines</td>
<td>51</td>
</tr>
<tr>
<td>2.5 Software</td>
<td>52</td>
</tr>
<tr>
<td>2.6 Buffers and Solutions</td>
<td>52</td>
</tr>
<tr>
<td>2.7 Basic Pneumococcal ELISA for Serum</td>
<td>54</td>
</tr>
<tr>
<td>2.8 Supernatant Screening ELISA</td>
<td>55</td>
</tr>
<tr>
<td>2.9 Subjects and Vaccinations</td>
<td>56</td>
</tr>
<tr>
<td>2.10 Mycoplasma Screening</td>
<td>56</td>
</tr>
<tr>
<td>2.11 Hybridoma Generation</td>
<td>58</td>
</tr>
<tr>
<td>2.12 Immunocytochemistry</td>
<td>59</td>
</tr>
<tr>
<td>2.13 Generation of Antibody Stocks</td>
<td>60</td>
</tr>
<tr>
<td>2.14 Antibody Avidity</td>
<td>60</td>
</tr>
<tr>
<td>2.15 Opsonophagocytosis</td>
<td>60</td>
</tr>
<tr>
<td>2.16 Sequencing</td>
<td>63</td>
</tr>
</tbody>
</table>
## 2.1 General Reagents

<table>
<thead>
<tr>
<th>Name, Chemical Formula, MWt</th>
<th>Supplier¹</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABI™ Prism Big Dye™ Terminator Cycle Sequencing kit</strong></td>
<td>Perkin-Elmer</td>
<td>4303152</td>
</tr>
<tr>
<td>Acetone</td>
<td>BDH</td>
<td>100033P</td>
</tr>
<tr>
<td>Agarose (Electrophoresis grade)</td>
<td>GibcoBRL</td>
<td>15510-019</td>
</tr>
<tr>
<td>Ammonium Thiocyanate, NH₄SCN</td>
<td>Sigma</td>
<td>A0302</td>
</tr>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP conjugated antihuman IgG for standard ELISA</td>
<td>Hybridoma reagent Lab</td>
<td>HP6043</td>
</tr>
<tr>
<td>Goat anti-human IgG</td>
<td>Sigma</td>
<td>I 9885</td>
</tr>
<tr>
<td>Goat anti-human IgA</td>
<td>Sigma</td>
<td>I 0844</td>
</tr>
<tr>
<td>Goat anti-human IgM</td>
<td>Sigma</td>
<td>I 761</td>
</tr>
<tr>
<td>Polyvalent Goat anti-human IgGAM</td>
<td>Sigma</td>
<td>A8775</td>
</tr>
<tr>
<td>HRP conjugated goat antihuman IgG</td>
<td>Sigma</td>
<td>A7032</td>
</tr>
<tr>
<td>HRP conjugated goat antihuman IgA</td>
<td>Sigma</td>
<td>A6907</td>
</tr>
<tr>
<td>HRP conjugated goat antihuman IgM</td>
<td>Sigma</td>
<td>A7164</td>
</tr>
<tr>
<td>Rabbit antihuman IgG</td>
<td>Sigma</td>
<td>A0423</td>
</tr>
<tr>
<td>Rabbit antihuman IgA</td>
<td>DAKO</td>
<td>A0425</td>
</tr>
<tr>
<td>Rabbit antihuman IgM</td>
<td>DAKO</td>
<td>A0262</td>
</tr>
<tr>
<td>Rabbit antihuman antikappa</td>
<td>DAKO</td>
<td>A0191</td>
</tr>
<tr>
<td>Rabbit antihuman antilambda</td>
<td>DAKO</td>
<td>A0193</td>
</tr>
<tr>
<td>Bovine Serum Albumin grade 1 (BSA)</td>
<td>Sigma</td>
<td>A4503</td>
</tr>
<tr>
<td>Cell Fix</td>
<td>Becton Dickinson</td>
<td>340181</td>
</tr>
<tr>
<td>Cell Wall Polysaccharide (CWPS)</td>
<td>Statens Seruminstitut</td>
<td>252 130</td>
</tr>
<tr>
<td>Citric Acid C₆H₈O₇+H₂O</td>
<td>Sigma</td>
<td>C 7129</td>
</tr>
<tr>
<td>Complement (sterile baby rabbit serum Pel-freeze 12.5%)</td>
<td>Statens Seruminstitut</td>
<td></td>
</tr>
<tr>
<td>di-sodium acetate (acetic acid)</td>
<td>BDH</td>
<td>10236</td>
</tr>
<tr>
<td>di-Sodium Hydrogen orthophosphate Na₄HPO₄</td>
<td>BDH</td>
<td>1024946C</td>
</tr>
<tr>
<td>Di-aminobenzidine (DAB)</td>
<td>Sigma</td>
<td>D-8001</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>BDH</td>
<td>103232J</td>
</tr>
<tr>
<td>DNA ladder 1KB</td>
<td>Gibco BRL</td>
<td>15615-016</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid di-sodium salt (EDTA) C₁₀H₁₄O₈Na₂.2H₂O</td>
<td>Sigma</td>
<td>E2515</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Gibco</td>
<td>15585-011</td>
</tr>
<tr>
<td>Ethyl Alcohol</td>
<td>Hayman</td>
<td>00/080/A9</td>
</tr>
<tr>
<td>ExtrAvidin® Peroxidase Staining Kit: Rabbit:</td>
<td>Sigma</td>
<td>Extra-3</td>
</tr>
<tr>
<td>Ficoll</td>
<td>Pharmacia</td>
<td>7-1440-02</td>
</tr>
<tr>
<td>Ficoll-hypaque separation medium: LYMPHOPREP™</td>
<td>Gibco</td>
<td>1001969N</td>
</tr>
<tr>
<td>Fluorescein Isothiocyanate (FITC)</td>
<td>Sigma</td>
<td>F-7250</td>
</tr>
<tr>
<td>First Strand cDNA synthesis kit</td>
<td>Pharmacia Biotech</td>
<td>27-9281-01</td>
</tr>
<tr>
<td>Foetal Calf Serum Myocline super plus</td>
<td>Gibco</td>
<td>10081-073</td>
</tr>
</tbody>
</table>

¹ See Appendix 1 for name and address of suppliers
<table>
<thead>
<tr>
<th>Material/Component</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungizone (Amphotericin B) 250μg/ml</td>
<td>Gibco</td>
<td>15290-026</td>
</tr>
<tr>
<td>GeneClean™</td>
<td>BIO101</td>
<td></td>
</tr>
<tr>
<td>Glycine (amino acetic acid) C₂H₅NO₂</td>
<td>Sigma</td>
<td>G-7403</td>
</tr>
<tr>
<td>Hanks Balanced Salt Soln (HBSS)</td>
<td>Gibco</td>
<td>14025-050</td>
</tr>
<tr>
<td>Histopaque</td>
<td>Sigma</td>
<td>1119-1</td>
</tr>
<tr>
<td>Hoechst Stain 500μg/l</td>
<td>ICN</td>
<td></td>
</tr>
<tr>
<td>HAT supplement (50x)</td>
<td>Gibco</td>
<td>21060-017</td>
</tr>
<tr>
<td>HT concentrate lyophilised (50x)</td>
<td>Gibco</td>
<td>41065-012</td>
</tr>
<tr>
<td>Hydrogen Peroxide (H₂O₂) 30%</td>
<td>Sigma</td>
<td>H1009</td>
</tr>
<tr>
<td>L-glutamine 200mM (100x)</td>
<td>Gibco</td>
<td>25030-024</td>
</tr>
<tr>
<td>Maysers haematoxylin</td>
<td>Sigma</td>
<td>MHS16</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Sigma</td>
<td>M6250</td>
</tr>
<tr>
<td>Hydrogen Peroxide (H₂O₂) 30%</td>
<td>Sigma</td>
<td>M5904</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>Sigma</td>
<td>MB-1</td>
</tr>
<tr>
<td>Non Essential Amino Acids (100x)</td>
<td>Gibco</td>
<td>11140-035</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Promega</td>
<td>U1240</td>
</tr>
<tr>
<td>Omni serum</td>
<td>Statens Serum Institut</td>
<td></td>
</tr>
<tr>
<td>Type 4</td>
<td></td>
<td>16747</td>
</tr>
<tr>
<td>Type 6B</td>
<td></td>
<td>16923</td>
</tr>
<tr>
<td>Type 18C</td>
<td></td>
<td>16958</td>
</tr>
<tr>
<td>Type 23F</td>
<td></td>
<td>16968</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Sigma</td>
<td>O 3125</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (10,000U/100ml: 100mg.ml)</td>
<td>Gibco BRL</td>
<td>15140-114</td>
</tr>
<tr>
<td>O-Phenylenediamine (OPD) Tablets</td>
<td>Sigma</td>
<td>P8287</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) Tablets</td>
<td>Oxoid</td>
<td>BR14A</td>
</tr>
<tr>
<td>Pneumococcal Polysaccharides: Serotype 4</td>
<td>ATCC</td>
<td>175-X</td>
</tr>
<tr>
<td>Serotype 6B</td>
<td></td>
<td>227-X</td>
</tr>
<tr>
<td>Serotype 9V</td>
<td></td>
<td>255-X</td>
</tr>
<tr>
<td>Serotype 14</td>
<td></td>
<td>199-X</td>
</tr>
<tr>
<td>Serotype 18C</td>
<td></td>
<td>247-X</td>
</tr>
<tr>
<td>Serotype 19F</td>
<td></td>
<td>207-X</td>
</tr>
<tr>
<td>Serotype 23F</td>
<td></td>
<td>219-X</td>
</tr>
<tr>
<td>Polyethylene Glycol (PEG 4000)</td>
<td>Gibco</td>
<td>14030-035</td>
</tr>
<tr>
<td>Pyrophenol Free Water</td>
<td>Parkfields Pharmaceuticals</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Gibco</td>
<td>11360-039</td>
</tr>
<tr>
<td>QIaprep Spin plasmid kit</td>
<td>Qiagen Inc.</td>
<td>27104</td>
</tr>
<tr>
<td>RNazol</td>
<td>Biogenesis Ltd.</td>
<td>90507-176</td>
</tr>
<tr>
<td>RPMI with 25mm Hepes and L-glutamine (R₁)</td>
<td>GibcoBRL</td>
<td>52400-025</td>
</tr>
<tr>
<td>Serum Control 89SF</td>
<td>Carl Frasch</td>
<td></td>
</tr>
<tr>
<td>Sodium Azide, Na₃N₅, FW 65.0</td>
<td>Sigma</td>
<td>S2002</td>
</tr>
<tr>
<td>Sodium Carbonate (Na₂CO₃)</td>
<td>BDH</td>
<td>30121</td>
</tr>
<tr>
<td>Sodium di-hydrogen Phosphate (NaH₂PO₄·H₂O)</td>
<td>BDH</td>
<td>102454R</td>
</tr>
<tr>
<td>Sodium Pyruvate100mM</td>
<td>GibcoBRL</td>
<td>11360-039</td>
</tr>
<tr>
<td>Streptococcus pneumoniae (freeze dried)</td>
<td>ATCC</td>
<td></td>
</tr>
<tr>
<td>Sulphuric Acid (H₂SO₄)</td>
<td>BDH</td>
<td>30307</td>
</tr>
<tr>
<td>Swine Serum</td>
<td>DAKO</td>
<td>X0901</td>
</tr>
<tr>
<td>Todd Hewitt Broth</td>
<td>Oxoid</td>
<td>X4547</td>
</tr>
</tbody>
</table>
2.2 Plastics/Glassware

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nunc 96 well maxisorp immuno plates</td>
<td>Life Technologies</td>
<td>442404A</td>
</tr>
<tr>
<td>Nunc 96 well U btm plates</td>
<td>Life Technologies</td>
<td>163320A</td>
</tr>
<tr>
<td>Nunc 96 well tissue culture plates</td>
<td>Life Technologies</td>
<td>167008A</td>
</tr>
<tr>
<td>Nunc 24 well tissue culture plates</td>
<td>Life Technologies</td>
<td>143982A</td>
</tr>
<tr>
<td>Glass tubes</td>
<td>Fisher</td>
<td>BTS-160-016G</td>
</tr>
<tr>
<td>Angled neck filter lid Flasks 25cm²</td>
<td>Life Technologies</td>
<td>I-36196A</td>
</tr>
</tbody>
</table>

2.3 Vaccines

Pneumococcal 23 valent plain polysaccharide vaccine (23V-PS, Pneumovax II™, Pasteur Mérieux, France) containing 25μg of each serotype. Pneumococcal 7 valent polysaccharide conjugate vaccine containing 2μg each of 4, 9V, 14, 18C, 19F, 23F and 4 μg of 6B, conjugated to a mutant diphtheria toxin (7V-CRM₁₉₇) (kindly donated by Wyeth Lederle Vaccines Paediatrics, NY, USA).

2.4 Cell lines

Murine myeloma cell line: OURI (P3-X63-Ag8-OURI) (Thompson et al, 1991) were a gift from Dr M Melamed, University of East London, UK. OURI was derived from the common fusion partner: P3-X63-Ag8 (Kearney et al, 1979) and was named for it’s high resistance to ouabain :OUabain RIsistant. Oubain is toxic to EBV-tranformed human B-cells (EBVTB) and was used to selectively kill unfused EBV transformed B cells in the production of hetero-hybridomas. Although ouabain resistance was not necessary with direct B cell-myeloma fusions, the line was still used as it remained an exceptionally good fusion partner.

Murine Peritoneal Macrophages were used as a feeder layer for hybridomas immediately after fusion. The macrophages were obtained by peritoneal irrigation of
BALB/c mice immediately post mortem. 10ml of tissue culture medium (R10) was injected into the peritoneum using a 21G butterfly needle and after 10 seconds of agitation, withdrawn back into the syringe. The macrophages were then irradiated (25 rads) and plated onto 96 well tissue culture plates at 100μl/well. 1 irrigation was used for 1x96 well plate.

2.5 Software

ELISA software: Revelations 2.0 (Dynatech, UK) used to calculate antibody titre. Cell Quest software (Beckton Dickinson, California, USA) was used to analyse flow cytometric data. Statistics software: Pascal Software program (Ludwig Cancer Institute, Sao Paulo, Brazil) used to calculate Odd's Ratios (OR) and 95% Confidence Limits (CL) DNASTAR software (DNASTAR, Madison, WI): used to analyse immunoglobulin heavy and light chain sequences. DNAPLOT used to align nucleotide sequences to search VBASE (http://www.mrc-cpe.cam.ac.uk) and assign the germline gene nomenclature according to accepted locus nomenclature described on VBASE. The corresponding canonical structure numbers are also given where known ('U' indicates that the canonical structure of this loop is currently unknown).

2.6 Buffers and solutions

Ammonium Isothiocyanate (NH₄SCN): Ammonium Isothiocyanate was dissolved in BSA-PBS-T to give a starting concentration of 4M. Five doubling dilutions were prepared in BSA-PBS-T. NH₄SCN is hydroscopic. Once diluted, it could be stored for up to 1 week at +4°C in the dark.

Antibody Dilution Buffer (BSA PBS-T): 1g Bovine Serum Albumen (BSA) was dissolved in 100ml of wash buffer to give 1% PBS-T-BSA

Carney’s Fixative: 1 part glacial fixative was added to 3 parts absolute methanol.

FPLC Buffers: All were prepared using double distilled water and filtered through 0.22μm filters prior to use.
Gluconic acid; 1L 0.1M Gluconic acid HCl was adjusted to pH 2.7 with concentrated HCl.

**Horse Radish Peroxidase (HRP) Substrate Buffer:** 20 mls 0.1M citric acid (21g/l) and 20ml 0.2M di-sodium hydrogen orthophosphate (28.5g/l) were mixed, 10mg OPD substrate was added and dissolved, 20μl 30% H$_2$O$_2$ was added immediately prior to use.

**HAT supplement:** Hypoxanthine 0.68g/l (5mM), Aminopterin 0.0088g/l (20mM), Thymidine 0.194g/l (0.8mM)

**Opsonophagocytosis Buffer:** HBSS-1% BSA

**Phosphate Buffered Saline (PBS):** PBS tablets were dissolved in 1 litre of double distilled water. Final composition: NaCl 8g/l, KCl 0.2g/l, Na$_2$HPO$_4$ 1.15g/l, KH$_2$PO$_4$ 0.2g/l. pH 7.3.

**Pneumococcal Antigen Polyvalent Coating Mix:** Heptavalent antigen mix was prediluted in phosphate buffered saline (PBS) comprising pneumococcal capsular polysaccharides (PPS) serotypes 4, 6B, 19F at 20μg/ml, 9V, 14 and 23F at 10μg/ml and 18C at 2μg/ml (ATTC)

**Pneumococcal Polysaccharide ELISA Coating Buffer:** Phosphate Buffered Saline Tablets were dissolved in 1 litre distilled water, azide was added to give final concentration of 0.02%.

**Running Buffer:** 200ml of 0.1M Na$_2$HPO$_4$.H$_2$O was mixed with 300ml of 0.1M NaH$_2$PO$_4$

**Tissue Culture Medium (R$_{10}$):** 5ml of 100x Non Essential Amino Acids, 5ml of 100mM Sodium Pyruvate and 5ml of 200mM L-glutamine were added to 500ml of RPMI1640 containing 25mM Hepes and L-glutamine. 10% FCS (mycoplasma free), penicillin (100 IU/ml), streptomycin (100 μg/ml) and Mercaptoethanol (ME)10⁻⁵M were also added.

**Todd Hewitt Broth:** 5g of Todd Hewitt Broth was dissolved in 500ml distilled water. This was autoclaved for 15 minutes with slow exhaust to sterilise and stored at RT.
Prior to use, 0.125ml of yeast extract (0.5%) and 2.5ml (5%) of human serum were added to 50ml of Todd Hewitt broth.

50X TAE Buffer : Tris : 242g, Acetic acid (BDH): 57.1 ml and EDTA : 23.25g (500mmol/100mls) were made up to 1 litre with distilled water to provide a 50XTAE stock solution. Stock solution was prediluted 50X with distilled water prior to use.

1M Tris: 20ml : volume required to neutralise 1ml of glycine at pH 2.7 was pre-determined.

Wash Buffer (PBS-T): 0.5ml Tween was added to 1 litre PBS. The final solution was 0.05%PBS Tween.

2.7 Basic Pneumococcal ELISA for Serum

Coating Plates
Antigen stock solution was prepared under laminar flow conditions. 10mg vials of lyophilised single serotype polysaccharide (obtained from American Type Tissue Collection, Rockville, MD) was dissolved in 10ml pyrogen free water by rotation overnight at +4°C or rapid agitation at room temperature. Dissolved polysaccharide then stored in 1ml aliquots of 1mg/ml at -80°C until required. Concentration required for coating plates was serotype dependent. Antigen was diluted in coating buffer to give the following final polysaccharide concentration: Type 18C at 2μg/ml, Type 9V,14,23F at 10μg/ml, Type 4,6,19C at 20μg/ml and then added at 100ml/well to Nunc 96 well maxisorp plates. Plates were incubated in a sealed humid chamber at 37°C for 5hr and then stored at +4°C for up to four weeks.

Pre-incubation with CWPS
Antisera were incubated with Cell Wall Polysaccharide (CWPS) to neutralise anti-CWPS antibodies prior to ELISA. CWPS (10mg lyophilised) was reconstituted in 10ml pyrogen free water and stored : 1mg/ml at -80°C. The concentration of CWPS required to neutralise cross reactive Abs was calculated on the basis of Ab dilution to
be used. The calculation was based on a 1/10 dilution of antisera requiring 5% CWPS (50μg/ml) to neutralise cross reactive Abs.

**ELISA**

The standard antisera :89SF (kindly supplied by Carl Frasch, Center for Biologics Evaluation and Research, Bethesda, MD (Quataert et al, 1995)) was used at a 1/20 dilution and test sera used at 1/40 dilution. PBS-T-BSA was the diluent used. Prediluted CWPS was incubated with pre-diluted sera for 30 min at RT. Doubling dilutions of the sera are made and added to the pre-coated plates (80μl/well) which were washed x4 in wash buffer prior to use. Plates were incubated in a humid chamber for 2hr at RT. Washed x 4. The HRP-conjugated second antibody was then added (HRP-goat anti-human IgG diluted 1/2000 in PBS-T-BSA) (80μg/ml) and the plates reincubated for 2hr at RT. Washed x 4. The chromogenic substrate buffer (HRP-substrate buffer) was then added (80μg/well) and left for up to 40 min at RT in the dark for colour to develop. The reaction was stopped by the addition of 40μl/well 4M Sulphuric acid (H₂SO₄). Absorbance was measured at 490nm using an automatic ELISA plate reader (Dynex) linked to a computer employing ELISA software (Revelations 2.0; Dynatech, UK). Immunoglobulin concentrations were calculated from the standard curve produced by 89SF. All samples were run in duplicate with a minimum of four dilutions. Positive and negative controls were included on each plate.

**2.8 Supernatant screening ELISA**

**Primary Screening**

Nunc 96 well maxisorp plates were coated for 5 hr at 37°C with a heptavalent antigen mix prediluted in phosphate buffered saline (PBS) comprising pneumococcal capsular polysaccharides (PPS) serotypes 4, 6B, 19F at 20μg/ml, 9V, 14 and 23F at 10μg/ml and 18C at 2μg/ml. Plates were then washed five times with PBS-T. Cell supernatants were diluted by 50% in BSA PBS-T, add directly to the coated plates (80μl/well) and incubated for 2 hr at room temperature (RT). Plates were then washed five times and the second antibody : Horse radish peroxidase conjugated goat antihuman polyvalent IgG ( Sigma, Dorset, UK) was added at a 1/2000 dilution in PBS-T-BSA. Following a further 2 hr incubation at RT, wells were rewashed five times with PBS-T and substrate added. After 10-40min at RT, 40 μl 4M H₂SO₄ was added to stop the reaction and absorbence read at 490nm was determined using an automatic ELISA plate reader.
Serotype and Isotype specificity
Positive wells were then rescreened on single serotype coated plates to determine serospecificity and isotype was determined using a panel of isotype specific HRP-conjugated goat anti-human IgG antibodies (Sigma, Dorset, UK). The second antibody: HRP-conjugated goat anti-human IgG, A or M (Sigma, Dorset, UK) was used at a dilution of 1/1000, 1/500 and 1/1000 respectively.

Inhibition Assay
Serospecificity of the Mabs was confirmed by inhibition assay. Non-specific binding to cell wall polysaccharide (CWPS) was excluded by pre-incubating supernatant diluted 50% in BSA PBS-T with CWPS at a final concentration of 50μg/ml as described above. Cross-reactivity with other serotypes was evaluated by preincubating antibody at a dilution pre-determined to give an OD of 1.0 on ELISA for 30 min with 50μg/ml (final concentration) of homologous or heterologous capsular polysaccharide and then performing the standard ELISA. Inhibition of binding of >90% by the homologous serotype and <30% by the heterologous serotype was considered to validate specificity.

Hib ELISA for cross reactivity
Cross reactivity of the polyreactive antibodies to another polysaccharide antigen (Hib) was evaluated by a standard ELISA as described by Phipps et al 1990.

2.9 Subjects and vaccinations
Healthy adult volunteers were recruited and given a single dose of pneumococcal 23 valent plain polysaccharide vaccine (23V-PS), or a 7 valent polysaccharide conjugate vaccine (7V-CRM197). Blood was taken immediately prior to vaccination and on day 7 & 28 post vaccination. Lymphocytes were isolated by ficoll density centrifugation from day 7 venesections while sera obtained at each time point was separated and stored at -80°C.

2.10 Mycoplasma screening
Cells to be tested were cultured through 3 passages with no antibiotics and left for continuous culture for 72 hr prior to sampling the supernatant for DNA hybridisation or culturing the cells in mycoplasma selective media.
Test 1. DNA Flourescence Stain

This assay involved staining cells with Hoechst Stain, a flourescent stain for DNA. Control positive and negative samples were required. Cells were grown on slides/coverstips in a petri dish until 50-80% confluent (complete confluence makes interpretation of results difficult). Hoechst stain was prepared by dilution 1/10 in 1X Hanks Balanced Salt Solution (HBBS) to give a concentration of 50µg/ml and then titrated to give optimum fluorescence (final concentration: 0.05-0.5 µg/ml).

Culture medium was aspirated from the petri dishes containing the slide monolayers of cells to be tested. Carnoys Fixative was added at a volume to fully cover the slide. Slides were left for 3 min, fixative removed and fresh fixative added and left for a further 10 min. Slides were air dried for 30min. Pre-diluted Hoechst Stain was added (1ml/slide) and left for 10-30min. The stain was aspirated and the slides washed x3 in distilled water, air dried and mounted using standard Mounting medium. Fluorescent staining was assessed using an Olympus BH2 flourescent microscope at 400-1000x magnification with a 360nm filter.

Test 2. DNA Hybridisation

A ^3H-labelled single stranded DNA probe complementary to the ribosomal RNA of all *Mycoplasma* and *Acoleplasma* species which commonly infect tissue culture was used to check OURI and EBV cell lines for mycoplasma contamination. The probe was obtained as part of the Gen-Probe MYCOPLASMA T.C. RAPID DETECTION SYSTEM (Gen-Probe, USA), a kit which supplies all the reagents required for detection of mycoplasma contamination in standard tissue culture cell lines. The kit was used as per manufacturers instructions. A background count was determined to confirm the kit was within its shelf life. The total count was determined for a sample of labelled probe solution. Positive and negative controls were supplied by the manufacturer. Tissue culture supernatant was taken from cells to be tested and centrifuged at 12-15,000g for 10 min to precipitate free nucleic acids. Supernatant was removed and the pellet resuspended in Probe solution. Hybridisation took place in a water bath at 72°C for 2 hr. Hybridised mycoplasma RNA was separated from unlabelled single strand RNA by resuspension in Separation Suspension and incubation at 72°C for 5 min. The samples were then centrifuged at 500g for 1 minute, supernatant discarded and the pellet washed twice in Wash Solution. Samples were finally resuspended in Scintillation Solution and analysed in a scintillation counter. Positive controls should produce >30%
hybridisation. Negative controls should produce <0.02% hybridisation. Total count should be >170,000 counts per minute (cpm).

Test 3. Tissue Culture
The company: Mycoplasma Experience was used to test for mycoplasma contamination of cell lines by direct culture on media which facilitate growth of mycoplasma. Cells were in exponential growth phase in antibiotic free media at time of testing. Tests for the following strains were performed: M. hyorhinis cultivar α 29052, M. orale T24.MM, M. pneumoniae 5167, M. synoviae 263.

2.11 Hybridoma Generation

Cell preparation
50 ml peripheral blood were collected into heparinized tubes from subjects on day 7 post-vaccination. Blood was diluted 50:50 with Rₐ. 25 ml ficoll-hypaque (allowed to warm to room temperature before use) was added to four x 50ml falcons and 25 ml of pre-diluted blood were gently overlaid in each tube. Tubes were centrifuged at 800g for 20 min with no brake. Lymphocytes were harvested from the ficoll-hypaque - Rₐ interface into a fresh Falcon and washed in Rₐ. Supernatant was discarded and the pellet resuspended in 1 ml Rₐ. A viable cell count was performed using Trypan Blue.

OURI myeloma cells (mycoplasma screened negative) were grown to confluence in 75cm² tissue culture flasks for 2 weeks prior to fusion. HAT sensitivity and non-secretor status of immunoglobulin was confirmed. Cells were harvested, washed and a viable cell count made.

OURI cells were then added to the isolated blood lymphocytes to give a 1:1 ratio. For each fusion, a maximum of 10⁷ lymphocytes in 0.5ml were used per fusion.

One day prior to fusion, 0.9ml Polyethylene Glycol (PEG 4000) was mixed with 100μl DMSO in a 25 ml glass tube, autoclaved and placed at 37°C. 4x50ml Rₐ and 4x50ml R₁₀ were held at 37°C 2 hr prior to fusion.

Fusion
All reagents were at 37°C and the whole procedure carried out in a sterile waterbath at 37°C. With continual agitation throughout the procedure, PEG-10%DMSO was added to the cell mix drop by drop over 90 sec, left for 10 sec, 5ml Rₐ added over 60 sec and
the residual 45 ml Rq over the next 3 min. Cells were pelleted by centrifuge at 200g for 5 min, washed in R10 and resuspended in 50ml of R10 with additives + HAT. This was plated out into 96 well plates ( precoated with mouse fibroblast feeder layer) at 100μl/well. The cells were incubated at 37°C in 5%CO2. Medium was changed every two to three days initially while all cells remained viable and then less often as unfused cells died, until heterohybridomas became established at three to four weeks. HT was gradually substituted for HAT as hybrodomas became established. Supernatants were screened (see below) against pneumococcal polysaccharide after two to three weeks as soon as proliferating clones are seen, and rescreened at weekly intervals for a total of six weeks after initial fusion. Pneumococcal serotype and isotype was determined for positive wells (see above) which were then subcloned twice by limiting dilution. Immunohistochemistry was used to determine whether >90% of cell population were secreting antibody. Positive clones were expanded into 24 well plates and then 25 cm² and 75 cm² tissue culture flasks, negative clones were recloned to rescue and expand high secretor colonies.

2.12 Immunocytochemistry

Once a clonal population of target antigen specific heterohybrids was established, secretor status was assessed. A cytospin slide was made of each hybrid clone, fixed in acetone for 30 min, and air dried. Cells were stained for isotype specific human Ig production by incubation in 100μl of primary antibody: rabbiti anti-human IgG, A or M (prediluted in swine serum at concentrations advised by manufacturer) for 1hr. Slides were washed in PBS, incubated with a biotinylated second antibody (goat anti-rabbit IgG) for 30 min, washed in PBS and incubated in peroxidase labelled extravidin antisera for 1hr. Peroxidase activity was developed by incubating in DAB solution (1 ml of 5% Di-aminobenzidine in Di-Methylformamide made up to 100mls with PBS plus 100μl of 30% H2O2) for 10 min. Slides were then washed in PBS and counterstained with Mayers haematoxylin for 30 sec, washed in tap water for 2 min, dehydrated (70% ethanol to 100% ethanol to xylene) and mounted using standard mounting medium. Using a light microscope, the percentage of cells staining brown (=antibody production) was counted. If >90% were stained, subcloning was considered adequate, if <90% are stained, further subcloning was required.
2.13 Generation of antibody stocks

Clonal cell populations were expanded from 96 well to 24 well tissue culture plates and then into 75cm² and 150cm² tissue culture flasks. Cells were grown to confluence, harvested and aliquots of 10⁷ cells cryopreserved at -160°C in 10% DMSO. To develop monoclonal antibody stocks, cells were grown to confluence in 150cm² flasks. Flasks were then turned upright and media topped up every three days until the flasks were full. Flasks were then left to stand for two weeks in 5% CO₂ at 37°C. Supernatants were collected into 50cm² V-bottomed tubes (FALCON) and centrifuged at 200g for 5 min to separate cells and cell debris.

Antibody concentration and purification

Antibody supernatant was concentrated using a 200 ml Amicon stir concentrator with a 150 kDa filter and purified by affinity chromatography on a protein-G Superose column (Pharmacia Biotech) using the FPLC system. Purified samples were then dialysed in PBS overnight x 3. Antibody titre was determined using the standard ELISA as described above and samples were stored at -80° for further use.

2.14 Antibody avidity

Antibody avidity was evaluated using a modification of the standard ELISA described above in which an incubation step using ammonium thiocyanate (NH₄SCN), a chaotropic agent which disrupts antigen-antibody binding, is incorporated into the assay. The method has been described in detail by Goldblatt et al (1993) and has been standardised for Hib (Goldblatt et al, 1998) and pneumococcal antibody avidity determination by colleagues in the Immunobiology Unit, ICH (D.Goldblatt, personal communication). In brief, antibodies were diluted in BSA-PBS-T to a concentration known to give an OD of 1 on the standard pneumococcal ELISA, plated out onto the appropriate serotype coated plate and incubated for 2 hours at RT. Plates were then washed x 4 in PBS-T and incubated in 5 doubling dilutions of ammonium thiocyanate (NH₄SCN) with a starting concentration of 4M. After 15 min incubation at room temperature, plates were washed x4 in wash buffer and incubated for a further 2 hr with the second antibody and the ELISA completed as described above.

2.15 Opsonophagocytosis

Method adapted from that described by Jansen et al 1998 (Jansen et al, 1998).
Neutrophil genotyping

Fc receptor (FcR) genotyping was done on all potential neutrophil donors to determine FcRII allotype. DNA was extracted from 5 ml of donor peripheral blood using the QIuAMP® DNA Blood Mini kit (Qiagen, Germany) and sent to Dr Nomdo Wessterdaal (Immunotherapy Laboratory. University Medical Center Utrecht. Wilhelmina Children's Hospital. Rm C2.085.2. Lundlaan 6. 3584 EA Utrecht. The Netherlands) where analysis was performed using the published protocol (Carlsson et al, 1998).

Culture of Encapsulated Bacteria

Bacterial strains of Streptococcus pneumoniae (ATCC, Rockville, MD) were incubated overnight on blood agar plates at 37 °C/5 % CO₂ and tested for the presence of polysaccharide capsule using the Quelling reaction (see below). If capsulation was poor, bacteria were re-cultured x 3 and re-tested. Well encapsulated strains exhibiting pure growth on the culture plate were expanded for labelling by inoculating colonies into 10 ml of Todd Hewitt broth (containing 0.5% yeast extract, 5% heat inactivated filtered human serum) at a concentration giving a change in OD of approximately 0.05 at 660nm. Tubes were incubated at 37 °C/5% CO₂ until the bacteria were at log phase growth as evaluated by an increase in OD to 0.5-0.6 at 660nm (4-5 hr)^2. Bacteria were then heat inactivated for one hour at 60 °C^2 and pelleted by centrifugation for 10 minutes at 2500g. The sample was then diluted in PBS to give an OD of 1.0 (equivalent to ~1x10^9 bacteria), transferred in 1 ml aliquots to eppendorf tubes, centrifuged for 10 minutes at 2500g and washed in HBSS-1% BSA. After washing, supernatant was removed from the bacteria.

FITC labelling (Nielsen et al, 1993).

A 0.5mg/ml solution of FITC in PBS was freshly prepared, undissolved FITC removed by centrifugation at 5000g, and the solution filtered (0.22 μm filter). 100μl FITC solution was added to the bacterial pellet, mixed thoroughly and left on a rotator for 30 min @ 4°C. Labelled bacteria were washed in 900 μl ice cold HBSS-1% BSA and centrifuged for 2 min at 5000g and washed again x2 in 1 ml ice cold HBSS-1% BSA. Labelled pneumococci were resuspended in 1 ml HBSS-1% BSA and aliquotted in 100 ul amounts to eppendorf tubes and stored at -70°C. Prior to use, % FITC labelling was

----

A sample of the bacteria was re-plated onto blood-agar for overnight culture to confirm colony purity.

---
checked by flow cytometry. 95-100% labelling with a mean fluorescence of greater than 800 was required for subsequent use in opsonophagocytic assays.

Quellung reaction
To test for the presence of polysaccharide capsule, cultured pneumococci were stained with anti-capsular omni-serum as follows: 10 μl of omni serum, 10μl of 0.3% methylene blue and 10μl of bacterial suspension (bacteria taken from blood agar plate after overnight culture and resuspension in PBS) were placed on a glass slide and carefully mixed. A coverslip was placed on top and the slide read under oil immersion. Bacteria were stained blue and capsules appeared as a clear halo surrounding the bacteria.

Isolation of human PMN using Ficoll/histopaque
Peripheral blood (50ml per assay) was taken from healthy adult volunteers into a sterile heparinised (10 IU/ml) tube. 12 ml histopaque were added to a 50 ml centrifuge tube and 10 ml of ficoll carefully layered on top. Blood was diluted 1:1 with PBS and 24 ml of diluted blood layered onto the ficoll/histopaque gradient. Samples were centrifuged for 20 min at 397g without brake to separate the different cell types. Upper layers were removed (plasma, monocytes, ficoll) by vacuum extraction and the PMN layer was transferred to a 50 ml tube and 25ml RPMI 1640 (with 25mM Hepes and L-glutamine) (Rq) was added. The sample was centrifuged for 10 min at 249g, maximum brake, supernatant was discarded and the PMN pellet loosened by tapping the tube gently. 9 ml of double distilled water was added to lyse erythrocytes. The sample was mixed gently and left for exactly 30 sec. 1 ml of 10X PBS was then added and the sample mixed again gently. The sample was washed again in Rq, the supernatant discarded and PMNs resuspended in a minimal volume of Rq. Cells were counted and adjusted to 5x10^6 cells/ml.

Stock reagents were kept sterile and aliquotted under laminar flow isolation. All reagents, including blood, were brought to room temperature before use unless described otherwise. The ficoll gradient was prepared immediately prior to use.

Flow cytometric opsonophagocytic assay
Human sera was heat inactivated for 30 min at 56°C before use. All samples were run in duplicate with and without complement. A minimum of four doubling dilutions were

3 The bacteria were confirmed to be non-viable by plating out on blood agar plates.
used. Non-opsonin, complement and cell controls were run in each assay. Specificity was evaluated by incubation with two other pneumococcal serotypes to which Mabs were not cross reactive on ELISA. Non-serotype specific antibody controls (10% FCS or Mabs to a different serotype) were also used. The Bacterial : PMN ratio was 2:1 unless stated otherwise.

**Opsonisation**

Test sera was serially diluted 2 fold in HBSS-1% BSA and aliquotted (40-80μl/well) onto 96 well U-bottom plates (Nunc). 40μl (approx. 1x10⁷) of prediluted FITC labelled bacteria and either 20μl of complement (sterile rabbit serum Pel-freez 12.5%) or 20 μl of HBSS-1% BSA were added to each well and plates were incubated at 37°C for 30 minutes on a shaker tray at 100 rpm.

**Phagocytosis**

80μl PMN (5x10⁸) were then added to each well and the plates were further incubated at 37°C for 30 minutes with shaking. To stop the reaction, 80μl of ice-cold HBSS-1% BSA were added to each well and the plates were spun for 10 min at 2160g. Cells were gently resuspended and fixed in 10% CellFix in PBS (~200μl) (Beckton Dickinson Benelux N.V. Belgium) and transferred to micronic tubes. Samples were then analysed by flow cytometry using the FACSCALIBUR cytometer (Beckton Dickinson. California, USA) and Cell Quest (Beckton Dickinson. California, USA) software. Neutrophils were gated on forward and side scatter and the level of opsonophagocytosis determined by the percentage of cells within the gated population demonstrating fluorescence (FL1-H) of greater than 20. Approximately 10000 cells were counted in each analysis.

**2.16 Sequencing**

**mRNA extraction**

mRNA was extracted from the hybridomas with RNAzol (Biogenesis Ltd, Bournemouth, UK) using the manufacturer's protocol. I vial of fresh or cryopreserved cells (10⁷) were resuspended in 2 x 1.5ml of sterile RNase free water and spun at 14,000 g for 5 min. Supernatant was discarded and cells were resuspended in the residual water. RNAzol (0.2mls/10⁸ cells) was added with chloroform(Sigma) (0.1ml/ml
Chapter 2 Materials and Methods

RNAzol) and samples were shaken for 15 sec, placed on ice for 5 min and spun at 14000g for 15 min. The aqueous phase was transferred to a new vial at 4°C and an equal volume of isopropanol (pre-cooled to 4°C was added). Samples were incubated for 15 min on ice and spun at 14000g for 15 min. At this stage the RNA pellet was visible. Supernatant was discarded and the pellet was washed in 75% ethanol (0.8ml/100µg RNA) by vortex. Samples were then spun at 8000g for 8 min and supernatant was removed. Final drying was performed under a laminar flow hood at room temperature. RNA was then frozen in 100% ethanol for later use or resuspended in nuclease free water at 4°C for immediate use.

cDNA synthesis

cDNA was made using the First Strand cDNA synthesis kit (Pharmacia Biotech supplied by Promega, Maddison, USA) with the pd(N)₆ primer as per the manufacturer’s instructions. The entire procedure was performed under a laminar flow hood. The air dried pellet of mRNA was resuspended in 20µl RNAse free water. The sample was incubated for 10 min at 65°C, for 2 min at 4°C than pulsed for 10 sec. Premixed cDNA synthesis reagents were then added to the RNA and the sample was incubated for 60 min at 37°C then put on ice or frozen at -20°C for subsequent analysis. The synthesis reagents comprised: 5µl of Bulk mix (supplied by the manufacturer and comprising murine reverse transcriptase, RNAguard (porcine), Rnase/Dnase free BSA, dNTP, dCTP, dGTP and dTTP in aqueous buffer, 1µl DTT, 0.2µg in 1µl pd(N)₆, oligo(dT)₁₅, or NotI(dT)₁₈ primer and 8µl RNA.

PCR

cDNA heavy and light chain genes were amplified in duplicate by PCR using a pooled primer mix. A DNA negative control was used in each experiment. All the primer sequences used have been described previously (Thompsett et al, 1999), (Hawkins et al, 1994) (Baxendale et al, 2000) and are shown in Table 2.1.
Chapter 2  Materials and Methods

Table 2.1 Primer sequences for PCR. Redundancies are symbolised in bold. R=A+G, Y=C+T, M=A+C, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, N=A+C+G+T, V=G+A+C.

<table>
<thead>
<tr>
<th>Cγ primers:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HMCR</td>
<td>TTTGGCGGTTTGCTGGAGC</td>
</tr>
<tr>
<td>HACR</td>
<td>CTTGGGAGAGGACAGTCCCT</td>
</tr>
<tr>
<td>HGCR</td>
<td>AGCTGGCGATGTTGAGTTTGG</td>
</tr>
<tr>
<td>Vγ primers:</td>
<td></td>
</tr>
<tr>
<td>Vγ,1/7 Lead</td>
<td>CTACACCGGGATGGGACCTGGAG</td>
</tr>
<tr>
<td>Vγ,2 Lead</td>
<td>AGGACCAATTTGTCACRCC</td>
</tr>
<tr>
<td>Vγ,3 Lead</td>
<td>CCACTGGGATTGGGTCAGCTGG</td>
</tr>
<tr>
<td>Vγ,4 Lead</td>
<td>ACACTGAAAGCTGTGCTTCC</td>
</tr>
<tr>
<td>Vγ,5 Lead</td>
<td>ATGCGGTCAACGCGATTTG</td>
</tr>
<tr>
<td>Vγ,6 Lead</td>
<td>ATGCTGGCTCTCTCTCTCT</td>
</tr>
<tr>
<td>Vδ primers:</td>
<td></td>
</tr>
<tr>
<td>Vδ,1FR1</td>
<td>CAGTCTGGATGAGCGCCCC</td>
</tr>
<tr>
<td>Vδ,2FR1</td>
<td>CAGCCTGTGCTGACTCGACCT</td>
</tr>
<tr>
<td>Vδ,3FR1</td>
<td>CACAGGTGCTGACTGACCT</td>
</tr>
<tr>
<td>Vδ,4FR1</td>
<td>CGGCTGTGCTGACTCGACCT</td>
</tr>
<tr>
<td>Vδ,5FR1</td>
<td>CAGCTGTCCTGACTGACCT</td>
</tr>
<tr>
<td>Vδ,6FR1</td>
<td>ATGCTGCCCTGACTGACCT</td>
</tr>
<tr>
<td>Vδ,7FR1</td>
<td>TCCTMTGATGAGCCSMYCYC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Jδ primers:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jδ,1</td>
</tr>
<tr>
<td>Jδ,2&amp;3</td>
</tr>
<tr>
<td>Jδ,4</td>
</tr>
<tr>
<td>Vκ primers:</td>
</tr>
<tr>
<td>Vκ,1&amp;4</td>
</tr>
<tr>
<td>Vκ,2&amp;6</td>
</tr>
<tr>
<td>Vκ,3</td>
</tr>
<tr>
<td>Vκ,5</td>
</tr>
<tr>
<td>Jκ primers:</td>
</tr>
<tr>
<td>Jκ,1&amp;2&amp;4</td>
</tr>
<tr>
<td>Jκ,3</td>
</tr>
<tr>
<td>Jκ,5</td>
</tr>
</tbody>
</table>

Table 2.1 Primer sequences for PCR. Redundancies are symbolised in bold. R=A+G, Y=C+T, M=A+C, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, N=A+C+G+T, V=G+A+C.

Primers were diluted in dH₂O prior to use to give a concentration of 40pmol/µl (200ng) for all V and C/J gene primers in the primer mix. 0.5µl (20pmol of each primer) was used per PCR reaction.

To 2µl DNA, 5µl 10X PCR buffer with Mg, 1µl 10mM dNTP (Promega) and 0.5µl primer mix (Genosys Biotechnologies, UK) were added. The sample was made up to 50µl volume with double distilled water (ddH₂O) and 1 drop of mineral oil (Sigma, UK) overlayed. The samples were then heated to 94°C for 3 min, 0.5µl Taq polymerase

* J4-6 are pseudogenes and primers for these genes were therefore not included.
(Promega, UK) introduced and the PCR cycling was started. At the end of the PCR the samples were run on an agarose gel, bands of amplified DNA isolated and purified as described below prior to sequencing. The annealing temperature was selected depending on the composition of the primer pool and was 5°C less than lowest melting temperature in the primer pool.

The PCR cycles used were:

\[ \text{V}_H \quad (45 \text{ sec at } 94^\circ C > 45 \text{ sec at } 56^\circ C^* > 60 \text{ sec at } 72^\circ C) \times 30 \]
followed by 10 min at 72°C

\[ \text{V}_k \text{ and } \text{V}_\lambda \quad (30 \text{ sec at } 94^\circ C > 30 \text{ sec at } 55^\circ C > 60 \text{ sec at } 72^\circ C) \times 30 \]
followed by 10 min at 72°C

**Agarose gel electrophoresis**

The PCR product was separated by agarose gel electrophoresis using ethidium bromide. A 1.5% solution of nusieve agarose was made by dissolving 2.25g of agarose in 150ml of TAE buffer by microwave on low heat for approximately 5 min. When the solution had cooled to 'hand warm' 5μl ethidium bromide were added and the gel was poured into an electrophoresis tank and left to set overnight. TAE buffer was then added to the gel to keep it moist prior to use.

**Extraction of PCR product**

Two methods were used to extract the PCR product from the agarose gel, the Geneclean™ protocol (BIO101, Anachem, Luton, UK) or the QIAprep Spin plasmid kit (Qiagen Inc, Surrey, UK) both used as per the manufacturer's instructions. No difference in DNA yield was observed between the two procedures and they were used interchangeably. The Geneclean™ protocol uses a high concentration salt solution to facilitate DNA binding to glass milk. Once extracted, the DNA is released from the glass by resuspension in water which results in DNA dissociation.
Chapter 2 Materials and Methods

DNA Terminator cycle sequencing reaction

Cleaned PCR products were directly sequenced in forward and reverse using the leader and constant region primers described in the PCR. The ABI™ Prism Big Dye™ Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington, UK) provided the reagents for the sequencing reaction. The polymerase used was AmpliTaqt™ DNA polymerase, FS, a variant of the standard Thermus aquaticus (Taq) DNA polymerase which had been mutated to produce a more thermally stable enzyme with better discrimination between different nucleotides. The terminator labels were 4 new highly sensitive fluorescein donor dyes linked to dRhodamine which emits light. On the electropherogram, terminator A emits green, C emits red, G emits blue and T emits black. The dye terminators, deoxynucleoside triphosphates, enzymes, magnesium chloride and buffer were provided in a single vial ready for use. 4µl of the d-Rhodamine (Big Dye™), 2.5µl sterile dH2O and 0.5µl of primer (3.2pmol) were mixed and 3µl of DNA (5-20ng) were added. The sample was then sequenced using the d-Rhodamine programme advised by the manufacturer. Each clone was sequenced a minimum of four times from a minimum of two separate PCR reactions to reduce PCR or sequencing error when making the final base assignments.

The sequenced product was then cleaned by precipitation. To the 10µl sequenced DNA, 2µl 3M sodium acetate and 50µl 100% ethanol were added. The sample was vortexed, put on ice for 10 min and spun at 14,000g for 30 min. Supernatant was carefully removed taking care not to disturb the visible pellet. 250µl of 70% ethanol was added, the sample was spun at 14,000g for 5-10 min, the supernatant removed and the cleaned sequencing product dried in air for 5 min. The sequenced DNA was then kept at -20°C for up to 48 hr or resuspended in loading solution (2µl formamide in dextran blue (5:1)), denatured at 90°C for 2 min and put on ice until ready to load onto a sequencing gel and run on an ABI sequencing machine (Perkin Elmer. UK).

Sequence analysis

Heavy and light chain sequences were analysed using the DNASTAR software program (DNASTAR, Madison, WI). Nucleotide sequences were then aligned using DNAPLOT to search VBASE and given the germline gene nomenclature as assigned by Tomlinson et al (http://www.mrc-cpe.cam.ac.uk).
Somatic mutation was analysed using the VBASE germline genes for reference and ratios of replacement to silent mutation (R/S ratios) in CDR1 and 2 were calculated for each clone using the method described by Chang and Casali., 1994 (Chang & Casali, 1994). Canonical structure was assigned using the tables generated by Vargas-Madrazo et al., 1997 (Vargas et al, 1997).

**Statistical analysis**
Fisher's exact 2-tailed t-test was used to compare the gene frequency of our sample with the frequency described in normal adult populations of circulating IgM positive B cells (Foster et al, 1997; Brezinschek et al, 1997) CD5 negative IgM positive B cells were used as the comparison group for the heavy chain genes (Brezinschek et al, 1997) and IgM positive B cells were used as the comparison group for the light chain genes (Foster et al, 1997) Odd's Ratios (OR) and 95% Confidence Limits (CL) were calculated using the Pascal Software program (Ludwig Cancer Institute, Sao Paulo, Brazil).
CHAPTER THREE
PRODUCTION OF HUMAN HETEROHYBRIDOMAS SECRETING
MONOCLONAL ANTIBODY SPECIFIC FOR STREPTOCOCCUS PNEUMONIA

3.1 Introduction .............................................................................................................70
3.1.1 Hybridoma technology .................................................................................................71
3.1.2 The dynamics of the B cell response post pneumococcal vaccination ...................73
3.1.3 Mycoplasma .................................................................................................................75

3.2 Methods ...................................................................................................................76
3.2.1 Mycoplasma assessment ...........................................................................................76
3.2.2 Hybridoma generation .................................................................................................78

3.3 Results .....................................................................................................................81
3.3.1 Mycoplasma screening ...............................................................................................81
3.3.2 Pre and post vaccination pneumococcal serotitres ..................................................84
3.3.3 Hybridoma Characteristics ........................................................................................86
3.3.4 Hybridoma cell lines ....................................................................................................90
3.3.5 Antibody Concentration and purification .........................................................92

3.4 Discussion ...............................................................................................................93
3.4.1 Mycoplasma Contamination .......................................................................................93
3.4.2 Hybridoma Yield ..........................................................................................................94
3.4.3 Hybridoma Serospecificity ..........................................................................................97
3.4.4 Antibody Concentration ...............................................................................................98
3.4.5 Isotype distribution ......................................................................................................98
3.1 Introduction

To evaluate the heterogeneity of the antibody response induced by conjugate and polysaccharide pneumococcal vaccines at a genetic level, previous studies have purified pneumococcal specific antibodies from post vaccination serum using affinity chromatography and amino acid sequences have been determined (Lucas et al., 1997; Abadi et al., 1998; Sun et al., 1999). These studies have shown the repertoire to be limited to 1-4 clonotypes per pneumococcal serotype with common $V_h$ and $V_l$ genes used in different individuals. However, using this technique, it is not possible to perform detailed genetic analysis of the antibodies or to correlate specific genotypes with antibody function. For such studies, production of clonal populations of immortalised pneumococcal specific B cells secreting monoclonal antibody (Mab) are required.

Epstein-Barr virus (EBV) has been used to produce human Mabs (Crawford et al., 1985) EBV selectively infects human B cells expressing the complement receptor 2 (CR2, CD21). Human and marmoset cell lines have been established which secrete infectious virus. EBV containing supernatant is then added to human B cells. Those which become infected will grow indefinitely in medium lacking exogenous growth factors. The immortalised human B cells may then be subcloned on the basis of antigen specificity. However, there are a number of problems associated with this technique. Lymphocytes exhibit a low rate of infection (crawford, 1985) and successfully transformed cells tend to show unstable growth and secrete low levels of antibody. Although the cell line will release antibody of all isotypes into the supernatant, antibody isotype is dominated by IgM (Tosato et al., 1985) which may be explained by the higher surface EBV receptor density on IgM producing B cells. Concerns have also been raised that EBV may preferentially stimulate a sub-population of B-cells which may not even be present in vivo (Tosato et al., 1986). In
addition, the therapeutic use of antibodies derived from EBV-infected cells raises various safety issues.

A more successful approach to produce human monoclonal antibodies to analyse then normal immune repertoire is to generate hybridomas in which B cells are immortalised by fusion with a myeloma cell line and then selected on the basis of antigen binding specificity of the Mabs they produce.

3.2.1 Hybridoma technology

The production of murine monoclonal antibodies against specific antigens by hybridomas is a well established technique. Since their original description (Kohler & Milstein, 1975), murine hybridomas have become widely used and valuable research tools. The application of murine monoclonal antibodies is widespread, from biological and medical research to clinical diagnostics. Monoclonal antibodies also have potential in human therapeutic applications (Scott & Welt, 1997). Unfortunately their usefulness is limited due to negative side effects including the recipient's immune response to the murine protein (human anti-mouse antibody response; HAMA).

To circumvent the problems associated with the HAMA, human-human hybridomas were first generated in 1980 (Olsson & Kaplan, 1980). However success has been limited by a lack of suitable fusion partners and although such hybrids avoid the problem of the immunogenicity that is seen with murine Mabs when used therapeutically, antibody production in generally unstable and the titre is low (Jahn et al., 1989).

Currently the most successful approach used to generate human monoclonal antibodies for analysis of the normal immune repertoire is to generate human-mouse heterohybridomas. The traditional fusion approach is used, in which hybridomas are formed by fusing spleen cells from an immunised mouse to a suitable mutant myeloma partner, but replacing murine spleen cells with human lymphocytes. The fusion of
human lymphocytes to murine myeloma partners is a simple process with an efficiency higher than human-human fusions but lower than murine-murine fusions. It makes use of the existing bank of murine myeloma cell lines and allows the use of the powerful HAT (hypoxanthine-, aminopterin- and thymidine) selection technique in which myeloma cell lines deficient in enzymes essential for salvage nucleic acid synthesis pathways are used as the fusion partner (de & Scheidegger, 1980) for lymphocytes which can use the salvage pathways. Following polyethylene glycol (PEG)-induced fusion, unfused lymphocytes cells cannot exist in long-term culture and die within two to three days. In selective HAT media, myeloma cells that have not found a spleen cell partner also die due to the presence of aminopterin which blocks normal nucleic acid synthesis. Only myeloma-lymphocyte hybrids, which can use hypoxanthine and thymidine for DNA and RNA synthesis via salvage pathways, survive.

Aggressive cloning and screening for antibody production results in the selection of "immortal" cell lines which stably express monoclonal antibodies. Although chromosomal instability is cited as a major problem associated with human-mouse heterohybrids, extensive subcloning may facilitate the isolation of stably secreting clones.

The quantity, purity and activation state of the B cells are all important to ensure fusion success. Unstimulated metabolically inactive cells may yield cytoplasmic hybridomas, but the nuclei often fail to fuse and the multi-nucleate giant cells do not divide. If the donor lymphocytes are in a proliferative state, cytoplasmic and nuclear fusion occurs more readily and the hybridomas replicate. In vitro stimulation of lymphocytes prior to fusion has been assessed as a method of increasing Ag specific B cell numbers. This can be done using antigen (eg. tetanus toxoid (Butler & Lane, 1983)), polyclonal mitogens (eg. pokeweed mitogen (Teng & Lam, 1983) or LPS (Yoshinari & Arai, 1995)). However specificities may be directed away from the in vivo response.
producing artificial bias in the profiles of hybridomas produced. *In vitro* stimulation is therefore of limited usefulness if the objective is to evaluate the circulating B cell repertoire.

In vivo stimulation through vaccination activates antigen specific circulating B cells eliminating the need for *in-vitro* stimulation prior to fusion. Human volunteers may be boosted against specific antigens and peripheral blood then collected to provide a readily available source of human lymphocytes with which to examine the elicited response. The optimal timing to retrieve lymphocytes post vaccination depends upon the antigen and the immune history of the individual. A detailed analysis of the dynamics of the immune response to the antigen under investigation must therefore be made to achieve optimal yield. This technique has been used successfully to analyse the response to Hib conjugate and PS vaccines (Gigliotti *et al.*, 1984; Adderson *et al.*, 1991). However, limited data have been published using this technique to analyse responses to the pneumococcus (Shaw *et al.*, 1995).

### 3.2.2 The dynamics of the B cell response post pneumococcal vaccination

Detailed analyses of the dynamics of vaccination induced circulation of human B cells secreting type specific antibodies against pneumococcal polysaccharides have been made by a number of groups (Heilmann & Pedersen, 1986; Heilmann *et al.*, 1987; Kehrl & Fauci, 1983b; Kehrl & Fauci, 1983a) Heilman *et al* (Heilmann & Pedersen, 1986; Kehrl & Fauci, 1983b) administered a polyvalent pneumococcal vaccine (23VPS) to healthy adult volunteers. Using ELISPOTs to quantify B cell numbers they found that B cells secreting specific antibody of IgM isotype peaked in the circulation on day six post vaccination (552 x 10^6/l MNC) and those secreting IgG and IgA isotypes peaked on day seven (625 x10^6/l MNC and 1691 x 10^6/l MNC respectively). By day eight post vaccination, 5-20% of peripheral B cells were antigen binding. Pneumococcal specific B cells were not found in the circulation before day four post
vaccination or after day 12-14 and numbers of IgG specific B cells declined rapidly following the day seven peak. Polyclonal activation of circulating IgG producing B cells occurred as demonstrated by an increase in the expression of B cell activation markers such as the interleukin 2 receptor (IL-2R) (Tvede et al, 1989), although only one third of these cells were Pnc antigen specific.

The frequency distribution of pneumococcal specific AbSC and the antibody increment post vaccination was also studied for four of the pneumococcal serotypes in the vaccine and for the non-type specific antigen CWPS (Heilman et al 1987b). The distribution of AbSC numbers and antibody increments were not uniform across the serotypes with the absolute numbers of pneumococcal specific IgG producing B cells differing by five to six fold depending on the pneumococcal serotype. Results are shown in Table 3.1.

A positive correlation was found between mean numbers of B cells secreting IgG and IgA antibody against a given PPS antigen and the corresponding geometric mean concentration of post-vaccination IgG and IgA. However, this was not found for IgM. Prevaccination titres to CWPS were high with minimal increment post-vaccination and IgG dominated the isotype of CWPS AbSC in the circulation.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>% of Ag specific AbSC</th>
<th>Absolute AbSC nos/10^6 MNC*</th>
<th>Dominant isotype</th>
<th>Ab increment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9</td>
<td>700 x 10^6 (73-516)</td>
<td>IgA</td>
<td>4.2</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>97 x 10^6 (62-927)</td>
<td>IgA</td>
<td>4.6</td>
</tr>
<tr>
<td>18C</td>
<td>6</td>
<td>54 x10^6 (8-369)</td>
<td>IgA</td>
<td>2.9</td>
</tr>
<tr>
<td>CWPS</td>
<td>5</td>
<td>131 x 10^6 (0-627)</td>
<td>IgG</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 3.1 Frequency, absolute numbers and isotype of circulating AbSC specific for different pneumococcal serotypes and CWPS from a pool of 8 subjects on day seven post-vaccination with 23V-PS vaccine (Heilman et al 1987b). *mean and range of frequency.
These studies used the simple polysaccharide vaccine and data are not available for 
Pnc specific AbSC cell numbers post pneumococcal conjugate vaccine. However, a 
similar analysis has been performed examining the response to Hib conjugate and 
polysaccharide vaccines and similar isotype distribution and dynamics of AbSC 
numbers were demonstrated for both vaccine types (Barington et al, 1990).

In the present study, human-mouse heterohybridomas against the immunising antigen 
(23V-PS or 7V-CRM, conjugate pneumococcal polysaccharide vaccine) were 
generated as described below. Following a complete lack of success in the initial 
attempts to generate heterohybridomas, the fusion partner (a mouse myeloma cell 
line, OURI) was assessed for mycoplasma contamination, a well recognised cause of 
fusion failure.

3.2.3 Mycoplasma

The Mycoplasma genus is a serious source of contamination of tissue culture cell 
lines. It can grow to high titres in cell cultures with concentrations of up to $10^8$ colony 
forming units per ml of supernatant. In addition it can be found attached to cell 
membranes (Uphoff et al, 1992a). Mycoplasma lack a cell wall, they do not produce 
turbidity in culture and contamination is often undetected. Once an infection has 
become established, it can spread rapidly to other cell lines in the laboratory.

The incidence and effects of mycoplasma contamination have been described by a 
number of authors (Uphoff et al, 1992a; Uphoff et al, 1992b; Barile, 1977; Barile et al, 
1978; Hay et al, 1989). Infection is common and can have a profound effect on cell 
functioning at a number of levels. Mycoplasma can induce chromosomal abnormalities, 
alter the antigenicity of cell membranes, affect cellular metabolism by competition for 
nutrients and influence cell fusion procedures. The latter was particularly relevant for 
this project. Heterohybridoma production involved the successful fusion of murine and 
human cells, an inherently sensitive system and once produced, cell lines were being
grown in continuous culture for a number of months. The lack of a cell wall confers resistance to many antibiotics and, once a cell line has become infected, treatment is expensive, time consuming and has a maximum success rate of ~75% (Uphoff et al, 1992b; Fleckenstein et al, 1994; Drexler et al, 1994).

The number of mycoplasma species exceeds 20 and of these six species have been shown to cause 98% of laboratory infection. These are: *Mycoplasma orale* (human throat commensal, cell culture isolate), *Mycoplasma arginini* (cell culture isolate), *Mycoplasma hyorhinis* (porcine origin, cell culture isolate), *Mycoplasma salivarium*, *Mycoplasma hominis* and *Acholeplasma laidlawii* (cell culture isolate).

It has been recommended that a combination of methods should be used to reliably determine cell contamination. Of the methods available, a direct culture and an indirect or non-culturable method should be used. Uphoff et al, 1992, have discussed the range of methods available to detect contamination. Three mycoplasma screening tests were used to evaluate the mycoplasma status of the OURI mouse myeloma cell line and subsequent hybridoma cell lines, as described below. Once certified clean, all cell lines and subsequent hybridomas were cultured in an exclusive mycoplasma free laboratory in which regular screening for contamination was performed.

### 3.3 Methods

(For detailed description of general methods see Chapter 2, Section 2.10-2.11)

#### 3.3.1 Mycoplasma assessment

Following the initial failure to generate successful heterohybridomas, the OURI cell line used and all subsequent cell lines and heterohybridomas were screened for *Mycoplasma* contamination using a minimum of two of the following three tests.
Test 1: DNA Fluorescent Staining

In this method, the cells to be tested were grown to confluence on a slide and then stained with a fluorescent stain (Hoechst stain) which stains the nuclear DNA of host cells and any contaminating DNA. The stain emits fluorescence at 490-500nm. Mycoplasma positive cells show nuclei surrounded by pinpoint fluorescence (0.1-3μm) at the cell wall (Figure 3.1) and negative controls show only nuclear staining. The morphology of mycoplasma contamination is variable and may be seen as spherical or filamentous bodies aggregating in clusters or rows. Transformed cell lines may show high cytoplasmic background fluorescence although nuclear debris stains brightly and tends to be larger than the mycoplasma.

Test 2: DNA Hybridisation

The DNA hybridisation assay is based on the ability of complementary nucleic acid strands of DNA to come together to form stable double stranded complexes. A $^3$H-labelled single stranded DNA probe was used which was complementary to the ribosomal RNA of all Mycoplasma and Acholeplasma species which commonly infect tissue culture.

The $^3$H-labelled DNA probe was allowed to combine with the RNA of the target organism to form a stable DNA-RNA hybrid. This was separated from the non-hybridised DNA probe. Scintillation solution was added to the labelled hybrids and counted in a scintillation counter. Test results were calculated as the percentage of input counts hybridised. Mycoplasma species known to be reactive in the test included: A.axanthum, A.granularum, A.oculi, M.arthritidis, M.fermentans, M.hominis, M.muris, M.pirum, M.salivarium, S.citri, A.laidlawi, A.morum, M.arginini, M.capricolum, M.genitalium, M.hyorhinis, M.oreale, M.pneumoniae, S.apis, S.floricola. The probe was designed to cover a broad range of Mycoplasma and Acholeplasma which may infect
tissue culture, although due to a divergence in the taxonomy of mycoplasma species, other bacterial species may also be detected with low sensitivity.

Test 3: Tissue Culture

These assays were performed by a commercial operator off site. Cells to be tested were sent in log phase growth. The cell samples are inoculated into a unique recovery media which was able to support the growth of \textit{M. hyorhinis} cultivar $\alpha$ and fastidious strains of \textit{M. orale} and \textit{M. fermentans}.

3.3.2 Hybridoma generation

The sequence of events to generate a panel of heterohybridomas pneumococcal specific monoclonal antibody (Mab) is shown in Figure 3.1a -c. Five healthy adult volunteers were bled for serum and whole blood and then vaccinated with the 23V-PS or the 7V-CRM$_{197}$ vaccine ($n=1$ and $n=4$ respectively). The 23V-PS contained 50$\mu$g of each serotype. The 7V-CRM$_{197}$ contained 2$\mu$g each of serotypes 4, 9V, 14, 18C, 19F, 23F and 4$\mu$g of serotype 6B. The vaccinees were re-bled on day (D)+7 to collect lymphocytes and on D+28 to collect serum.

D7 lymphocytes were isolated from peripheral blood by density gradient centrifugation (Figure 3.1b), washed and fused to a mouse myeloma cell line (OURI) using PEG mediated fusion. Fused cells were washed, resuspended in HAT medium and plated out onto a 96 well plate pre-coated with an irradiated mouse fibroblast feeder layer and incubated for 2-3 weeks before screening.
Chapter 3 Hybridoma Production

Vaccinate

- 23V-PS (Pneumovax®)
- 7V-CRM₁₉₇ (7 valent)

D₀
Bleed: serum

D+7
Bleed: lymphocytes

D+28
Bleed: serum

Figure 3.1a. Vaccination schedule and post vaccination bleeds. Serum and whole blood was taken on day 0 (D₀) and subjects were vaccinated with either the 23V-PS or the 7V-CRM₁₉₇ vaccine. Subjects were re-bled on day 7 and day 28 post-vaccination for lymphocyte and serum separation respectively.

1. Lymphocyte isolation
2. Lymphocyte/OURI fusion
3. incubate 2-3/52 on fibroblast feeder layer

Figure 3.1b Heterohybridoma production. D7 lymphocytes were isolated by density gradient centrifugation on ficoll. Washed lymphocytes were then mixed with OURI mouse myeloma cells and fused using polyethylene glycol (PEG). Fused cells were then plated out onto a 96 well plate pre-coated with a mouse fibroblast feeder layer and incubated for 2-3 weeks prior to screening for antibody production.
After two to three weeks of culture, supernatants were screened by ELISA for pneumococcal specific antibody production (Figure 3.1c). Supernatant was initially screened on a heptavalent coated plate. Serospecificity was then determined using a plate coated with single serotypes. Positive wells were subcloned at least twice by limiting dilution. Persistent high secretor clones were then grown in standard tissue culture flasks, samples of each clone were cryopreserved and stocks of Mab were generated.

Figure 3.1c Screening heterohybridoma supernatants by ELISA for pneumococcal specific antibody production, subcloning and generation of antibody stocks.
Immunocytochemistry was performed to determine light chain use and the secretor status of the clone. Mab isotype, serotitre and \textit{in vitro} functional activity were determined and antibody stocks were then concentrated and purified.

Five healthy adult volunteers were vaccinated. Four received the 7V-CRM$_{197}$ conjugate vaccine (subjects A-D) and one (subject E) received the 23V-PS vaccine.

3.4 Results

3.4.1 Mycoplasma screening

\textit{DNA Fluorescent Staining}

The original source of OURI cells stained positive for mycoplasma contamination as shown in Figure 3.2 in which the nuclei are surrounded by pin point fluorescence in the cytoplasm and the cell wall. This was subsequently validated with the DNA hybridisation and tissue culture assays as described below.

\textit{DNA HYBRIDISATION}

Results of the DNA hybridisation assay are shown in Table 3.2. The total count represents the radioactivity in the labelled probe and the background represents activity in the other reagents used. The negative control is buffer containing preservative where-as the positive control is buffer, non-infectious mycoplasma nucleic acid and preservative.

The first batch of OURI cells screened strongly positive for mycoplasma contamination using the DNA hybridisation test in accordance with the results from the Hoechst stain. A new source was located, a cell line established and subsequently screened. The results obtained are shown in Table 3.3.
Figure 3.2 Hoechst stain of Mycoplasma contaminated OURI cell line. Nuclei stain uniformly brightly where-as Mycoplasma stain as particulate matter in the cytoplasm and along the cell membrane.
### Table 3.2 Results of mycoplasma DNA hybridisation test for batch 1 OURI cells.

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Counts per minute (CPM)</th>
<th>% hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>total count</td>
<td>126936</td>
<td>100</td>
</tr>
<tr>
<td>background</td>
<td>5</td>
<td>NA</td>
</tr>
<tr>
<td>negative control</td>
<td>107</td>
<td>0.08</td>
</tr>
<tr>
<td>positive control</td>
<td>99231</td>
<td>78</td>
</tr>
<tr>
<td>OURI 1</td>
<td>49721</td>
<td>39</td>
</tr>
</tbody>
</table>

CPM represents counts per minute of tritiated thymidine from radiolabelled mycoplasma detection probe. % hybridisation represents % of DNA labelling positive relative to positive control. % hybridisation > 0.4% indicates mycoplasma contamination.

### Table 3.3 Results of DNA hybridisation test for batch 2 OURI cells.

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>CPM</th>
<th>% hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>total count</td>
<td>120658</td>
<td>100</td>
</tr>
<tr>
<td>background</td>
<td>15</td>
<td>NA</td>
</tr>
<tr>
<td>negative control</td>
<td>393</td>
<td>0.003</td>
</tr>
<tr>
<td>positive control</td>
<td>117968</td>
<td>98</td>
</tr>
<tr>
<td>OURI 2</td>
<td>488</td>
<td>0.004</td>
</tr>
</tbody>
</table>

This second batch of OURI cells tested negative for mycoplasma contamination and were used for all subsequent hybridisations. This test was re-validated with the tissue culture test performed by a commercial operator.
3.4.2 Pre and post vaccination pneumococcal serotitres

Serum was taken on the day of vaccination in the pre-vaccination bleed and at day 28 post vaccination and analysed in the standard pneumococcal ELISA for serotype specific antibody (method described in Chapter 2). The results obtained are shown in Table 3.4. Subjects A-D received the 7V-CRM\textsubscript{197} vaccine and subject E received the 23V-PS vaccine.

All subjects demonstrated pre-existing IgM and IgG to the seven serotypes tested although pre-vaccination titres were generally low. Good seroconversion was demonstrated post vaccination with IgG and IgM levels increasing up to 30 fold to all serotypes tested with the exception of subject A who demonstrated weak seroconversion overall, subject B who produced low levels of IgG for serotypes 9V and 18C and subject E who produced low levels of IgM for serotype 23F.

Pre-vaccination IgA titres were generally low with the exception of subjects C and E having measurable IgA to serotype 6B, and subjects C and D having IgA to serotypes 4 and 18C respectively. Overall there was poor seroconversion of IgA post vaccination with the exception of subject D who demonstrated a very large increase in IgA titre to serotype 4. The polysaccharide vaccinee demonstrated a slight increase in IgA titres to serotype 4 and 6B IgA levels remained elevated but have not been quantified further. The conjugate vaccinees demonstrated a rise in titre in only two or the five serotypes tested (4 and 6B).
Table 3.4 Serum antibody concentration (μg/ml) of IgM, IgA and IgG to 5 pneumococcal serotypes pre- and day 28 post-pneumococcal vaccination. Isotypes and serotypes from subjects for which hybridomas were grown are in bold italics.
3.4.3 Hybridoma Characteristics

22 hybridomas secreting antibody to Pnc PS were derived from the five vaccinees. The serotype and isotype distribution of the hybridomas is shown in Table 3.5.

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>VACCINE</th>
<th>CLONE</th>
<th>SEROTYPE</th>
<th>ISOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7V-CRM</td>
<td>AbA5</td>
<td>18C</td>
<td>G1</td>
</tr>
<tr>
<td>A</td>
<td>7V-CRM</td>
<td>AbA6</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>B</td>
<td>7V-CRM</td>
<td>DMF4</td>
<td>4</td>
<td>A2</td>
</tr>
<tr>
<td>B</td>
<td>7V-CRM</td>
<td>DM5</td>
<td>6B</td>
<td>G2</td>
</tr>
<tr>
<td>B</td>
<td>7V-CRM</td>
<td>DM17</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>C</td>
<td>7V-CRM</td>
<td>CbH4</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>C</td>
<td>7V-CRM</td>
<td>CbE2</td>
<td>23F</td>
<td>G2</td>
</tr>
<tr>
<td>C</td>
<td>7V-CRM</td>
<td>CbB2</td>
<td>4</td>
<td>G2</td>
</tr>
<tr>
<td>C</td>
<td>7V-CRM</td>
<td>CbG8</td>
<td>6B</td>
<td>A2</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db3G9</td>
<td>6B</td>
<td>G2</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db7D4</td>
<td>4</td>
<td>G2</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db8C11</td>
<td>4</td>
<td>A1</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db2C10</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db3B5</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db5F9</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db7A9</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>7V-CRM</td>
<td>Db7F1</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>E</td>
<td>23V-PS</td>
<td>6bC3</td>
<td>9V</td>
<td>A1</td>
</tr>
<tr>
<td>E</td>
<td>23V-PS</td>
<td>6b53</td>
<td>18C</td>
<td>G3</td>
</tr>
<tr>
<td>E</td>
<td>23V-PS</td>
<td>6b5D7</td>
<td>6B</td>
<td>M</td>
</tr>
<tr>
<td>E</td>
<td>23V-PS</td>
<td>6b5B12</td>
<td>9V</td>
<td>M</td>
</tr>
<tr>
<td>E</td>
<td>23V-PS</td>
<td>6B1A7</td>
<td>P</td>
<td>M</td>
</tr>
</tbody>
</table>

Table 3.5. Serotype and Isotype distribution of 22 hybridoma clones. Subjects A-D received the 7V-CRM, vaccine and subject E received the 23V-PS vaccine. 17 clones were derived from the 7V-CRM vaccinees and five from the 23V-PS vaccinee. Clone identification is shown in column 3. Serospecificity and isotype are shown in columns four and five respectively. P = polyreactive to all serotypes tested. IgG and IgA subclasses are also given with the exception of five serotype 4 IgA clones.

Four of the IgA isotypes (DMF4, CbG8, Db8C11 and 6bC3) were analysed to determine whether they were monomeric or dimeric by Dr J Reinholdt (Copenhagen, Denmark) using Superose column separation, an IgA alpha chain-specific sandwich
ELISA and an ELISA to assess the ability of the Mabs to bind free secretory component (Russell et al., 1989). All Mabs were found to be in monomeric form.

Of the 22 clones, 17 came from the four conjugate vaccine recipients of which eight were from one individual (subject D) and five came from the single recipient of the simple polysaccharide vaccine. All clones came from subjects who had demonstrated high post-vaccination antibody levels of the same isotype with the exception of Subject A (IgG Mab to serotype 18C) who had low post vaccination serum levels to all serotypes and subject E (IgA Mab to serotype 9V) (Table 3.4). Subject D demonstrated marked elevation in IgG, A and M to serotype 4 and of the eighth clones produced from this vaccinee, seven were serotype 4 specific and although only two have been analysed to date (Db8C11 and Db7D4: discussed in Chapter 4) they may all be products of the same serotype 4 specific clone.

14 of the antibodies secreted by the hybridomas obtained from the conjugate vaccine recipients were IgG or IgA and serotype specific. Three were polyreactive demonstrating binding to all serotypes tested on ELISA, and of IgM isotype. Of the five antibodies produced by the plain polysaccharide, four were serotype specific however only two were isotype switched (6bC3, 6b53).

Of the 22 hybrids selected by initial screening, 15 underwent more detailed analysis to verify serospecificity and analyse genotype. This included two of the polyspecific IgM secreting clones. Pre-incubation with pneumococcal CWPS did not inhibit binding of these Mabs to PS and no cross-reactivity with a capsular polysaccharide derived from an unrelated organism (Hib, data not shown) was demonstrated. These two antibodies were classified as pneumococcal specific polyreactive Mabs (P). The 13 clones which appeared serospecific on standard ELISA were re-evaluated using a competitive inhibition ELISA, results are shown in Figure 3.3.
Figure 3.3: Specificity of the 13 serotype specific monoclonal antibodies as determined by competitive inhibition ELISA with homologous capsular polysaccharides or a combination of six heterologous pneumococcal capsular polysaccharides. Results are expressed as percentage inhibition of binding to the putative capsular polysaccharide target.
High specificity was demonstrated by the Mabs derived from individuals receiving the conjugate vaccine. The specificity of the antibodies derived from the individual receiving the PS vaccine (these included two IgM Mabs) was more difficult to define and significant cross reaction and less complete inhibition was demonstrated.

### 3.4.4 Hybridoma cell lines

Immunocytochemistry was performed on a number of the hybridomas to determine the percentage of cells producing human antibody and to distinguish between kappa and lambda light chain usage of the hybridomas. These results are shown in Table 3.6.

<table>
<thead>
<tr>
<th>Clone</th>
<th>% cells staining positive for isotype specific heavy chain</th>
<th>% cells staining positive for light chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF4</td>
<td>80%</td>
<td>75% kappa</td>
</tr>
<tr>
<td>DM17</td>
<td>100%</td>
<td>NA</td>
</tr>
<tr>
<td>DM5</td>
<td>100%</td>
<td>NA</td>
</tr>
<tr>
<td>AbA6</td>
<td>50%</td>
<td>50% kappa</td>
</tr>
<tr>
<td>AbA5</td>
<td>95%</td>
<td>NA</td>
</tr>
<tr>
<td>CbB2</td>
<td>80%</td>
<td>50% kappa</td>
</tr>
<tr>
<td>Db7D4</td>
<td>85%</td>
<td>40% kappa</td>
</tr>
<tr>
<td>CbE2</td>
<td>70%</td>
<td>40% kappa</td>
</tr>
<tr>
<td>Db3G9</td>
<td>5%</td>
<td>5% kappa</td>
</tr>
<tr>
<td>6b53</td>
<td>85%</td>
<td>5% kappa</td>
</tr>
</tbody>
</table>

Table 3.6: Immunohistochemistry of selected hybridomas to evaluate the percentage (%) of cells producing human Mab as determined using biotinylated anti-human heavy chain and light chain antibodies.

The majority of clones assessed demonstrated a high percentage (%) Ig production when stained with anti-Cₙ antibody. A representative photograph of the cytospin slide of hybridomas from clone ABA5 stained with HRP labelled anti human IgG is shown in Figure 3.4.
Fig 3.4 Immunocytochemistry. Cytospin of AbA5 hybridoma cells stained with peroxidase labelled antihuman IgG. Human IgG stains brown and is seen in the cytoplasm of the hybridoma cells.
The anti-\( C_L \) antibodies did not stain as well with a maximum of 50% of cells staining for human light chain production. However the anti-\( C_L \) antibodies were effective at distinguishing kappa from lambda light chain usage and no cross reactivity was demonstrated. Of the seven clones stained by immunohistochemistry for light chain usage, kappa light chain usage predominated with only one clone (DM5) using the lambda chain.

High secreting clones were then expanded into tissue culture flasks and stocks of Mab were produced. IgA and IgM clones were cryopreserved within a month of successful cloning and IgG clones were maintained in tissue culture to build up cell and antibody stocks. The low secretor clone Db3G9 was re-subcloned and rescreened but secretion was lost within three months of continuous culture. Eleven of the hybridomas were grown in continuous culture for six months. Over this time two clones (6bC3 and 6b53) lost secretion despite repeated subcloning and three demonstrated a marked reduction in secretion as determined by antibody concentration in the hybrid supernatants.

The success of reconstituting cryopreserved hybrids was also assessed. 18 of the 22 lines were reconstituted and 16 of these including the Db3G9 clone continued to secrete antibody (data not shown). Antibody secretion was lost in two of the clones: 6bC3 and 6b53 and despite repeated subcloning secreting cells could not be rescued. Although useful for subsequent genotype analysis these clones did not produce sufficient antibody to facilitate subsequent assessment of antibody functional activity (Chapter 5).

3.4.5 Antibody Concentration and purification

The antibody titre in the hybridoma supernatants and/or concentrates were
determined by the pneumococcal ELISA standardised for measuring IgG (Chapter 2, section 2.7).

Antibody titres in fresh hybridoma supernatants varied greatly and despite concentrating 50 fold, maximum concentrations of $72 \mu\text{g/ml}$ were achieved as measured by ELISA. CbB2 supernatants were concentrated and purified using a protein G-column. The titre of concentrated G-column purified Mab was compared with the concentrated Mab and no increase in concentration was achieved through purification.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Serotype</th>
<th>Isotype</th>
<th>supernatant/concentrate</th>
<th>Ab concentration by ELISA $\mu\text{g/ml}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbA5</td>
<td>18C</td>
<td>G1</td>
<td>concentrate</td>
<td>18</td>
</tr>
<tr>
<td>DM5</td>
<td>6B</td>
<td>G2</td>
<td>concentrate</td>
<td>0.8-3</td>
</tr>
<tr>
<td>DM4</td>
<td>4</td>
<td>A2</td>
<td>concentrate</td>
<td>40</td>
</tr>
<tr>
<td>CbE2</td>
<td>23F</td>
<td>G2</td>
<td>concentrate</td>
<td>33</td>
</tr>
<tr>
<td>CbB2</td>
<td>4</td>
<td>G2</td>
<td>concentrate</td>
<td>23</td>
</tr>
<tr>
<td>CbG8</td>
<td>6B</td>
<td>A2</td>
<td>fresh</td>
<td>7.7</td>
</tr>
<tr>
<td>CbG8</td>
<td>6B</td>
<td>A2</td>
<td>concentrate</td>
<td>72</td>
</tr>
<tr>
<td>Db3G9</td>
<td>6B</td>
<td>G2</td>
<td>concentrate</td>
<td>4.5-35</td>
</tr>
<tr>
<td>Db7D4</td>
<td>4</td>
<td>G2</td>
<td>concentrate</td>
<td>1</td>
</tr>
<tr>
<td>Db8C11</td>
<td>4</td>
<td>A1</td>
<td>fresh</td>
<td>4.6</td>
</tr>
<tr>
<td>Db8C11</td>
<td>4</td>
<td>A1</td>
<td>concentrate</td>
<td>45</td>
</tr>
<tr>
<td>6b53</td>
<td>18C</td>
<td>G</td>
<td>concentrate</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>6b5B12</td>
<td>9V</td>
<td>M</td>
<td>concentrate</td>
<td>0.4</td>
</tr>
<tr>
<td>6bC3</td>
<td>9V</td>
<td>A1</td>
<td>concentrate</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 3.7 Antibody concentration in fresh supernatants and supernatant concentrates from IgG producing serotype specific hybridomas as determined by ELISA.
3.5 Discussion

3.5.1 Mycoplasma Contamination

The Hoechst stain was the first mycoplasma test used and although rapid to perform, was not as reliable as either the DNA hybridisation or tissue culture diagnosis. It was therefore not incorporated into the routine screening protocol. The DNA hybridisation assay was highly reliable and the most useful screening assay since the results were available on the day of the test. Tissue culture diagnosis was a useful complement to the DNA hybridisation technique. It was used as a back up for screening cells in continuous culture which had been tested mycoplasma negative by DNA hybridisation at the outset.

Both original stocks of the OURI cell line and B95.8 tested positive for mycoplasma contamination as shown by the Hoechst stain (Figure 3.1), DNA hybridisation (Table 3.1) and tissue culture diagnosis. New sources of both cell lines were obtained and they both tested negative by DNA hybridisation (Table 3.2) and tissue culture testing. A separate laboratory was established ‘Mycoplasma Free’ and all subsequent tissue culture for hybridoma production was performed in this exclusive facility. (Protocol for lab use: Appendix 2).

3.5.2 Hybridoma Yield

The yield of antigen specific hybridomas achievable post vaccination varies depending on the number of antigen specific AbSCs circulating at the time of post vaccination bleed, the fusion rate of the B cell / myeloma cell mix, the sensitivity of the supernatant screening assay and the success of sustaining hybridomas in tissue culture through the process of subcloning and cell line expansion.
The enhanced immunogenicity of the conjugate vaccine may induce more AbSCs and thus improve hybridoma yield although a direct comparison between circulating Day seven AbSC numbers induced post conjugate or simple PS vaccine has not been described. No difference in hybridoma yield was demonstrated between the conjugate and polysaccharide vaccinees in this study although the number of vaccinees was small.

The age of the vaccinee and their immune history may affect hybridoma yield. AbSC numbers have been quantified following Hib conjugate vaccination in adults and infants (Barington et al 1994). Adults receiving a single dose of Hib-conjugate vaccine produced ten times more AbSCs/ml of blood than infants receiving two to three doses of the vaccine in accordance with the infant immunisation schedule. However, in the same study AbSCs were also quantified in adults receiving a second vaccination four weeks later and AbSC numbers were shown to drop to one tenth of infant levels following the second dose. The phenomenon of epitope suppression (Heyman et al 1990) or tolerance (Pichichero 1985, Kearney and Johnstone 1985, Klaus and Humphrey 1976b, Klaus 1976a) was demonstrated and suggests that repeated immunisation of adults will not be useful for boosting circulating AbSC numbers. Although AbSC numbers may be lower in infants this does not equate with a reduced chance of fusion success. Results from a collaborative laboratory in which infants and adults were vaccinated with tetanus toxoid demonstrated that age of the vaccinee may influence the success rate of the actual fusion process (Jessup et al 2000). In this study one infant provided 13 hybridomas producing antibody to tetanus toxoid from 2ml of blood compared to an adult yield of an average of three hybrids from 10ml of blood. The data suggests that this high yield was a result of an increase in fusion efficiency overall rather than an increase in the relative proportion of circulating
AbSC. The superior fusion potential of infant B cells may be enhanced by infant serum. Westerwoudt and Blom (1983) cite human cord serum as having a strong growth-promoting activity on hybridomas. The use of human cord blood lymphocytes as feeder cells for human hybridomas has also been reported (Kurpisz & Simon 1987).

Variables such as PEG concentration (30-50%), the addition of DMSO to the fusion mix, pH or temperature are all considered important in determining heterohybridoma fusion efficiency. However a comparative analysis of two methods using different PEG concentrations +/-DMSO and different speeds of fusion (Jessup et al 2000) suggest that these variables may not have had a great impact on hybridoma yield.

The addition of growth factors to the culture medium is known to improve growth rates and cloning efficiency of both murine and human-mouse hybridomas. Supernatant from a human tumour line (5637) has been shown to further assist growth of heterohybridomas (Jessup et al 2000), perhaps by providing further factors of human origin. Similarly, Zhu and Jin (1993) found the use of conditioned media from various human fibroblast and lymphoblastoid lines improved heterohybridoma growth, cloning efficiency and antibody secretion. In this study, the use of Hybrimax, a cocktail of mouse macrophage derived growth factors either alone or as a fibroblast supplement offered no advantage in hybridoma yield over a mouse fibroblast feeder layer alone which proved most successful for hybridoma yield and viability. It is recognised that direct contact of heterohybrids with the fibroblasts facilitates hybridoma growth and survival (Murti et al 1996, MacMillan & Gonda 1994, Long 1986, Ishii et al 1981, Epstein & Kaplan et al 1979) and it may be that a combination of a feeder layer and additional growth factors would provide the optimal growth conditions.
Chromosomal instability is a characteristic of heterohybridomas and may be reflected in the irregular morphology shown within heterohybridoma colonies. Heterohybrids preferentially lose human chromosomes (Kozbor & Croce 1985) and hence the human Ig genes may be quickly lost in culture. This accords with the data presented above and the experience of others (Schlom et al 1980) in which the antibody production of at least some clones reduced over time in continuous culture. Secretion was lost completely in three of the fifteen clones and reduced considerably in a further three clones over a period of six to nine months of continuous culture. Of these six cell lines, four were successfully resurrected from cryopreserved samples to secrete antibody at good titre.

Chromosomal loss is not random and chromosomes 14 and 22 (containing heavy and λ chains respectively) tend to be retained while chromosome 2 (containing the κ chain) is lost more easily (Croce et al 1980, Erikson et al 1981). This may result in bias when hybridomas are generated to analyse light chain use and would explain the majority of reports where the antibodies produced almost exclusively contain the λ chain (Krenn et al 1999). However, kappa chain dominance was clearly demonstrated by the pneumococcal hybrids described and there have been numerous other reports of heterohybrids producing antibodies to a variety of antigens, containing the κ chain (Lucas et al 1998, Lane et al 1998, Schlom et al 1980).

Extensive subcloning with regular immunohistochemical analysis to maintain high secretor status may facilitate the isolation and maintenance of a representative population of heterohybridomas stably secreting high levels of Ig (Westerwoudt & Bloom 1983, Thielmans et al 1984). However, the procedures to retain high levels of secretion are time consuming and a range of cell division inhibitors, anti-cancer drugs and agents which lengthen cell doubling times have been investigated to reduce
this problem (Yoshinari et al 1996). Oubain and cytochalasin B are known to stimulate and prolong the production of κ chain antibody-producing heterohybrids but the growth rate of heterohybridomas may be reduced. Perhaps the answer is a complex heterohybridoma medium supplement containing human cytokines, growth factors (including those derived from infant B cells) and selective agents designed to facilitate the retention of human chromosomes.

3.5.3 Hybridoma Serospecificity

The poor specificity of hybrids derived from the PS vaccinee compared to those derived from the conjugate vaccinees is difficult to explain. IgM is recognised as having less specificity compared with isotype switched antibodies (Baccala et al 1989). IgM is produced early in the immune response and is regarded as part of the pre-immune repertoire having not undergone the process of receptor editing associated with BcR ligation (Hertz & Nemazee 1997) which results in selective expansion of high affinity clones with improved specificity. This process occurs concurrently with isotype switching resulting in improved specificity of isotype switched clones. However, although the 2 IgM antibodies (6b5D7 and 6b5B12) performed most poorly in the inhibition assay, the IgG and IgA Mabs from the PS vaccinee also demonstrated incomplete inhibition of binding by the homologous serotype although no cross reactivity was demonstrated. Evaluation of these Mabs in opsonophagocytic assays will clarify whether functional activity is serotype specific or demonstrated across a number of serotypes.

3.5.4 Antibody Concentration

The ELISA used to screen the hybrid supernatants was highly sensitive and able to detect antibody concentrations of 1-20 ng/ml depending on serotype. This
facilitated selection and subcloning early after fusion. Antibody concentration in hybridoma supernatants after two weeks incubation ranged from 4-11 μg/ml (DbSCI1 and CbG8 respectively). These levels were comparable to those secreted by Hib heterohybridomas produced using similar techniques (Gigliotti et al 1984, Martin et al 1988).

3.5.5 Isotype distribution

Of the 22 hybridomas produced, 18 were serotype specific of which 16 were isotype switched reflecting the peak in switched antigen specific AbSC in the circulation 7-8 days post vaccination (Heilmann & Pedersen 1986, Heilmann 1987, 1990. All of the serotype specific clones from the conjugate vaccine recipients were of IgG or A isotype compared to two out of four of the clones from the PS vaccinee. This may be a reflection of vaccine formulation which is known to influence the dynamics of the immune response (Barington et al 1991, Granoff et al 1993, Makela et al 1987). The immune response to the conjugate peptide is an integral part of the humoral response to conjugate polysaccharides. Hib conjugate vaccine studies have demonstrated that prevaccination immunity to tetanus toxoid carriers can result in earlier peaking of IgG and IgA AbSC to the polysaccharide and higher circulating AbSC numbers. This may be explained by the existence of a primed population of B and T cells to the carrier resulting in more efficient antigen presentation and greater T cell help. In contrast, prevaccination immunity to the PS has not been shown to influence the dynamics of the response (Heilmann & Pedersen 1986) and, as has been discussed, an inverse relationship may even exist with reduced AbSC numbers documented after second or subsequent antigen challenge (Barington et al 1994). A positive correlation between seroconversion and hybridoma yield was suggested (Table 3.7). This was most
notable for subject D who demonstrated high post-vaccination antibody levels to serotype 4 and yielded eight serotype 4 hybridomas. The association of high seroconversion and high circulating AbSC numbers is supported by data published by Heilmann et al (1990) and Lue et al (1988) in which circulating IgA AbSC numbers on day 7 post vaccination correlated with a high IgA Ab concentration on day 15.

In summary, human-mouse heterohybridoma production represents an effective and valuable technique for sampling the immune response to vaccine antigens. The advantage of heterohybridoma production over more efficient cell sampling techniques such as single cell PCR and ELISPOT analysis is that immunoglobulin gene usage can be directly correlated with antibody function. The disadvantage is that the yield is relatively small and the process is very labour intensive. Yield may be improved by modification of the methods described using B cell enrichment techniques such as panning lymphocytes on antigen prior to fusion or passing lymphocytes through a MACS (Magnet activated cell sorter) column pre-coated with the antigen of interest. The addition of specialised cytokines and growth factors to optimise heterohybrid viability and antibody secretion rates is under investigation in a number of laboratories with the caveat that any *in vitro* manipulation of the B cells prior to fusion or post fusion may bias the profile of heterohybridomas produced.

Detailed genotypic analysis of 15 of the heterohybridomas produced will be presented in the next chapter and *in vitro* assays of Mab functional activity are presented in Chapter 5.
CHAPTER FOUR

GENETIC ANALYSIS OF PNEUMOCOCCAL HYBRIDOMAS

4.1 Introduction ................................................................. 101

4.2 Methods ........................................................................... 103

4.3 Results ........................................................................... 108
  4.3.1 V gene use .................................................................. 108
  4.3.2 CDR3 and Somatic Mutation ..................................... 114
  4.3.3 Canonical Classification ............................................ 114

4.4 Discussion ....................................................................... 122
  4.4.1 V Gene Use ............................................................... 122
  4.4.2 Canonical Genes ......................................................... 124
  4.4.3 Mutation and Memory .............................................. 125
4.1 Introduction

The molecular basis of the immune response to bacterial polysaccharides is poorly understood. Studies to determine the evolution of immunoglobulin germline genes and diversity in both mice and man have suggested that the response to bacterial antigens such as the phosphorylcholine (PC) hapten and capsular polysaccharides is restricted both within and between individuals with specific immunoglobulin V genes dominating the response (Yang et al, 1994; Adderson et al, 1993). Molecular analysis of the antibody response to *Haemophilus influenzae* type b (Hib) has dominated the studies in man. Serological studies have demonstrated that the immune response within an individual is oligoclonal (Adderson et al, 1993; Insel et al, 1985). In addition gene families Vh3 and Vk2 are over represented and, within these families specific genes Vh3-15 (Vh26), Vh3-23 (Vh9.1) and Vk2A2 appear to dominate both within and between individuals (Adderson et al, 1993; Lucas et al, 1994; Silverman & Lucas, 1991). While complementarity determining region 3 (CDR3) characteristics are less well defined, some investigators have demonstrated selective VDJ gene use and identical joining sites within and between individuals (Adderson et al, 1993). Preferential use of Vh3 and Vk2 gene families has also been noted for other polysaccharide antigens (Pirofski et al, 1995; Azmi et al, 1994).

A dramatic change in Vλ region use in the response to Hib conjugate vaccine as a function of age has been demonstrated (Lucas et al, 1993). This is supported by murine studies in which the pattern of restriction to PC also appears to change with age (Yang et al, 1994). Studies of responses to vaccines consisting of native Hib capsular polysaccharide (polyribitol ribosyl phosphate, PRP) of different lengths, PRP conjugated to protein and pneumococcal di- and tri-saccharides have revealed that the nature of the particular form of vaccine can influence both the repertoire and the
functional activity of antibody generated (Pillai et al, 1991; Adderson et al, 1998; Alonso-De et al, 1993). The significance of this has been emphasised by further Hib studies in which antibody fine specificity, avidity and protective capacity correlate with the expression of particular clones and V regions (Granoff et al, 1993; Lucas & Granoff, 1995; Chung et al, 1993).

Repertoire diversity, if a function of age and vaccine formulation, may have important implications for vaccine efficacy in the infant population and influence the immune repertoire of adults receiving polysaccharide conjugate vaccines in infancy. Despite recent progress in both vaccinology and immunology relatively little is known of the molecular characteristics of antibodies to Streptococcus pneumoniae. It has been shown by means of isoelectric focusing and light chain analysis that the antibody response to pneumococcal polysaccharides is generally oligoclonal and within individuals is dominated by either κ or λ (Lucas et al 1997). This dominance may be determined by serotype with antibodies to serotype 6B demonstrating selective lambda chain and antibodies to serotypes 14 and 23F selective kappa chain usage (Lucas et al, 1997). As is the case for antibodies to other encapsulated bacteria, gene families Vh3 and Vk2 are over-represented and a number of individuals appear to use similar genes (Park et al, 1996). In a recent study analysing Vh gene family use following 23V-PS vaccine in HIV infected and non-infected individuals, a significant difference in the distribution of Vh gene families was demonstrated between the two groups and it was proposed that the reduced expression of Vh3 genes in the HIV infected group may reflect in a poor immune response to pneumococcal vaccination (Chang et al, 2000).

Sequence data for the variable region genes of anti-pneumococcal antibodies is extremely limited. Sequencing of the N terminal amino acids of a purified anti-type 14 antibody (Lucas et al, 1997) and two human IgM monoclonal antibodies (Shaw et al, 1995) revealed all three Abs used Vh3. A more recent study using phage display
immunoglobulin expression from spleen lymphocytes post vaccination with a 23 valent polysaccharide vaccine (23V-PS), described heavy and light chain gene usage for eight pneumococcal specific antibodies cross-reactive with double stranded DNA (Kowal et al, 1999). V\textsubscript{H}3 and V\textsubscript{K}1 dominance was demonstrated. However, these families dominate in the normal repertoire however which suggests that these data do not represent a bias in V-family use (Brezinschek et al, 1997; Foster et al, 1997). More detailed analysis of repertoire including serotype specific V gene usage and the role of vaccine formulation in shaping the immune response at a genetic level has not been described.

The objective of this study was to examine the diversity of the immune response to the 23V-PS and a new heptavalent pneumococcal-mutant diphtheria toxin (CRM\textsubscript{197}) conjugate vaccine (7V-CRM\textsubscript{197}) in an adult population. 15 of the human heterohybridomas described in Chapter 3 were analysed for V,D and J gene use, somatic mutation, canonical structure and CDR3 characteristics. These results were then correlated with serospecificity and isotype to determine whether selective V gene usage within and between individuals and across isotypes was demonstrated.

4.2 Methods

15 hybridomas were selected for molecular analysis. Two of these produced antibody which was pneumococcal specific but polyreactive to all serotypes tested, 13 were serotype specific. Of the 15 clones, three produced IgM, four produced IgA and eight produced IgG. Details of the standard methods used are given in Chapter 2. A summary of the procedures to sequence the hybridoma V genes is given below (Figure 4.1).

10\textsuperscript{7} clonal cells were used per reaction. RNAzol\textsuperscript{TM} disrupted the cell membrane and nuclear membrane to release the nuclear contents. The addition of chloroform to the
cell/RNAzol™ mix separated the RNA from DNA, proteins and cell membranes. cDNA was synthesised from the RNA using a Moloney murine leukaemia virus (M-MuLV) reverse transcriptase and primers specific for the polyA tail characteristic of RNA.

$10^7$ clonal cells washed and resuspended in residual medium

RNA extracted using RNAzol™

cDNA produced with First Strand cDNA synthesis kit

\[ \downarrow \]

ssDNA amplified by PCR using a pooled primer mix of human Ig primers

PCR product cleaned and sequenced directly using the ABI™ Prism Big Dye™ Terminator Cycle Sequencing reagents

\[ \downarrow \]

Sequences analysed using DNASTAR software and aligned to germ line immunoglobulin V genes using VBASE

Mutation rate determined

Canonical structure assigned

Statistical analysis performed

**Figure 4.1** Summary of methods used to sequence to V region DNA from hybridomas
The cDNA provided the template for the PCR reaction in which specific primers were used to selectively amplify human immunoglobulin V (D) J genes across the V region. The reaction involved the use of pooled primers containing a mix of oligonucleotides to match all possible functional V and C or J genes (see Table 2.1 for V gene primer sequences). The temperatures and duration of the reactions in the PCR differed depending on the primer melting temperature (Tm). Each clone was sequenced a minimum of four times from at least two separate PCR reactions to minimise PCR error.

PCR products were then run on an agarose gel to confirm success and allocate kappa or lambda chain usage. Samples of the $V_h$ and $V_l$ PCR bands on agarose gels are shown in Figures 4.2 and 4.3.

![Gel 1](image)

**Figure 4.2** Agarose gel demonstrating $V_h$ gene PCR products from a panel of pneumococcal hybridomas. In the first lane of each gel, the ladder (L) is seen. Each channel to the right of the ladder represents the cleaned PCR product of a clone of hybridomas. Arrows show the bands of amplified V regions. Not all the PCRs worked in the first round as demonstrated by empty channels. The top row demonstrates heavy chain PCR and the bottom row demonstrated light chain PCR.
Figure 4.3 Agarose gel demonstrating $V_L$ gene PCR products from a panel of pneumococcal hybridomas. In the first lane of the gel, the ladder (L) is seen. Each channel to the right of the ladder represents the cleaned PCR product of a clone of hybridomas. Light chain PCR products using kappa or lambda $V$ region primers are shown for DM5 and AbA5 clones. DM5 used a lambda light chain and AbA5 used a kappa light chain.

The PCR products were cleaned using the Geneclean™ protocol or Qiagen spin columns as described in Chapter 2. No significant difference in yield was observed between the two methods and the variation in method used was a reflection of the different preferences in the two laboratories in which the work was undertaken. Samples were then sequenced using the ABI™ Prism Big Dye™ Terminator Cycle Sequencing kit according to manufacturer’s instructions and run on an ABI sequencer (Perkin Elmer Biosystems. UK).

A PCR based method of restriction digestion (White, 1998) was then used to detect amplification of the $V$ genes used by the hybrid clones in D7 and D28 peripheral blood samples taken from the vaccinees. An outline of the procedure is shown in Figure 4.4 and a detailed description of the methodology is given in White. H.N., 1998.
Figure 4.4 Isotype specific restriction PCR of whole blood cDNA. cDNA was amplified using isotype specific primers. The PCR products were then digested using restriction enzymes selected to cut at sites pre-determined from the heterohybridoma sequence data such that clonal expansions of selected V genes could be identified. The restriction digest fragments were labeled with $^{33}$P and separated by electrophoresis to provide a restriction fingerprint of expressed Ig V genes.
4.3 Results

The molecular profiles of the monoclonal antibodies analysed are shown in Table 4.1. V(D)J gene assignments of for both heavy and light chains and the % homology of Mabs to the germline V genes are shown. CDR3 length and the ratio of replacement to silent mutation (R/S ratio) of nucleotides within the CDR1 and 2 of both heavy and light chains are also given.

Figures 4.5 and 4.6 give the amino acid sequences for both heavy and light chains of the 15 Mabs with alignment to their respective V genes. Silent and replacement mutations are shown in lower case and in bold respectively. Sequence analysis demonstrated that two of the Mabs produced (Db8C11 and Db7D4) were isotypic variants of the same clone (Figures 4.5-4.8) and for statistical analysis of gene frequency use were considered as one.

Nucleotide sequence alignment data for light and heavy chains of the clonal isotypic variants Db8C11 and Db7D4 are shown in Figures 4.7 and 4.8. The corresponding amino acid data is also shown with discordant mutations highlighted in red and blue.

4.3.1 V gene usage

The frequency of specific V genes and gene families used by the hybridomas isolated in this study was compared with that published for a reference population. The reference population is derived from the single cell PCR of V genes from an unselected sample of circulating IgM B cells taken from blood collected from two adult volunteers (Foster et al, 1997; Brezinschek et al, 1997).

Vh3 and Vk2 gene families dominated the repertoire and were used by 10/15 and 7/15 hybridomas respectively. Statistical analysis to compare this frequency with the normal repertoire revealed that there was not a significant deviation in Vh gene family usage compared to the normal repertoire. Of the Vh3 genes used, two specific genes, Vh3-48
and $V_{\mu}3-7$ were each used by three of the switched hybrids. While the numbers of antibodies are small, the frequency of the use of $V_{\mu}3-48$ in the monoclonal pool and specifically within the isotype switched somatically mutated antibodies was higher than in the reference population ($p=0.025$ and 0.01 respectively).

14/15 of the Mabs were given kappa or lambda chain assignments and 13 of these were sequenced. 13/14 of the light chains were kappa ($\kappa$), and $V_{\kappa}2A17$, $V_{\kappa}2A19/A3$ and $V_{\kappa}3A27$ genes were each used by three Mabs. The dominant use of $V_{\kappa}2$ is not a feature of the normal repertoire ($p=0.017$) and although a bias in the use of particular $V$ genes was suggested, only in the isotype switched clones was this bias significant for the single gene $V_{\kappa}2A17$ ($p=0.023$).
<table>
<thead>
<tr>
<th>Subject</th>
<th>Mab</th>
<th>Isotype</th>
<th>Serotype</th>
<th>V&lt;sub&gt;H&lt;/sub&gt; gene</th>
<th>Homology %</th>
<th>D&lt;sub&gt;h&lt;/sub&gt; gene</th>
<th>J&lt;sub&gt;h&lt;/sub&gt; gene</th>
<th>CDR3 length</th>
<th>R/S ratio</th>
<th>R/S ratio</th>
<th>V&lt;sub&gt;I&lt;/sub&gt; gene</th>
<th>Homology %</th>
<th>J&lt;sub&gt;i&lt;/sub&gt; gene</th>
<th>CDR3 length</th>
<th>R/S ratio</th>
<th>R/S ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AbA5</td>
<td>G1</td>
<td>18</td>
<td>V3-48</td>
<td>97</td>
<td>N/A</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4/5b</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>Vk3A27</td>
<td>93</td>
<td>N/A</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>DMF4</td>
<td>A</td>
<td>4</td>
<td>V1-46</td>
<td>93</td>
<td>D5-24</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>Vk3A27</td>
<td>93</td>
<td>Jk4</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DM5</td>
<td>G2</td>
<td>6B</td>
<td>V1-3</td>
<td>92</td>
<td>N/A</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>Vk3A27</td>
<td>94</td>
<td>N/A</td>
<td>14</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>CbG8</td>
<td>A</td>
<td>6B</td>
<td>V3-30</td>
<td>93</td>
<td>D5-5**</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>Vk2A19/A3</td>
<td>97</td>
<td>Jk1</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CbB2</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>95</td>
<td>N/A</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;6c</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>Vk2A17</td>
<td>96</td>
<td>Jk3</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CbE2</td>
<td>G2</td>
<td>23</td>
<td>V3-48</td>
<td>96</td>
<td>N/A</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;2</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>Vk</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CbH4</td>
<td>M</td>
<td>P*</td>
<td>V3-p1</td>
<td>100</td>
<td>D6-13</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;6b</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>Db8C11</td>
<td>A</td>
<td>4</td>
<td>V3-7</td>
<td>94</td>
<td>N/A</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>Vk2A17</td>
<td>95</td>
<td>Jk5**</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Db7D4</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>90</td>
<td>D3-16</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>Vk2A17</td>
<td>92</td>
<td>NA</td>
<td>8</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Db3G9</td>
<td>G2</td>
<td>6B</td>
<td>V3-15</td>
<td>86</td>
<td>D1-26**</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>Vk3A27</td>
<td>94</td>
<td>Jk3</td>
<td>12</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>6b1A7</td>
<td>M</td>
<td>P*</td>
<td>V4-39</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>17-25</td>
<td>0</td>
<td>0</td>
<td>Vk3A27</td>
<td>100</td>
<td>Jk5</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6b5B12</td>
<td>M</td>
<td>9V</td>
<td>V1-02</td>
<td>100</td>
<td>D1-7</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;6b</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>Vk2A18/A3</td>
<td>100</td>
<td>Jk1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6b5D7</td>
<td>M</td>
<td>6B</td>
<td>V1-e</td>
<td>100</td>
<td>D6-6**</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;6b</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>Vk1012/02</td>
<td>100</td>
<td>Jk2</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6bC3</td>
<td>A</td>
<td>9V</td>
<td>V3-48</td>
<td>93</td>
<td>D1-1**</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;3b</td>
<td>13</td>
<td>2</td>
<td>9</td>
<td>Vk2A19/A3</td>
<td>99</td>
<td>NA</td>
<td>7</td>
<td>2.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6b53</td>
<td>G3</td>
<td>18C</td>
<td>V3-7</td>
<td>93</td>
<td>D6-06</td>
<td>J&lt;sub&gt;i&lt;/sub&gt;4b</td>
<td>14</td>
<td>4</td>
<td>2.5</td>
<td>Vk3B1**</td>
<td>72</td>
<td>Jk4**</td>
<td>9</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.1 Molecular profiles of 15 Human Monoclonal Antibodies (Mab) from five subjects: Subjects A-D received the 7V-CRM<sub>197</sub> conjugate vaccine and subject E the 23V-PS vaccine. Isotype, serotype, V(D)J gene alignments and V gene % nucleotide homology to germline are given for heavy and light chains. CDR3 amino acid length and R/S ratios (replacement to silent mutations) in CDR1&2 are given. * P = polyreactive to all serotypes tested. **Alignment to germline poor. N/A: No alignment to germline.
<table>
<thead>
<tr>
<th>FR1</th>
<th>CDR1</th>
<th>FR2</th>
<th>CDR2</th>
<th>FR3</th>
<th>CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vα3-7</td>
<td>EVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YWSM</td>
<td>WVRQAPGKGLAEWVA</td>
<td>NIKQ---DGSEKYYVDSVYKQ</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>Cbν2</td>
<td>-------------------</td>
<td>A---A---P---F---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dd6C11</td>
<td>W------------</td>
<td>N---R---N</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>DbD7D4</td>
<td>EIV------F---I</td>
<td>I---R---N</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6b53</td>
<td>---------------</td>
<td>------S---</td>
<td>T---C---F</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα3-48</td>
<td>EVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YSMN</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>AbA5</td>
<td>------H---------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Cbν2</td>
<td>------H---------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Cbν3</td>
<td>Q------------</td>
<td>------G---S---</td>
<td>S---FG---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα3-15</td>
<td>EVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>DbD3G9</td>
<td>D------F---R---K---T------L---S---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα3-30</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YGHM</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>Cbν8</td>
<td>R---Q---------</td>
<td>------T---S---</td>
<td>------F---H---N---T---E---</td>
<td>------R---1------V---D---</td>
<td>------SFPTTDYRFY---WGRGTLV</td>
</tr>
<tr>
<td>Vα1-e</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>6b5d7</td>
<td>SYAIS</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα1-03</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>DMS</td>
<td>------S---------M---S---t---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα1-02</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>G---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>6b5B12</td>
<td>------G---------M---S---t---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα1-46</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>DMSF</td>
<td>------I---------N---S---T---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vα1-39</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>6bIA7</td>
<td>SSSYYWG</td>
<td>WIRQPPGKLGEMIG</td>
<td>ISTY---YSGTSTYNSLKS</td>
<td>RVTISDVSNKQPSLKLSSVTAADTAVYCAR</td>
<td>------IIRRGGGGJRGGYDEAGVTEAGIT</td>
</tr>
<tr>
<td>Vα3-64</td>
<td>QVQLVESGGGLVQPGGLSLCVRASGFTFS</td>
<td>S---YAMH</td>
<td>WVRQAPGKGLAEWSV</td>
<td>YISS---SSSTIYYADSVYK</td>
<td>RFTISRARNKSNLYLQMNSLRAEDTAVYCAR</td>
</tr>
<tr>
<td>Cbν4</td>
<td>------G---------M---S---t---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

**Figure 4.5** Vₚ gene amino acid alignments for the 15 Pneumococcal specific Mabs. Silent mutations are shown in lower case and replacement mutations in bold.
Figure 4.6 V\textsubscript{L} gene amino acid alignments for the 13 pneumococcal specific Mabs. Silent mutations are shown in lower case and replacement mutations in bold.
Figure 4.7 Nucleotide sequence alignments of the light chain of Db8C11 and Db7D4 aligned to the VJI- A17 germline gene. The corresponding amino acid sequence of the germline gene is also shown. Nucleotide mutations resulting in amino acid replacement are shown in bold. Discordant mutations in Db8C11 and Db7D4 are shown in blue and red respectively.
Figure 4.8 Nucleotide sequence alignments of the heavy chain of Db8C11 and Db7D4 aligned to the Vh3-07 germline gene. The corresponding amino acid sequence of the germline gene is also shown. Nucleotide mutations resulting in amino acid replacement are shown in bold. Discordant mutations in Db8C11 and Db7D4 are shown in blue and red respectively.
4.3.2  CDR3 and Somatic Mutation

The CDR3 characteristics of the hybrids were highly heterogeneous both in V(D)J junctional characteristics and in length (Table 4.1 and Figures 4.5-6). Alignment to germline was obtained for 8/15 of the D genes and 24/29 of the J genes. J_{4b} was used by 7/14 of the heavy chains for which assignments could be made, but preferential VDJ joining was not seen and CDR3 length was variable. There was no inverse correlation between CDR3 length and the level of somatic hypermutation in the V(D)J region.

Isotypic variation in the levels of somatic mutation was seen. The five IgM Mabs aligned 100% to germline V genes whereas all the isotype switched Mabs derived from both the conjugate and polysaccharide vaccinees were highly mutated. There were three cases of a common replacement mutation between two distinct clones using a common V gene (Figures 4.5 and 4.6). All were found in Mabs derived different individuals vaccinated with the 7V-CRM_{197} vaccine. AbA5 and CbE2, both specific for pneumococcal serotype 23F, shared a single amino acid replacement mutation (serine to threonine) within the CDR2 of V_{3-48} (Figure 4.5). CbB2 and Db8C11/Db7D4, all specific for pneumococcal serotype 4, shared a single amino acid replacement mutation (alanine to valine) within the FWK3 of V_{3-7} (Figure 4.5). DMF4 and Db3G9, specific for pneumococcal serotypes 4 and 6B respectively shared a replacement mutation within the CDR2 (serine to threonine) of the V_{3A27} gene (Figure 4.6).

4.3.3  Canonical Classification

Variable chain canonical loop assignments demonstrated the dominant H1-H2 conformations: 1-3 and 1-2 characteristic of polysaccharide binding antibodies in 13 of the 15 clones assigned (Table 4.2). Of those not assigned to this group, one (polyreactive IgM, 6b1A7) had the 3-1 and one (serotype 6B specific IgG, Db3G9) had the 1-U(4) conformation. Db3G9 was the most highly mutated of all the genes identified (Table 4.1).
<table>
<thead>
<tr>
<th>Mab</th>
<th>Isotype</th>
<th>Serotype</th>
<th>Canonical $V_H$</th>
<th>$V_H$ clan</th>
<th>Canonical $V_L$</th>
<th>$V_K$ clan</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbA5</td>
<td>G1</td>
<td>18</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>DMF4</td>
<td>A</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>6-1-1</td>
<td>1</td>
</tr>
<tr>
<td>DM5</td>
<td>G2</td>
<td>6B</td>
<td>1-3</td>
<td>1</td>
<td>N/A</td>
<td>NA</td>
</tr>
<tr>
<td>CbG8</td>
<td>A</td>
<td>6B</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>CbB2</td>
<td>G2</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>CbE2</td>
<td>G2</td>
<td>23</td>
<td>1-3</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CbH4</td>
<td>M</td>
<td>P</td>
<td>1-3</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Db8C11</td>
<td>A</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>Db7D4</td>
<td>G2</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>Db3G9</td>
<td>G2</td>
<td>6B</td>
<td>1-U(4)*</td>
<td>11</td>
<td>6-1-1</td>
<td>1</td>
</tr>
<tr>
<td>6b1A7</td>
<td>M</td>
<td>P*</td>
<td>3-1</td>
<td>11</td>
<td>6-1-1</td>
<td>1</td>
</tr>
<tr>
<td>6b5B12</td>
<td>M</td>
<td>9V</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>6b5D7</td>
<td>M</td>
<td>6B</td>
<td>1-2</td>
<td>1</td>
<td>2-1-1</td>
<td>1</td>
</tr>
<tr>
<td>6bC3</td>
<td>A</td>
<td>9V</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
</tr>
<tr>
<td>6b53</td>
<td>G3</td>
<td>18C</td>
<td>1-3</td>
<td>1</td>
<td>5-1-1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.2 Canonical Structure classification of Mabs. N/A = not available. ND = not determined. *Unassigned.

L1, L2 and L3 assignments were made for all the $V_K$ genes sequenced, a database for $V_\lambda$ is not available. Light chain canonical structure is considered to be less antigen restricted and most antibodies in the circulation have the 4-1-1 conformation. In our sample, light chain
canonical loops were more heterogeneous. 4-1-1 was used by nine of the clones. 2-1-1, 5-1-1 and 6-1-1 were used by the remaining five.

4.3.4 Restriction PCR fingerprints

Figure 4.9 represents the restriction digest PCRs of six of the hybridoma cDNAs (two using Vh1 and four using Vh3 genes) to identify the location of amplification bands for later comparison with whole blood isotype specific restriction PCR profiles. New restriction sites (\(\phi\)) introduced through somatic mutation are identified. Amplification bands are identified by their size (base pairs) and the restriction enzyme used (Hpall and Rsal for Vh1 and FokI and Rsal for Vh3 genes). Identical fragments were produced from Db7D4 and Db3G9, using Vh3-07 and Vh3-15 genes respectively. This suggests that the CDR3 length was similar for the two hybridomas and the V gene restriction site shared the same location in the two genes. Sequence analysis confirmed this with the FokI recognition motifs lying in the middle of the CDR1 of both genes. Different sized fragments were produced from CbB2, Db7D4 and 6b53 hybridomas all of which used Vh gene 3-07. This reflects variation in the CDR3 length of the different clones. The clonal PCR products are only partially digested as indicated by persistent undigested bands at the top of the gel and multiple fragments within one clone. This limits the potential of this technique to provide a quantitative estimate of clonal size.
Figure 4.9 Restriction PCR profile of six Mabs. The first two columns on the left represent the restriction digests of V\textsubscript{H}1 PCR products from Mabs DM5 and 6b5D7. The next four columns represent the digests of V\textsubscript{H}3 PCR products. The first three Mabs used V\textsubscript{H}3-07 and the fourth Mab used V\textsubscript{H}3-15. Each fragment is labeled with size and the restriction enzyme used. $S$ represents a new restriction site introduced through somatic mutation. C3 represents a restriction site in the CDR3 region.
Day 7 peripheral blood mononuclear cells from the donors from which the hybridomas were derived were then analysed to determine whether clonal expansions of the genes used by the hybridomas could be identified in whole blood restriction PCR. Each isotype was analysed separately to determine whether clonal expansions were restricted to or shared between isotypes. Figure 4.10 shows a sample of the restriction-PCR profile seen for V₃3 genes. PCR restriction digests of IgA and IgG2 isotypes are shown on the left and right respectively. Subject D demonstrates amplification bands of FokI digests 241bp long in both IgA and IgG2 lanes (red asterisk). These are the same size as the Db7D4 and Db3G9 IgG hybridoma digests (Figure 4.9) and represent amplification of V₃3-07+/−V₃3-15 genes in whole blood of that individual.

An amplification band (FokI 232) representing the V gene used by the IgG Mab CbB2 (V₃3-07) is demonstrated in both IgA and IgG2 lanes of subject C although the amplification was much weaker in the IgA clone.

A new Rsal restriction site (Rsal 107) created by mutation from germline in one of the hybridomas derived from subject C was predicted by sequence analysis and was also demonstrated in the restriction PCR. This mutation was only seen in the IgG2 column however and not demonstrated in whole blood IgA (Figure 4.10).

Figure 4.11 shows the isotype specific restriction PCR double digest using V₁1 primers and restriction enzymes Rsal and HpaII of day 7 peripheral blood mononuclear cells from subjects A-D and an unvaccinated control (N). Of the Mabs produced, four used genes from the V₁1 family (Figure 4.5). The marker lane (M) shows the PCR digest products from 6b5D7 and DM5 clones, using genes V₁1-e and V₁1-03 respectively. Rsal cuts 6b5D7 producing bands 202 base pairs long and HpaII cuts DM5 producing bands 268 & 235 base pairs long. Strong bands representing an amplification at the HpaII 235 site were observed in the IgA and IgG2 lanes of subject B. This represents clonal expansions in two isotypes of the gene used by the
Mab derived from this donor. In addition, the restriction digests from subject C demonstrated a marked expansion at the HpaII 235 site in the IgA column and a very weak band in the IgG2 column.

Strong amplification bands were noted for subject B at the new restriction sites Rsal 60 and HpaII 268 identified in the DM5 Mab. They were present in the IgG1+/IgG2 isotype lane of whole blood. The Rsal 60 site was identified from sequence analysis (data not shown) to occur in the CDR3 of the clone (C3). The HpaII 268 site was created by two somatic mutations at the start of the CDR1. Amplification bands created by these new restriction sites are not seen in the IgM or IgA columns.
Figure 4.10 Restriction PCR of D7 peripheral blood from four vaccinees (A-D) and a non-vaccinated age matched control. Two restriction enzymes were selected to pull out the $V_{\gamma3}$ genes expressed in seven of the IgG clones. The marker lane represents restriction digests produced from the seven hybridomas. The red asterisks * mark IgG2 and IgA amplification bands seen in whole blood from subjects C and D which share the same location as that of the specific hybridomas generated from those individuals. The yellow asterisks show large IgG2 and IgA $V_{\gamma3}$ gene amplification in blood from subjects A and D that are not seen in the hybridoma marker lane. A new mutation site found in a hybridoma from subject C is also found in the IgG2 but not the IgA column derived from whole blood from this individual.
Chapter 4 Genetic Analysis

Res-PCR of PB Ig VH1
HpaII/RsaI double digest

Figure 4.11 Isotype specific restriction PCR of Day 7 peripheral blood from four vaccinees (A-D) and a non-vaccinated age matched control (N). Two restriction enzymes (RsaI and HpaII) were selected to pull out the $V_{\mu}1$ genes expressed in two of the IgG clones. The marker lane (M) represents restriction digests produced from the two hybridomas. The red asterisks * represent clonal amplifications in subject D seen across all the isotypes. The yellow asterisks represent clonal amplifications in subject B across two or more isotypes at three restriction sites known to be present in hybridomas produced using blood from this individual. One of the restriction sites was created by a new somatic mutation (HpaII268s) and another was located in the CDR3 (Rsa60C3). The blue asterisk * denotes an amplification band in subject C in IgA and IgG1 lanes, but not in the other isotypes analysed.
Chapter 4 Genetic Analysis

The Rsal 202 site representing the V\(_h\)1-6 gene used by the IgM Mab: 6b5D7 was weakly amplified in the IgG1 columns of subjects A-C (Figure 4.11) although similar levels of amplification were demonstrated by the control suggesting that this clonal expansion was not the result of vaccination. Day 7 post-vaccination blood was not available from subject E from whom this Mab was derived.

Clonal expansions of identical V\(_h\)3 and V\(_h\)1 genes across the isotypes and between individuals but not used by any of the hybrid clones were also seen (Figures 4.10 & 4.11). The specificity of these clones was unknown.

4.4 Discussion

The restricted nature of the human B cell response to bacterial polysaccharide has been the subject of ongoing investigation. It is manifest at several levels including the poor response of young children to polysaccharide antigens, the limited immunogenicity of these antigens at all ages, the absence of immunological memory after encounter with the antigen and the apparently restricted nature of the immune repertoire at a molecular level. Attempts to study diversity at the molecular level have been hampered by the technical difficulties associated with producing stable human heterohybridomas. Thus data on pneumococcus is scarce. In this study a number of human heterohybridomas secreting antibody to a variety of pneumococcal serotypes have been generated. The hybrids are all derived from antigen specific peripheral blood B cells (PBMC) taken in the day 7 post immunisation window when these cells are found at their peak in the peripheral circulation.

4.4.1 V gene usage

While the number of pneumococcal specific hybridoma clones produced in this study were relatively small, the data suggest a bias in immunoglobulin V gene usage in the adult immune
response to pneumococcus with an overrepresentation of $V_h$3-48 and $V_k$2A17. This bias is not serotype specific and is demonstrated by different individuals. Oligoclonality is also suggested in the response of subject D to serotype 4 in which two of the three clones analysed from this individual were isogenic variants (IgG and IgA) of the same clone.

The sequence data is supported by the preliminary results of the restriction PCR studies. The initial study produced a restriction fingerprint of the hybridoma clones. Sequence analysis identified the location of restriction sites specific for different V genes and for new restriction sites introduced through somatic hypermutation. Isotype specific restriction PCR was then performed on day 7 whole blood. Amplification bands at the same location represented clones sharing an identical restriction site and CDR3 length. This was found for Db7D4 and Db3G9. Other clones sharing the same V gene, but with distinct CDR3s, produced amplification bands at different positions. New restriction sites introduced through somatic hypermutation and predicted from sequence analysis were also demonstrated.

Subsequent restriction PCR fingerprints of Day 7 whole blood demonstrated clonal expansions of the $V_h$ genes in the same location as those demonstrated by the hybridoma clones. Within individuals, these expansions were seen across the isotypes and identical restriction sites were demonstrated between individuals. In addition, new restriction sites identified in hybridoma clones CbB2 and DM5 were seen in the whole blood PCR from the donors. The amplification bands were not seen across all isotypes. For example, the CDR1 mutation in clone CbB2 was demonstrated only in IgG2 and the CDR1 and CDR3 mutations in clone DM5 were demonstrated only in IgG1 and IgG2.

These data highlight how this method can be used to follow the process of somatic mutation through isotype switching and provides a strong argument for clonal identity between different isotypes.
The preferential use of specific V genes has been described for a number of antigens including autoantigens in both human (Smith et al., 1995) (Pinchuk et al., 1995) and murine responses (Press & Giorgetti, 1993) but of particular relevance is the restriction that has been described in association with Hib capsular polysaccharide, although different specific V genes are used (Lucas et al., 1994). While the precise mechanism governing such restricted usage is not entirely clear, it has been suggested that the observed restriction may be explained by canonical V genes (Kowal et al., 1999) (Kohsaka et al., 1996).

4.4.2 Canonical Genes

The dominance of specific canonical V-genes that are of high affinity and yet remain unmutated has been described for Hib (Hougs et al., 1999b) and these genes are considered to be the prototypic genes for optimal antigen binding. They show selective association with specific J genes and homology in the CDR3. In this current pneumococcal study, the majority of antibodies analysed (13/15) used the H1-H2 conformation 1-2 and 1-3 which is characteristic of murine anti-polysaccharide antibodies (Vargas-Madrazo E., 1995) and human responses to Hib (Adderson et al., 1991). Unlike the canonical genes described for Hib, all of the isotype switched clones in this sample were highly mutated and a number of common mutations within the CDR were seen between hybridomas using the same V genes but derived from different individuals (Figures 4.5 and 4.6).

The selective use of the canonical Vh3-48 gene by hybridomas derived from three different individuals and each recognising a different capsular polysaccharide suggests that Vh3-48, whilst not sharing all the characteristics of canonical genes, may be an important non-serotype specific canonical gene in the response to pneumococcal polysaccharide in adults. The differing serospecificity may be attributed to differences in the CDR3 and the light chain pairing which was quite distinct and the distinct mutations within the CDR2.
Two of the clones using Vh3-48, AbA5 and CbE2, also shared a common somatic mutation in the CDR2 region. Site directed mutagenesis or molecular modeling of antigen binding is required to determine whether this demonstrates selective mutation on the basis of superior antigen binding.

Of two clones (Db3G9 and 6b1A7) that used non-canonical Vh genes, Db3G9, the serotype specific isotype switched clone, was the most highly mutated of the entire group (86% homology to germline gene) with extensive mutation throughout both the framework and the CDR regions. Whether the use of this non-canonical V gene use is associated with low avidity of antigen binding will be explored in the next chapter.

4.4.3 Mutation and Memory

Plain polysaccharide vaccines are limited by their poor immunogenicity in the young and by their inability to induce immunological memory in vaccinees of all ages. Conjugate vaccines overcome these limitations because the polysaccharide component of the vaccine is rendered a T dependent antigen by conjugation to a protein. The nature and extent of hypermutation in the clones obtained from the conjugate and plain polysaccharide vaccinees was compared and subsequently correlated with antibody function. The level of random replacement mutation within individual V genes and CDR hot spots is known to differ widely (Chang & Casali, 1994) and R/S ratios of >2.9 can no longer be considered to be indicative of selection of somatic hypermutation, a feature of the memory response. However extrapolating from detailed kinetic studies of murine splenic B cells, the level of somatic hypermutation seen in all the isotype switched hybridomas was inconsistent with their derivation from naïve B cells. Even using a high estimation of the germinal center mutation rate ($10^{-3}$
substitutions/nucleotide/cycle) (McKean et al, 1984; Clarke et al, 1985) and a short doubling time (6h) (Zhang et al, 1988) it would take more than seven days to produce the level of mutation seen in most of our isotype switched clones had they come from a naïve precursor pool. Using the mutation rate described above, the two highly mutated class switched clones (progeny of the same precursor) derived from subject D would each have taken a minimum of 9-14 days in the spleen to achieve the level of mutation demonstrated. Only 1 clone, AbA5 mutating and dividing at a high rate even in murine kinetics could have achieved the level of mutation it demonstrated by day 7 post vaccination were it derived from a naïve precursor. This suggests that all the subjects had been primed prior to vaccination and the switched clones were derived from the B cell memory pool. In contrast the IgM clones, all of which demonstrated 100% germline gene alignment, are indicative of a de novo primary response.

As none of the volunteers in this study had been previously vaccinated, immunological memory to the capsular polysaccharide must have been induced by previous encounters with *Streptococcus pneumoniae* or cross-reacting antigen. It is well recognised from nasopharyngeal carriage, serological studies and ELISPOT analysis of circulating AbSC numbers as discussed in Chapter 3 that most adults have previously encountered pneumococcal polysaccharide. However, capsular polysaccharides are T independent and do not induce memory. If memory is induced through wild type pneumococcal colonisation, it is likely that the original encounter with capsular polysaccharide involved T and B cells “seeing” *Streptococcus pneumoniae* derived capsular polysaccharide naturally conjugated to protein, possibly cell wall components. In this context, the encapsulated bacteria may be capable of inducing a T-dependent immune response to their capsular polysaccharide with the features of somatic hypermutation and the generation of memory. This may explain the declining incidence of invasive pneumococcal disease with age. Once immunological memory is established, subsequent challenge with conjugate or plain polysaccharide is able to elicit a
memory response, as suggested by this study. While the day 7 characteristics of the immune response to plain polysaccharide or conjugate may appear similar, it is clear that conjugate vaccines will induce further immunological memory in contrast to plain polysaccharide vaccines which, although stimulating some pre-existing memory B cells, fail to induce further memory (Konradsen, 1995; Goldblatt, 1999).

Hougs et al (1999) have generated data which support this suggestion. They have recently shown that the immune response of an adult to a single dose of Hib PS-TT conjugate vaccine is dominated by cells derived from a memory pool, with the progeny of a single rearranged B cell accounting for over 50% of the clones. Analysis of the frequency of somatic mutation and the clone size suggested that this population must have been derived from a pre-existing population of B-cells which had already been highly selected, mutated and clonally expanded prior to vaccination.

If mucosal colonisation can prime for immunological memory, the poor response of infants to plain polysaccharides as compared to that of adults may be explained not only by the delayed ontogeny of the B cell response to plain polysaccharides, but also by the relative lack of exposure to encapsulated bacteria which results in delayed priming. This may have implications for vaccine development since the immune repertoire induced by conjugate vaccine in a naive individual may not be the same as that induced in an individual primed through colonisation with the encapsulated bacteria.

In summary, our data suggests that the Day 7 post vaccination PBMC V gene repertoire used in the adult response to pneumococcal polysaccharide and conjugate vaccines may be determined by pre-existing immune memory to the wild type pneumococcus. A bias in the use of $V_H$ and $V_K$ genes is suggested and although the numbers are small, this bias becomes apparent as the immune response matures and isotype switching occurs. The dominant
genes used are canonical genes known, from murine studies, to be selective for polysaccharide.

More detailed analysis of whole blood by isotype specific restriction PCR fingerprinting would help clarify a number of the issues discussed. Analysis of sequential blood samples by restriction PCR would identify the temporal course of emergence of clones identical to the pneumococcal specific clones produced using the hybridoma technology. The combination of these techniques would permit following clonal expansions through the process of isotype switching and acquisition of somatic mutations. The early appearance of mutated isotype switched clones would provide strong support for the presence of memory clones. Having identified important clones within a few individuals restriction PCR finger printing could then be used to screen population samples for common V gene usage and shared mutations.

In the next chapter, in vitro assessments of Mab function are described and correlated with V gene usage and somatic mutation.
CHAPTER FIVE

IN VITRO FUNCTIONAL ASSESSMENT OF ANTIBODY ACTIVITY:

AVIDITY AND OPSONOPHAGOCYTIC ACTIVITY

5.1 Introduction ..................................................................................................130
5.1.1 Nature of antigen-antibody interaction ..............................................................130
5.1.2 In vitro assessment of antibody function ..........................................................134

5.2 Methods ........................................................................................................139
5.2.1 Avidity ...................................................................................................................139
5.2.2 Opsonophagocytosis .........................................................................................139

5.3 Results ..........................................................................................................141
5.3.1 Avidity ...................................................................................................................141
5.3.2 Opsonophagocytosis ..........................................................................................143

5.4 Discussion ....................................................................................................151
5.4.1 Avidity of Mabs and serum IgG .........................................................................151
5.4.2 Opsonophagocytic activity of Mabs and serum ..................................................152
5.4.3 Correlation of Avidity and Opsonophagocytic activity .......................................155
5.4.4 Correlation of in vitro functional analysis and genetic analysis .........................155
Chapter 5

Antibody Function

5.1 Introduction

The functional activity of antibody may be influenced by immunoglobulin V gene use and the level of somatic hypermutation at the antigen binding site (Granoff et al, 1993). Although the functional implications of repertoire variation have not been studied in detail a number of studies of Hib have demonstrated a correlation between V gene use and antibody avidity, specificity and in vitro bactericidal activity (Lucas et al, 1998; Lucas et al, 1994; Nahm et al, 1995; Lucas & Granoff, 1995a). In this chapter antibody avidity and opsonophagocytic activity, both \textit{in vitro} surrogate tests of antibody function, are used to determine the functional activity of the monoclonal antibodies described in Chapter three. To ascertain whether an association between specific V gene use, somatic hypermutation and \textit{in vitro} tests of antibody function is demonstrated, these data are then compared with the genetic data described in Chapter four.

5.1.1 Nature of antigen-antibody interaction

The strength of binding of a monovalent ligand to a single antigenic determinant, a hapten, is the intrinsic affinity of the antibody. Antigens are rarely haptenic however, and most pathogens bind both of the antibody binding sites of a single antibody molecule. Bacterial polysaccharides consist of multiple identical repeating epitopes and antibody binding to such antigens is generally polyvalent. IgG and monomeric IgA demonstrate divalent binding, dimeric IgA demonstrates quadravalent binding and IgM, a pentamer demonstrates decavalent binding. For both dimeric IgA and pentameric IgM the apparent strength of antigen-antibody interaction is thus increased and the overall strength of binding of each antibody to an antigen becomes a more biologically relevant measure of antibody-antigen interaction. This is known as the avidity (or functional affinity) of the antibody.
Chapter 5  
Antibody Function

The affinity and avidity of antigen binding is variable and determined mainly by V gene usage, V(D)J combination and somatic hypermutation in the hypervariable regions (HV). The V gene used determines the overall shape of the antigen-binding site (canonical structure) and as discussed in Chapter 1, V genes are grouped according to the type of conformation they produce. Fine specificity and affinity of interaction are determined by V(D)J gene combination, N and P additions at the V(D)J joining site and somatic mutation within the three hypervariable regions (HV) in addition to the V gene used.

The mechanism of binding between antibody and antigen is a reversible non-covalent interaction which involves electrostatic, Van der Waals and hydrophobic forces and hydrogen bonds. Antibodies are unusual in that they possess many aromatic amino acids in their antigen binding sites which participate in these bonds. The Van der Waals and hydrophobic interactions only operate over a very short range and pull together 2 regions that are complementary in shape. The electrostatic interactions and hydrogen bonds which occur between antigen and the amino acid side chains in the hypervariable (HV) regions strengthen the overall interaction and increase both the specificity and the affinity of the Ag-Ab binding interaction. Close approximation of antigen and antibody is required for these bonds to form and as the overall fit improves, the bonds become stronger.

It is a combination of the overall surface shape together with the electrostatic and hydrogen bonds which determine antibody specificity and affinity. With time post antigen challenge, somatic mutation and clonal selection of B cells producing high affinity antibody is demonstrated (Eisen & Siskind, 1964; Griffiths et al., 1984; Claflin et al., 1989).

The strength of the antigen-antibody interaction is assessed by a variety of techniques. Affinity of an antibody for a haptenic antigen can be measured directly by equilibrium
Chapter 5  
Antibody Function

dialysis. A known amount of antibody is separated from differing concentrations of hapten by a dialysis membrane through which antigen but not antibody can move. Antigen diffuses across the membrane, binds antibody and an equilibrium between bound and unbound antigen is established. The concentration of antigen either side of the dialysis membrane is then determined and an affinity constant is derived from the data using the Scatchard analysis ([van, Kramer, et al. 1998 ID: 1467].

Avidity of binding is measured by a variety of techniques which disrupt the antigen-antibody interaction and involves the use of solid phase assays in which polystyrene plates are coated with antigen. Antibody is then added and a variety of physical methods are used to cause dissociation of binding (Goldblatt, 1997). There may be number of problems associated with the techniques. These include conformational changes in the antigen induced by solid phase binding and alteration in antibody binding to antigen on solid phase. Affinity heterogeneity in serum may also result in masking of high affinity antibody in low concentration by more abundant low affinity antibody.

Despite the many theoretical problems, good correlations between results using solid phase assays and equilibrium dialysis have been demonstrated for monoclonal antibodies and their haptns (Friguet B & Chaffotte AF, 1985; Nieto A & Gaya A, 1984). In addition, similar ranking of polyvalent serum samples has been demonstrated (Rath S & Stanley CM, 1988). The measurement of antibody avidity, although not measuring ‘true affinity’, does provide useful information for ranking antibodies specific for the same antigen. High avidity antibodies are known to demonstrate superior functional activity in both in vitro and in vivo tests of antibody activity (Usinger & Lucas, 1999) and avidity assessment of serum antibody post vaccination is becoming a useful surrogate for vaccine efficacy (Granoff & Lucas, 1995; Konradsen, 1995; Usinger & Lucas, 1999).
There are two main types of assay for the measurement of avidity: the competitive inhibition assay and the elution assay. The former uses different concentrations of free antigen in solution to compete with solid phase bound antigen for antibody binding. The latter uses chaotropic agents to disrupt the antigen-antibody interaction. Both methods give a measure of the average antibody avidity and the value is relative. Elution assays have been applied to a number of antigens and are becoming increasingly popular. The principle of the assay is that the higher concentrations of chaotrope are required to disrupt high avidity binding. An avidity index (AI) is derived which represents the molar concentration of chaotrope required to reduce binding by 50%. A number of different chaotropes have been used including potassium, sodium or ammonium thiocyanate, urea and diethylamine. The effect of latter 2 are both influenced by pH whereas the chaotropic effect of thiocyanate has been shown to be independent of pH and the thiocyanate elution assay is considered to be more reliable (Goldblatt et al, 1993).

The objective of this study was to determine the avidity of the IgG and IgA monoclonal antibodies using the ammonium thiocyanate (NH₄SCN) avidity ELISA. This assay has been previously described and validated in some detail (Goldblatt et al, 1993; Feldman et al, 1994; Anttila et al, 1998; Poirier et al, 1997; Pullen et al, 1986; McCloskey et al, 1996; Goldblatt et al, 1998). Mab avidity was compared with that of the pre- and post-vaccination sera from the five vaccinees. The IgM Mabs were not studied. Although IgM generally has low binding affinity at each of its antigen binding sites, it demonstrates pentameric binding and the avidity is consequently very high.

Avidity was also correlated with V gene use and level of somatic mutation within the variable region. Due to the small number of vaccinees involved a meaningful comparison of avidity between antibodies derived from conjugate and polysaccharide
vaccine recipients could not be made. This would be more usefully assessed in large scale population studies.

5.1.2 In vitro assessment of antibody function

Serotype specific IgG protects against invasive pneumococcal disease as discussed in Chapter 1. It fixes complement (Winkelstein, 1981) and acts as an opsonin facilitating Fc mediated phagocytosis and the subsequent intracellular killing of the bacterium (Figure 1.2).

Studies of vaccine and non-vaccine related immunity in man have demonstrated that the titre of type specific antibody may not always correlate with protection from invasive disease (Musher et al, 1986). Consequently a variety of methods to study the effectiveness of post-vaccination sera and monoclonal antibody in killing pneumococci were developed. However the earlier studies had not taken into account the effect of anti-CWPS antibodies. These antibodies are known to be present in high concentration in serum and cross react with pneumococcal polysaccharide on ELISA giving falsely high titres for serotype specific antibody. Following pre-adsorption of CWPS antibodies, in vivo studies in mouse and rat have demonstrated a close correlation between pneumococcal IgG antibody titre and protection from invasive disease (Alonso-De et al, 1995) and this is supported by in vitro studies using post-vaccination serum in killing and opsonophagocytic assays (Gardner et al, 1982; Esposito et al, 1990b; Esposito & Clark, 1990a; Vidarsson et al, 1998; Romero et al, 1997; Jansen et al, 1998; Martinez et al, 1999).

In vitro functional activity is now most commonly assessed using the opsonophagocytic assay. Radioactive $^3$H- or fluorescein-labelled bacteria are incubated with serum +/- complement to allow opsonisation to occur and neutrophils are then added as phagocytes. A major problem associated with the technique is the neutrophil source. There are two main IgG Fc receptors constitutively expressed on PMNs: Fc$\gamma$RIIa
(CD32) and FcγRIIb (CD16). Both receptors can bind human IgG1 and IgG3 containing complexes but only FcγRIIa can effectively interact with complexed IgG2, the dominant isotype in the immune response to bacterial polysaccharide (Warmerdam et al., 1993), to induce opsonophagocytosis of serum opsonised pneumococcus (Freijd et al., 1984; Lortan et al., 1993). A bi-allelic genetic polymorphism (FcγRIIa-R131 and FcγRIIa-H131) exists and determines whether or not opsonophagocytosis can be mediated through this receptor. The FcγRIIa-H131 variant has been shown to have higher phagocytic capacity (Sanders et al., 1995a; Jansen et al., 1999) and individuals homozygous for the -R131 variant have an increased risk of invasive pneumococcal disease (Sanders et al., 1995b). A bi-allelic genetic polymorphism of FcγRIIIb also exists but this appears not to influence IgG2 mediated opsonophagocytosis (Sanders et al., 1995a).

The optimal neutrophil source best able to express the phagocytic function relevant to anti-polysaccharide responses are fresh human neutrophils from heterozygous or -H131 homozygous donors. However, although appropriate for small studies it is not practical or possible to use such fresh neutrophils in large scale serological studies. Culturable granulocytes (HL60 cells) have been developed for this purpose (Romero et al., 1997). HL60 cells are not ideal as they express only the low affinity Fc receptor allelic variant: FcγRIIa-R131. However the use of HL60 cells avoids the lengthy process of neutrophil isolation from whole blood and the problem of interassay variation due to the use of different donors and opsonophagocytic data produced using these cells show good correlation with in vivo protection in mouse studies (Johnson et al., 1999). This makes the line particularly suited for use in large scale vaccine efficacy studies where interassay and interlaboratory variation must be minimised.

An alternative is a more recently developed neutrophil line in which cells are transfected with the -H131 gene (Van-den-Herik et al., 1995; Warmerdam et al., 1993).
In pneumococcal opsonophagocytic assays a good correlation has been demonstrated between these cells and fresh PMNs (Rodriguez et al., 1999). However, these cells are not yet commercially available.

In the studies described below, fresh neutrophils from -H131 homozygous or heterozygous donors were used after FcR genotyping of the donor pool. Pre and post vaccination serum and five of the seven IgG Mabs were assessed for avidity and opsonophagocytic activity. These data were then compared with the genetic data presented in Chapter 4.

The functional activity of three of the IgA Mabs was also assessed. The role of IgA in systemic immunity is unclear (Kilian et al., 1988; Mestecky et al., 1999) and there are few publications describing its in vivo functional activity. Although avidity of IgA has been measured in response to a number of other infectious agents such as influenza (Barington et al., 1996) and Plasmodium falciparum (Vajdy & Lycke, 1995) and is reported to be in the same range as that of IgG, there have been few studies on the avidity of IgA antibodies to capsular polysaccharide antigens and there are no data comparing the avidity of IgA and IgG anti-pneumococcal antibodies. The opsonophagocytic activity of IgA has been difficult to study and data on the opsonophagocytic activity of anti pneumococcal IgA and IgM are not yet available.

An IgA Fc receptor for monomeric and polymeric IgA: FcαR (CD89) is constitutively expressed on monocytes, neutrophils and eosinophils and is capable of mediating phagocytosis and transduction signals for oxidative metabolism and granule release (Morton et al., 1996). FcαRs are heterogeneous and variably glycosylated. Cytokines such as IL-8 or TNFα upregulate expression of the receptor on neutrophils, thus enhancing the phagocytosis of IgA opsonised bacteria (Nikolova & Russell, 1995; Shen et al., 1994). IgA mediated opsonophagocytosis has been demonstrated by mouse lung alveolar macrophages (Richards & Gauldie, 1985) and human serum IgA.
and slgA has been shown to induce phagocytosis of *Staphylococcus aureus* (Gorter *et al*, 1987). However, human IgA may also inhibit phagocytosis (Wilson, 1972; Van & Williams-RC, 1978) and in competing with IgG and IgM for antigen binding may have an anti-inflammatory role. This may be particularly significant in the case of the pneumococcus. *S.pneumoniae* produces IgA1 proteases which cleave IgA1 but not IgA2 into antigen binding (Fab) and constant region (Fc) portions. The Fab alone although antigen binding cannot interact with other cells of the immune system and may even mask the antigen from IgG mediated opsonophagocytosis (Kilian *et al*, 1988). In vitro experiments looking at IgA function suggest that neutrophil source and activation status are important variables which may explain the dual function of IgA. Suboptimal concentrations of IgA have been shown to promote phagocytosis and to enhance phagocytosis mediated by oral human PMNs but not blood PMNs (Fanger *et al*, 1983). IgA mediated inhibition of IgG associated complement-mediated opsonophagocytosis has been associated with resting PMN and only on PMN activation with IL-8 and consequent upregulation of FcαR expression are opsonisation and phagocytosis of IgA coated bacteria observed (Nikolova & Russell, 1995).

The interaction of IgA with complement is unclear and remains controversial (Mestecky *et al*, 1999). Human IgA when complexed with antigen does not activate the classical complement pathway (Heremans, 1974; Russell & Mansa, 1989a) and interferes with complement activation by IgG and IgM (Russell *et al*, 1989b; Griffiss & Jarvis, 1987). However polymeric IgA, aggregated secretory IgA (slgA), immune precipitates of IgA + Ag and aggregated Fcα has been claimed to activate complement via the alternative pathway (Burritt *et al*, 1977; lida *et al*, 1976; Pfaffenbach *et al*, 1982; Rits *et al*, 1987) and promote phagocytosis (Hiemstra *et al*, 1988; Hiemstra *et al*, 1987; Benedetti *et al*, 1998).
It has recently been reported (Johnson et al, 1996) that, unlike the response to Hib, meningococcus and various protein antigens, IgA is preferentially and persistently secreted in polymeric form following vaccination with pneumococcal capsular polysaccharide or infection with 
*S. pneumoniae*. The persistence of the polymeric form of IgA post wild type infection or immunisation may indicate some role in defense against pneumococcal disease. It has been argued that due to the low affinity of anti-polysaccharide antibodies and the limited affinity maturation that is reported to occur post polysaccharide vaccination, polymeric IgA is functionally advantageous improving the avidity of Ab-Ag binding and opsonophagocytosis of invading bacteria in the blood, liver and spleen.

To evaluate the function of IgA in the response to pneumococcus, three of the IgA Mabs (DMF4, CbG8, Db8C11) were included in both avidity and opsonophagocytic assays. Two of these were IgA2, one was IgA1 and Db8C11 - the IgA1 isotypic variant of IgG clone Db7D4 was included.

In collaboration with Dr Uffe Skov Sorenson (University of Aarhus, Aarhus C, Denmark) the valency of the antibodies was also determined.

Three of the IgM Mabs : 6b5D7 - the serotype 6B specific Mab, 6b1A7 and CbH4 - the two polyreactive IgM Mabs, were also assessed for opsonophagocytic activity with particular reference to a comparison of the ability of the serotype specific antibody and the polyreactive Mabs to induce opsonophagocytosis. The polymeric binding of IgM is known to produce very high functional affinity (avidity) and this was confirmed for 6b5D7 only.
5.2 Methods

5.2.1 Avidity

The ammonium thiocyanate (NH₄SCN) avidity ELISA was used as described in Chapter 2, Section 2.14. Results were expressed as the log percentage (%) reduction in binding of antibody (Y axis) against molar concentration of NH₄SCN (M). An avidity index (Al) was given to each antibody and corresponded to the molar concentration of NH₄SCN required to reduce the absorbance at 490nm (A₄₉₀) induced by antibody binding by 50%.

The percentage change in absorbance at different molarities of NH₄SCN was calculated using the following equation:

\[ 100 \times \left( \frac{\text{mean } A_{490} \text{ with Ab in BSA-PBS-T} - \text{mean } A_{490} \text{ with Ab plus NH₄SCN}}{\text{mean } A_{490} \text{ of wells with Ab in BSA-PBS-T}} \right) \]

The log % reduction in absorbance was plotted against molarity of NH₄SCN (Figure 5.2). At log 50% (1.699) a line was drawn on the graph and a perpendicular dropped from the point where the log 50% line crossed the Ab curves. This gave value x which was taken as the avidity index (AI).

5.2.2 Opsonophagocytosis

A detailed description of the method used is given in Chapter 2, Section 2.15. The basic principle is outlined below (Figure 5.1). All experiments were done in duplicate with non-opsonin controls (neutrophils and bacteria only) and specificity was evaluated by incubation with two other pneumococcal serotypes to which Mabs were not cross reactive on ELISA. Non-serotype specific antibody controls (10% FCS or Mabs to a different serotype) were also used.
The type 6B Mabs were defined serotype 6B specific by standard and inhibition ELISA. However, a well encapsulated strain of 6B was not available for use in the opsonophagocytic assay. Cross reactivity within serogroup 6 has been well documented (Nahm et al, 1997) and pneumococcal serotype 6A bacteria were used to assess the opsonophagocytic activity of these Mabs.

**Figure 5.1** Outline of procedure to evaluate opsonophagocytosis of pneumococci by serum and Mabs.
5.3 Results

5.3.1 Avidity

Figure 5.2 shows an example of the avidity data for one of the eight Mabs analysed: Db3G9 from donor D, and pre and post vaccination serum data from the same donor. The AI is the molar concentration of ammonium thiocyanate causing a 50% log reduction in absorbance.

![Avidity Graph]

**Figure 5.2.** Avidity of binding of Mab Db3G9 from donor D and D0 (Da) and D28 (Dc) post vaccination serum to PncPS serotype 6B as determined by ammonium thiocyanate elution ELISA. Log % reduction of binding is plotted on the Y axis and molar concentration of ammonium thiocyanate is plotted on the X axis (M). A horizontal line is drawn crossing the Y axis at 1.699 log % reduction representing a 50% reduction in absorbance. Vertical lines are dropped from this line where crossed by the absorbance curves for the different antibody samples. The avidity index (AI) is the molar concentration of ammonium thiocyanate producing a log 50% reduction in absorbance.
Serotype specific avidity indices were determined for nine of the monoclonal antibodies (five IgGs, three IgAs and one IgM) and compared with avidity for pre and post vaccination serum (Table 5.1) and the level of somatic mutation in the CDR1 & 2 of heavy and light chains (Table 4.1).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Mab</th>
<th>Isotype</th>
<th>Antibody specificity</th>
<th>Antibody avidity index (AI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre-immunisation serum</td>
</tr>
<tr>
<td>B</td>
<td>DMF4</td>
<td>IgA2</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>C</td>
<td>CbB2</td>
<td>IgG2</td>
<td>4</td>
<td>0.85</td>
</tr>
<tr>
<td>D</td>
<td>Db7D4</td>
<td>IgG2</td>
<td>4</td>
<td>1.84</td>
</tr>
<tr>
<td>D</td>
<td>Db8C11</td>
<td>IgA1</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>B</td>
<td>DM5</td>
<td>IgG2</td>
<td>6B</td>
<td>1.68</td>
</tr>
<tr>
<td>C</td>
<td>CbG8</td>
<td>IgA2</td>
<td>6B</td>
<td>NA</td>
</tr>
<tr>
<td>D</td>
<td>Db3G9</td>
<td>IgG2</td>
<td>6B</td>
<td>0.4</td>
</tr>
<tr>
<td>E</td>
<td>6b5D7</td>
<td>IgM</td>
<td>6B</td>
<td>NA</td>
</tr>
<tr>
<td>A</td>
<td>AbA5</td>
<td>IgG1</td>
<td>18C</td>
<td>0.75</td>
</tr>
<tr>
<td>E</td>
<td>6b53</td>
<td>IgG3</td>
<td>18C</td>
<td>0.7</td>
</tr>
<tr>
<td>C</td>
<td>CbE2</td>
<td>IgG2</td>
<td>23F</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Table 5.1.** Avidity indices (AI) measured by modified ELISA from seven pre and post vaccination serum samples for serotype specific IgG, five IgG, three IgA and one IgM Mab. NA: not available. *AI<0.25.

Six out of seven of the serum samples demonstrated an increase in avidity from pre to post vaccination. Subject C pre-vaccination serum demonstrated high avidity to serotype 23F and this did not increase post vaccination despite a six fold increase in antibody titre. Serum avidity indices ranged from 0.4 - 2.55 and four of the five IgG Mabs had avidity indices within this range. CbB2 was the exception, with an AI less than the minimum concentration of NH\textsubscript{4}SCN used (0.25M). It was assigned a value of half this minimum concentration: 0.125. Two of the three IgA Mabs analysed (both
IgA2) demonstrated ALs in the same range as serum and IgG Mabs. However
Db8C11 (IgA1) had an Al over four fold greater than its isotypic variant Db7D4. 6b5D7, the single IgM Mab analysed in the assay had an Al above the limits of detection of the assay.

5.3.2 Opsonophagocytosis

Twelve Mabs were analysed for in vitro functional activity by opsonophagocytic assay, they included six IgGs (one IgG1 and five IgG2), three IgAs (one IgA1 and two IgA2) and three IgMs. All assays were done in duplicate with a minimum of four doubling dilutions. A minimum of two non opsonin (antibody negative) controls (NOC) were included in each assay.

Figure 5.3 shows a sample of three histograms representing opsonophagocytosis of pneumococci by Mabs DM5, CbG8 and Db7D4.

Figures 5.4 and 5.5 show dot plot graphs of mean % phagocytosis (Y axis) of duplicate samples against antibody concentration (X axis) for the IgG, IgA and IgM Mabs respectively and paired 28 day serum from the vaccinee from whom the Mab was generated. Post vaccination serum antibody concentration (Ac, Bc, Cc, Dc, Ec) refers to donor serotype specific IgG only although IgA and IgM may have also contributed to the opsonophagocytic activity of these samples.

Figure 5.6 shows the opsonophagocytic activity (mean % PMN fluorescence (Y axis) of duplicate samples against antibody concentration (X axis)) for the polyreactive IgM Mabs against four of the pneumococcal serotypes included in the vaccines. The concentration of polyreactive IgMs was arbitrarily assigned on the basis of their performance in ELISA on serotype 4 coated plates using the standard serum 89SF.

Great interassay variation was demonstrated in the NOCs reflecting variable activation of the PMNs during the isolation process.
Chapter 5  Antibody Function

Figure 5.3 Histogram plots showing percentage opsonophagocytosis of FITC labeled pneumococci by peripheral blood PMNs after pre-incubation with three pneumococcal Mabs. The gated neutrophil count is shown along the Y-axis and fluorescent intensity (FL-1) along the X-axis. The sample population is shaded, the non-opsonin controls are unshaded. % opsonophagocytosis is determined by the percentage of the gated population lying within the marker M-1.
Figure 5.4. Opsonophagocytosis of pneumococci by IgG Mabs and post vaccination sera. %PMN Fluorescence (Y axis) is plotted against antibody concentration (µg/ml) (X axis). Non opsonin (ie. No serum added) controls (NOC) are shown in yellow.
Figure 5.5 Opsonophagocytosis of pneumococci by three IgA and one IgM Mabs and post vaccination sera. % PMN Fluorescence (Y axis) is plotted against antibody concentration (µg/ml) (X axis). * points extrapolated from curve: no data for this concentration. Non opsonin (ie. No serum added) controls (NOC) are shown in yellow.
Figure 5.6 Opsonophagocytosis of four different pneumococcal serotypes by polyreactive IgM Mabs. % PMN Fluorescence (Y axis) is plotted against antibody concentration (µg/ml) (X axis). Non opsonin (ie. No serum added) controls (NOC) are shown in yellow.

The opsonophagocytic index (OI) is a measure of the concentration of antibody (µg/ml) giving 50% of maximum opsonophagocytosis. It has been found to be one of the most reproducible measures of serum opsonophagocytic activity and is currently being used in the evaluation of new pneumococcal vaccines (Martinez et al, 1999, Vernacchio et al, 2000). However, the OI gives no indication of the absolute level of opsonophagocytic activity. Thus two antibodies may have the same OI with each
demonstrating a 50% reduction in opsonophagocytosis at 0.5 μg/ml but one produces 40% opsonophagocytosis at 0.5 μg/ml and the other produces 20%. A low OI represents an antibody or serum sample that achieves maximum opsonophagocytic activity at low concentration.

The OIs for IgG, IgA and IgM Mabs are shown in Table 5.2.

<table>
<thead>
<tr>
<th>Ab</th>
<th>Ig Isotype</th>
<th>Serotype</th>
<th>OI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbA5</td>
<td>IgG</td>
<td>18C</td>
<td>0.1</td>
</tr>
<tr>
<td>CbB2</td>
<td>IgG2</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>CbE2</td>
<td>IgG2</td>
<td>23F</td>
<td>0.25</td>
</tr>
<tr>
<td>Db3G9</td>
<td>IgG2</td>
<td>6B</td>
<td>0.125</td>
</tr>
<tr>
<td>Db7D4</td>
<td>IgG2</td>
<td>4</td>
<td>0.02</td>
</tr>
<tr>
<td>DMF4</td>
<td>IgA2</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Db8C11</td>
<td>IgA1</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>CbG8</td>
<td>IgA2</td>
<td>6A</td>
<td>0.7</td>
</tr>
<tr>
<td>6b5D7</td>
<td>IgM</td>
<td>6B</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Table 5.2 Opsonophagocytic indices (OI) for Mabs**

All antibodies analysed induced opsonophagocytosis to various degrees. The OI for the IgG and IgM Mabs were lower than for the IgA Mabs. This was particularly notable for the Db8C11, the IgA isotype switch variant of IgG clone Db7D4. Db7D4 demonstrated markedly lower OI than Db8C11. For all of the Mabs, the level of opsonophagocytosis was dose dependent.
The serotype 6B Mabs were analysed for opsonophagocytic activity on a 6A serotype due to the unavailability of a well encapsulated 6B strain. The data suggests that Db3G9, CbG8 and 6b5D7 all recognise and can induce opsonophagocytosis of serotype 6A pneumococci suggesting that these antibodies are serogroup 6 and not serotype 6B specific. If a well encapsulated serotype 6B pneumococcal strain becomes available it would be interesting to compare opsonophagocytic activity within serogroup 6. Crossreactivity to other serogroups (groups 4 and 14 were tested) was not demonstrated (data not shown) which supported the ELISA specificity data described in Chapter 3.

6b5D7, the serotype 6B Mab demonstrated opsonophagocytic activity (Figure 5.5) with a low OI. The polyreactive Mabs (CbH4 and 6b1A7) were also assessed. CbH4 was tested against four serotypes and demonstrated low levels of opsonophagocytosis to serotypes 18C and 23F and no opsonophagocytosis of serotypes 4 and 6A. 6b1A7 was tested against serotypes 18C and 23F only and produced high levels of opsonophagocytosis to both (Figure 5.6).

Avidity indices and opsonophagocytic activity of the IgG and IgA Mabs were then compared to determine whether the same ranking was demonstrated by the two different assays. Results are shown in Figures 5.7 and 5.8. The IgG Mabs demonstrated the same ranking order for each assay despite having different serospecificities. The two IgA2 Mabs (DMF4 and CbG8) demonstrated the same ranking. However, the IgA1 Mab Db8C11 specific for serotype 4, which was of high avidity produced very low levels of opsonophagocytosis compared with the medium avidity IgA2 Mabs.
Figure 5.7 Correlation of Avidity Index (AI) and opsonophagocytic activity of IgG Mabs at 0.5 μg/ml.

Figure 5.8 Correlation of Avidity Index (AI) and opsonophagocytic activity of IgA Mabs at 0.5 μg/ml.
5.4 Discussion

Studies with Hib have demonstrated that the avidity of antigen-antibody binding is variable (Schlesinger & Granoff, 1992a; Lucas & Granoff, 1995a) and may correlate with antibody bactericidal activity (Amir et al, 1990b; Amir et al, 1990a; Usinger & Lucas, 1999) and V gene use (Lucas et al, 1994; Lucas & Granoff, 1995a; Nahm et al, 1995). The level of somatic hypermutation within the three HV loops have also been shown to influence avidity and mutation of specific V gene regions and codons have been associated with marked improvement in AIs, especially for peptide antigens.

As the immune response matures, clonal selection of B producing high affinity antibodies occurs, a process known as affinity maturation (Griffiths et al, 1984) and B cells that have acquired mutations conferring improved affinity are selectively expanded. However, this process of affinity maturation is only seen in the response to TD antigens (Konradsen, 1995). This is confirmed by recent data looking at the change in avidity of antibodies to meningococcal and pneumococcal polysaccharides induced by PS (Tl) and conjugate vaccines (TD). It has been shown that in young, naïve vaccinees the avidity of antibodies induced by pneumococcal conjugate vaccines (TD) demonstrates the phenomenon of affinity maturation. This is not seen in the response to the plain polysaccharide vaccines for the same bacteria (Goldblatt et al: personal communication).

5.4.1 Avidity of Mabs and serum IgG

The avidity indices of all the IgG Mabs with the exception of CbB2 were in the same range as post vaccination serum IgG. Of the 3 IgA anti-pneumococcal Mabs analysed all had AIs in the same range as serum IgG with the exception of DbSCII, the IgA isotypic variant of IgG clone Db7D4 which demonstrated an AI over 4 fold greater than the IgG (3.9 and 0.9 respectively). Although Db8C11 and Db7D4 originate from the
same clone and share common mutations, they are also discordant for a number of
mutations in both CDR and framework regions (Figures 4.7 and 4.8). The two
replacement mutations identified within the CDR1 and 2 which are not shared
between the two clones may be responsible for altering the avidity of antigen binding.
Fish et al (1991) demonstrated that specific point mutations within the CDR2 of murine
VH genes can alter the specificity and avidity of antibody-hapten binding with a specific
mutation leading to amino acid replacement increasing the affinity of binding over five
fold. To clarify whether the variation in the CDR2 is responsible for the marked
differences in avidity demonstrated between the isogenic variant Mabs, site directed
mutagenesis of the discordant nucleotides and subsequent avidity studies are
required.

An alternative explanation for the difference in avidity between these two Mabs may lie
within the constant region. Although antibody affinity is primarily determined by the
immunoglobulin variable region structure, variation in the constant region between IgG
subclasses has been shown to influence both the affinity and avidity of antigen binding
(McCloskey et al, 1996; Sutliff and Finland u, 1932) and the kinetics of the antigen
antibody interaction can be altered considerably by switching the IgG subclass
(Francis & Tillett, 1930). Murine studies suggest that the heavy chain constant domain
can influence the binding of antibody to streptococcal polysaccharide (Shinefield et al,
1999; Cooper et al, 1993) and the level of glycosylation of the heavy chain has been
shown to modify Fc related function (Wright et al, 1914). These studies have generally
involved IgG subclass comparisons and it is not known how isotypic differences in Fc
regions may influence binding kinetics.

5.4.2 Opsonophagocytic activity of Mabs and serum

The opsonophagocytic activity of antibody is known to be influenced by the isotype and
subclass of immunoglobulin (Vidarsson et al, 1998; Anttila et al, 1999). In addition,
studies using clonal purified antibodies to Hib have demonstrated that V\_k gene use can influence both antibody avidity and bactericidal activity (Nahm et al, 1995). Due to the high interassay variability demonstrated by the non-opsonin controls (NOCs) comparative analysis of opsonophagocytic potential of the different Mabs was limited. However nine of the eleven Mabs were analysed paired with own donor D28 post vaccination serum and this permitted comparison of Mab function with that of polyclonal serum at similar antibody concentration. The serum concentration of IgG was used although it is recognised that the IgA and IgM in the serum samples may also have contributed to the opsonophagocytic activity of these samples. Only if Mabs sharing a common serospecificity were run on the same day using PMNs from the same source could direct comparisons be made between the Mabs.

Opsonophagocytic activity was demonstrated in all of the Mabs analysed (n=11) of which two were the polyreactive IgMs. All of the IgG Mabs demonstrated greater opsonophagocytic activity at the same Pnc specific IgG concentration as donor matched serum. This was not unexpected as mutation analysis revealed that all these clones demonstrated high levels of somatic mutation consistent with a secondary immune response (Chapter 4). In contrast, the serum IgG was polyclonal and it is anticipated that it would consist of antibody derived from a mixture of naïve and primed B cells with a wide range of opsonophagocytic activity.

All the IgA clones, including the high avidity binder Db8C11, demonstrated less opsonophagocytic activity at the same concentration as serum IgG. Correlations between Ig V gene use, somatic mutation and antibody function have not been reported for IgA and the role of IgA in systemic immunity is poorly defined. There is no published data comparing the opsonophagocytic activity of anti-pneumococcal IgG and IgA. However, post vaccination serum has been analysed to determine which Ig isotypes and subclasses correlate best with opsonophagocytic activity (Vidarsson et al,
The best correlation was found for total IgG concentration but no correlation was found for IgA. This is not unexpected as the major role of IgA is to protect mucosal surfaces from colonisation and invasion and studies attempting to analyse the opsonophasocytic activity of IgA have been problematic (Kilian et al, 1988). There are many possible explanations for the difference in opsonophasocytic activity between the isotypic variants of an identical clone. The most likely explanation lies in the functional interaction of Fcα with the FcαR and the source and activation state of the PMNs both of which are known to influence the opsonophasocytic activity of IgA.

Further investigations are underway to evaluate the function of the IgA Mabs in a mucosal epithelium *in vitro* model and an *in vivo* mouse model of pneumococcal bacteraemia. These studies are being performed in collaboration with Dr R. Read (Dept Microbiology, University of Sheffield, UK) and Professor I Jonsdottir (Dept Immunology, National University Hospital, Reykjavik, Iceland) respectively.

The serotype 6B Mabs - including the IgM Mab 6b5D7, were able to induce opsonophasocytosis of a well encapsulated serotype 6A Pnc strain. Cross-reactivity and cross protection within this serogroup has been documented previously (Nahm et al, 1997).

Opsonophasocytic activity demonstrated by the two polyreactive Mabs: CbH4 and 6b1A7 (Figure 5.6) was not uniform for all serotypes tested and no opsonophasocytosis of Pnc serotype 4 was demonstrated by CbH4. 6b1A7 induced higher levels of opsonophasocytosis against the two serotypes :18C and 6A for which comparative data is available. 6b1A7 does not use the canonical genes characteristically used by PS binding antibodies and further studies are required to identify the epitope to which both 6b1A7 and CbH4 bind.

A clear correlation between IgG2 titre and opsonophasocytosis was demonstrated despite the fact that IgG2 has been shown to bind only weakly to FcγRs (Kurono et al,
1999; Amir et al., 1990b). This was consistent with published studies demonstrating that serotype specific IgG2 titres in serum post Pnc conjugate vaccination show the best correlation with in vitro opsonophagocytic activity (Vitharsson et al, 1994).

5.4.3 Correlation of Avidity and Opsonophagocytic activity

Conflicting data exists regarding the correlation between antibody avidity, opsonophagocytic activity and in vivo protection (devey, 1997; Usinger & Lucas, 1999; Amir et al, 1990a; Fine et al, 1988; Granoff & Lucas, 1995; Schlesinger & Granoff, 1992b). In addition, the contribution of V gene usage and somatic mutation to these functional activities is unclear (Lucas & Granoff, 1995b; Chung et al, 1995).

Comparison of ranking order for AI and opsonophagocytic activity as determined by % PMN Fluorescence at 0.5mcg/ml Mab concentration demonstrated similar ranking for the IgG Mabs (Figure 5.7) despite heterogeneity of serospecificity and subclass. The IgA2 Mabs showed similar ranking between the two assays however, Db8C11 the IgA1 Mab of high avidity performed poorly in the opsonophagocytic assay within the antibody concentration range of 0.06-2 μg/ml (Figure 5.8).

5.4.4 Correlation of in vitro functional analysis and genetic analysis

Two of the pneumococcal hybridomas (AbA5 and CbE2) specific for different pneumococcal serotypes and derived from different donors, shared the same Vh3 gene (Vh3-48) and a replacement somatic mutation at position 55 in the CDR2 Figure 4.5). The Mabs secreted by these clones are both of high avidity and demonstrate good opsonophagocytic activity.

As has been discussed in Chapter 4, Vh3-48 is not commonly expressed in the normal repertoire. The probability of a replacement mutation in this common gene arising by chance is low suggesting that there is positive selection of such B cell clones in both vaccinees and that Vh3-48 may be an important V gene in the response to a number
of pneumococcal serotypes. The replacement mutation arises from the same base change in the codon. The sites within this V gene which confer the different antigen binding specificities are not known. In the panel of murine hybridomas described by Fish et al (1991) and discussed above, a mutation which conferred specificity of antigen binding within the CDR2 was demonstrated adjacent to the common mutation conferring high binding avidity. Mutation analysis of the two high affinity Mabs (AbA5 and CbE2) described, demonstrates an amino acid substitution in AbA5 at position 54 which is not shared with CbE2. This too is adjacent to the common mutation site. However, without more detailed analysis through site directed mutagenesis and structural modeling of the antigen binding site it is unknown whether these mutations are significant. Although the CDR2 may be important in conferring the differing specificities of the two clones it is also well recognised that the CDR3 plays a major role in conferring specificity of antigen binding (Xu & Davis, 2000) and the CDR3s differ considerably in these two clones. With regard to the many other non-shared mutations in these two high avidity antibodies, Fish et al (1991) demonstrated that the magnitude of the influence of the recurrently observed amino acid substitutions appeared to be unaffected by substitutions at other positions.

Two of the other hybridomas, Db8C11 and Db7D4, isogenic variants of the same clone with the same serospecificity differed considerably in antigen binding avidity. It is also recognised that the Fc region can contribute to the avidity of antigen binding (Feldman et al, 1994; Anttila et al, 1998) and the difference in binding avidity may be a function of differences in the Fc region between the Two clones. Clonality was identified by the presence of a number of common mutations in framework and CDRs of both Vh and Vl genes and identical CDR3s. However distinct mutations were also demonstrated (Figures 4.7 & 4.8). Within the CDR2 specifically, in addition to a shared amino acid mutation at position 56, additional replacement mutations were seen in Db8C11, the
clone producing high avidity antibody. The difference in binding avidity between the two clones may be attributable to the variation in somatic hypermutation found within this region. The positive and negative effects of somatic mutation on antibody function have been studied using saturation mutagenesis of anti-phosphocholine (PC) antibodies in vitro (Chen et al, 1995). Mutation of residues contacting the haptenic group, as determined by molecular modelling, did not improve binding. Instead, productive mutations occurred in residues that either contacted carrier protein or were distant from the antigen binding site, possibly increasing binding site flexibility through long-range effects. Site directed mutagenesis at the CDR2 site where the common mutation was demonstrated and subsequent avidity and opsonophagocytic assays are required to determine whether this mutation contributed to the relatively high avidity and opsonophagocytic activity of these antibodies. Subsequent molecular modeling would then be useful to determine how the mutation may affect the conformation of the antigen binding site and nature of the antigen-antibody interaction.

No correlation was observed between the ranking order of the Mabs and light chain V gene usage, the absolute level of somatic mutation (R:S ratios in CDR1&2 of $V_H$ and $V_L$) in heavy or light chain V genes or CDR3 length. All isotype switched clones demonstrated R:S ratios consistent with affinity maturation (Table 4.1) (Chang & Casali, 1994). Notably, the low avidity Mab, CbB2 demonstrated a relatively high R:S ratio and the high avidity Mabs (AbA5 and CbE2) demonstrated relatively low levels of somatic mutation.

In summary, in vitro functional activity has been demonstrated in all the Mabs analysed (n=12) with a good correlation between avidity and opsonophagocytic activity across a range of serotypes. The two Mabs which ranked most highly in both assays shared a
common \( V_h \) gene: V3-48, and a common mutation in the CDR2 region despite being derived from different individuals and demonstrating different Pnc serospecificities. Further functional assays are underway to evaluate the role of the IgA Mabs in nasal mucosa protection models and whether the IgG Mabs can protect against invasive pneumococcal disease in mice.

The next stage in this work would be to evaluate the contribution of the \( V_h 3-48 \) gene in the immune response to pneumococcus. In addition, the effect of the common somatic mutation in the CDR2 region on antibody specificity and function should be assessed. The use of restriction PCR as described in Chapter 4, an alternative to the development of anti-idiotypic antibodies, would determine whether \( V_h 3-48 \) is indeed an important gene in the anti-pneumococcal antibody response at a population level (Lucas & Granoff, 1990; Lucas et al, 1991; Adderson et al, 1993). If this proves to be the case, site directed mutagenesis at the site of the common CDR2 mutation (Chen et al, 1995; Chen et al, 1992) and subsequent functional assays may then be performed to determine whether this mutation has important functional consequences.
CHAPTER SIX

DISCUSSION

6.1 Molecular basis of the immune response to pneumococcal antigens........160

6.2 Isotype, mutation and memory........................................................................164

6.3 The future............................................................................................................167

6.4 Concluding remarks...........................................................................................168
6.1 Molecular basis of the immune response to pneumococcal antigens

As the introduction of successful vaccination strategies eliminates a range of infectious diseases, the possibility of vaccine associated morbidity (predominantly allergy and autoimmunity) is coming under more intense scrutiny (White et al, 1985; Retalliau et al, 1980; Schoenberg, 1978; Ward, 2000; Miller & Pisani, 1999; Scholtz & Duclos, 2000) and public concern regarding the potential for vaccine related disease is escalating. There are concerns that this anxiety has already compromised the efficacy of the MMR vaccine at a population level (Afzal et al, 2000) and a more detailed understanding of the immune response to vaccine antigen is now required.

There are many serological studies in which the antibody response to pneumococcal vaccines has been characterised in detail. However the molecular basis of the immune response to both the polysaccharide vaccines and the bacterium is poorly defined and the mechanisms involved in the generation of memory to polysaccharides are not well understood.

Data, mainly from Hib PS and conjugate vaccine studies, have suggested that the B cell response to polysaccharide and conjugate vaccines is of limited diversity (Silverman & Lucas, 1991; Carroll et al, 1995; Sun et al, 1999; Kowal et al, 1999; Lucas et al, 1997; Insel & Anderson, 1986; Scott et al, 1989) with specific V genes or antibody idiotypes dominating the response (Adderson et al, 1993; Pinchuk et al, 1995). Dominant genes have been associated with superior antigen-antibody binding (Lucas et al, 1994; Heilmann, 1990; Nahm et al, 1995; Fish et al, 1991).

A number of factors have been shown to influence the antibody repertoire generated including the age of the vaccinee (Lucas et al, 1993) and the method of manufacture ie. conjugate protein used, method of conjugation and poly- or oligosaccharide chain length (Granoff et al, 1993; Lucas & Granoff, 1995; Lucas & Granoff, 1990; Pillai et al,
However, the effect of co-administration of multiple antigens on the antibody repertoire induced has not been evaluated however although simultaneous vaccination with combination vaccines has been shown to influence antibody titre to the individual vaccine components (Paradiso, 1995; Lagos et al, 1998).

In this thesis the generation and characteristics (molecular and function) of a number of pneumococcal heterohybridomas induced by polyvalent conjugate and polysaccharide vaccines has been described. The sample size for this study was small (5 vaccinees, 15 hybridomas), reflecting the time required to produce stable antibody secreting heterohybridomas. This limited the interpretation of the hybridoma data. In collaboration with Dr H. White (ICH), hybridomas and whole blood from the vaccinees were also analysed by isotype specific restriction PCR. This facilitated placing the sequence data for the isolated hybrids in the context of the B cell repertoire in whole blood. Clonal expansion of B cells using V genes used by the hybridomas could be seen and new restriction sites identified in the hybridoma clones, introduced through somatic mutation could also be seen in the Day 7 blood restriction PCR. This provided a strong argument for clonal identity and demonstrated how the combination of heterohybridoma production and restriction PCR could be used to follow the emergence of clones through different B cell compartments. It also demonstrated how the combination of heterohybridoma production and restriction PCR could be used to probe the dynamics of the development of memory to vaccine antigens in man.

The results showed that a few genes dominated the pneumococcal antibody repertoire and in the case of $V_{\mu}3-48$, a gene not commonly used in the general repertoire, this was not demonstrated to be serotype dependent. The two antibodies (AbA5 and CbE2) using $V_{\mu}3-48$ came from different donors and demonstrated serotype specific high avidity binding and good opsonophagocytic activity. In addition to using the same V gene, they both demonstrated an identical amino acid replacement mutation (serine
to threonine) at position 56 in the CDR2 (H2). There were a number of other non-shared mutations proximal to this site and in the other CDRs and FR regions. The significance of this homology in V gene usage and shared and distinct mutations in the CDR2 is not known and warrants further investigation. Serine and threonine are both uncharged polar amino acids and it is not clear what the effect of this mutation on the antigen binding site would be. However, the likely-hood of this homology arising by chance is small, which suggests that this feature may be important in pneumococcal antigen binding.

The two clones: Db8C11 and Db7D4 also provided interesting sequence data. They were Isotype variants of the same clone with identical $V_h$, $V_l$ genes and CR3s but they demonstrated marked differences in antigen binding avidity and opsonophagocytic activity. Such isotype switch variants provided ideal reagents to look at the effect of isotype on antibody function. A number of distinct mutations were demonstrated in both FR and CDR regions in addition to common mutations. Although the non-shared mutations through the variable region may contribute to the functional differences seen between the two clones, it is considered more likely that the use of distinct Fc regions accounts for the heterogeneous results.

As discussed in Chapter 5, further studies are underway in collaboration with groups in Iceland and Sheffield, to evaluate the role of the IgA Mabs in protection against disease. Dr I.Jonsdottir (University of Reykjavik, Iceland) is analysing the potential for mucosaly applied IgA to protect rats from pneumococcal pneumonia and Dr R Read (University of Sheffield, UK) is investigating the effect of IgA on adherence of encapsulated pneumococci to nasal epithelia. In addition, Dr J Reinholdt (Aarhus University, Denmark) is studying the effect of IgA proteases on the IgA Mabs. Results from these studies are important in the context of the relatively high proportion of IgA found in adult but not infant serum post pneumococcal vaccination. It may be that the
isotype dominance post vaccination has a significant impact on the clinical efficacy of a
vaccine, the mechanism of protection from invasive disease and the subsequent
mucosal flora.

To evaluate the functional significance of shared mutations in the Mabs generated, site
directed mutagenesis at the mutation sites is required. Saturation mutagenesis of anti-
phosphorylcholine antibodies has been used to study the positive and negative effects
of somatic mutation on antibody function (Chen et al, 1995). Mutation of residues
contacting the haptenic group, as determined by molecular modeling, did not improve
binding. Instead productive mutations occurred in residues that either contacted the
carrier protein or were distant from the antigen binding site, possibly by increasing
binding flexibility through long range effects.

Sequential mutagenesis of the discordant replacement mutations could also be used to
locate residues conferring serospecificity. Using the information derived from such
data, a structural model of the antigen binding site for these two serotypes could then
be constructed with the locations of critical residues for high avidity and serotype
specific polysaccharide binding predetermined. This information would be useful for
the construction of a new generation of synthetic vaccines in which designer antigens
such as peptide mimics (mimotopes) are used to induce protective immunity.

The use of capsular polysaccharide mimotopes to elicit protective immunity has been
described in the literature and such technology has already been applied to other
encapsulated bacteria such as N.meningitidis, E.coli, and Cryptococcus neoformans
(McConnell et al, 1994; Grothaus et al, 2000; Jahn et al, 1995; Young et al, 1997) and
to a number of tumour antigens (Kieber et al, 2000; Luo et al, 1998; Kieber et al,
1997). The preliminary findings of limited diversity in the response to pneumococcus
with shared V genes and mutations in the antigen binding site of antibodies even with
different serotype specificities suggests that there may be a place for such peptides in
polyvalent pneumococcal vaccine design. In collaboration with Dr I Feavers (NIBSC, Potters Bar, UK) preliminary screening of peptide libraries for mimotope antigens is underway using a sample of the 6B specific Mabs produced.

6.2 Isotype, mutation and memory

The immune response to unconjugated polysaccharides is characterised by the absence of immunological memory. In contrast, conjugated polysaccharides do induce immunological memory. However, the nature of the response to infection or colonisation with the encapsulated bacteria remains unclear and there is no published data comparing the repertoire induced by vaccination with that induced through nasopharyngeal colonisation or systemic infection with the encapsulated bacteria.

Despite the presence of pneumococcal specific antibody in many unvaccinated adults most of the published studies analysing the immune repertoire following pneumococcal vaccination of adults assume the immune response to be primary and thus dictated largely by vaccine formulation. This conflicts with the results of this study. The hybridoma sequence data described in Chapter 4 demonstrates that 12/15 of the 15 hybridoma clones were isotype switched and highly mutated, consistent with previous exposure to the antigen. The level of mutation bore no relation to vaccine type and as none of the vaccinees had been vaccinated previously, antigen exposure must have come through earlier infection or nasopharyngeal carriage of S.pneumoniae or through cross-reactive antigens such double stranded DNA (dsDNA), Klebsiella spp or Ecoli K7 (K56). This is supported by data recently published by Hougs et al (1999) looking at the immune response to Hib vaccine. They demonstrate that clonally selected, affinity matured B cells secreting IgA2 dominate the immune response to the first dose of Hib conjugate vaccine in an adult vaccinee which suggest that, as for pneumococcus, the
immune repertoire induced in adults post Hib conjugate vaccine is influenced by pre-vaccination immunity with memory for the polysaccharide.

It is well recognised that up to 30% of adults may carry the pneumococcus at any one time so prior exposure is expected but carriage has not been thought to trigger an immune response and even if humoral immunity was induced, it has been assumed that the response would be T independent. These data provide a strong case for the response in an adult vaccinee to the first dose of Pnc PS being secondary, dominated by isotype switched, clonal selected, affinity matured B cells - the classic characteristics of memory cells.

A number of serological studies in adults and infants provide data which supports this hypothesis. The immune profile of the infant response - a naïve group - is shown to be markedly different from that of adults. Immunoglobulin isotype distribution is dominated by IgA in adults but this isotype remains low in infants despite boosting suggesting that adults have been primed via the mucosa prior to vaccination (Sigurdardottir et al, 1997; Vidarsson et al, 1998). The IgA subclass distribution studies have demonstrated that IgA2, the subclass normally found at low concentration in serum and at higher concentration in mucosal secretions forms a relatively high proportion of the serum IgA Ab to pneumococcus. The hybridoma data presented in this study supports this with two of the four IgA AbSC belonging to the IgA2 subclass. The isotype distribution alone, however, cannot distinguish whether these cells represent a long lived primary B cell population or a memory population and it is only with the additional mutation analysis that the argument for memory has been strengthened.

The presence of a pre-primed population of pneumococcal specific B cells in most adults clearly has important implications for immune repertoire analysis and vaccine design. The B cell repertoire induced post-vaccination will be shaped by pre-vaccination immunity and may be different from that induced in naïve individuals. It
may also explain the repertoire differences seen between adult and infant vaccinees, the latter not having been primed by the encapsulated bacteria. The implications of this for vaccine efficacy are not known although the overwhelming success of infant immunisation with Hib conjugate vaccine in eliminating invasive disease in the vaccinees suggests that vaccine efficacy may not be hindered although carriage rates and the mechanism of protection between the two groups may be different.

The influence of immune history in shaping the immune repertoire to vaccination may also have implications for vaccine related morbidity. The importance of microbial infection as a trigger for the induction of autoimmune diseases such as systemic lupus erythematosis (SLE) is frequently debated and it is recognised that anti-viral and anti-bacterial responses are often accompanied by self reactivity. Anti-pneumococcal antibodies elicited in both SLE patients and non-autoimmune individuals by pneumococcal vaccine have been shown to express lupus-associated anti-DNA idiotypes (Ray et al, 1996; Kowal et al, 1999). Whether these antibodies are pathogenic is unclear but it has been demonstrated in non-autoimmune mice that antibodies induced to phosphocholine - an integral part of the cell wall of *S. pneumoniae* - which do not have the idiotype most commonly used by anti-phosphocholine antibodies, may be cross reactive with dsDNA and be pathogenic (Limpanasithikul et al, 1995). The authors suggest that idiotypically restricted responses may serve to prevent the expression of Abs that react with both foreign and self Ags and so help maintain self-tolerance. This further emphasises the importance of developing a detailed understanding of the relationship between the timing of vaccination, immune history and the antibody repertoire induced.
6.3 The Future

The development of human heterohybridomas producing human monoclonal antibody has provided useful reagents with which to sample the B cell repertoire in response to vaccine antigens in man and to correlate genetic characteristics of antibody directly with antibody function. The combination of heterohybridoma production with restriction PcR fingerprinting has demonstrated how these techniques can be used to screen serial samples of blood for clonal expansions of target genes in response to vaccine antigens. The numbers of target genes identified by heterohybridoma production are small reflecting the time consuming process involved. Combining these techniques with the use of anti-idiotype antibodies for the genes thought to be important would facilitate analysis of the diversity of the immune repertoire and the factors that influence it at a population level.

A detailed understanding of the antibody repertoire to pneumococcal polysaccharides, the genes and mutations associated with superior functional activity and the factors controlling their selection would contribute to the serum data already accumulating to facilitate preferential selection of vaccines inducing an antibody profile known to be associated with superior antibody functional activity.

The incorporation of cytokines and receptor ligands to target specific lymphocyte populations may then be studied. The addition of cytokines to generate a polarised immune response has already been described for a number of antigens in which the addition of IFN gamma +IL-2 or IL-4+IL-5+IL-10 have been successfully used to elicit improved cell mediated and humoral immunity respectively (Golding & Scott, 1995). Receptor ligands such as anti-CD40 antibodies have also been studied. The co-administration of anti-CD40 antibodies with plain pneumococcal polysaccharide successfully induced a TD response to the polysaccharide in murine studies (Dullforce et al, 1998a; Dullforce et al, 1998b).
6.5 Concluding remarks

A detailed understanding of the molecular basis of the antibody response to the pneumococcus and the nature of the cellular interaction required for memory to polysaccharides is essential to facilitate the production of new more sophisticated vaccines. The response may then be manipulated using a combination of specific antigens with various cytokines and receptor ligands to permit the targeting of specific B cell populations to achieve a well regulated and highly defined immune response to a diverse spectrum of pathogens.


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Goodman D. J., C. Gaff, and S. Gerondakis. 1993. The IL-4 induced increase in the frequency of resting murine splenic B cells expressing germline Ig heavy chain gamma 1 transcripts correlates with subsequent switching to IgG1. Int Immunol 5(2) 199-208.


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Liu YJ, C Barthelemy, O de Bouteiller, C Arpin, I Durand, J Banchereau and YJ Liu. 


1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. Immunity. 2:239-248.


Lue C., A. Tarkowski, and J. Mestecky. 1988. Systemic immunization with pneumococcal polysaccharide vaccine induces a predominant IgA2 response of
Bibliography

Peripheral blood lymphocytes and increases of both serum and secretory anti-


McHeyzer-Williams, MG McHeyzer-Williams, MJ McLean, PA Lalor, and GJV Nossal.
Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography

against Streptococcus pneumoniae in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. *Clin Infect Dis* 29 (2) 281-8.


Bibliography


Smith G., M. Spellerberg, F. Boulton, D. Roelcke, and F. Stevenson. 1995. The immunoglobulin VH gene, VH4-21, specifically encodes autoanti-red cell antibodies against the I or i antigens. Vox Sang 68 (4) 231-5.


Bibliography


Bibliography


Vajdy M. and N. Lycke. 1995. Mucosal memory B cells retain the ability to produce IgM antibodies 2 years after oral immunization. *Immunology* 86 (3) 336-42.


Bibliography


Bibliography


Xu J. L. and M. M. Davis. 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13 (1) 37-45.


APPENDIX

Names and Addresses of Suppliers: UK unless otherwise stated

American Type Tissue Collection (ATTC)
12301 Parklawn Drive
Rockville Maryland
20582 USA

Amersham International
Amersham Place
Little Chalfont
Buckinghamshire

Amicon Ltd
Upper Mill Stonehouse
Gloucestershire

BDH (Merck Ltd)
Merck House
Poole Dorset

BIO101
Anachem
Luton

Biogenesis Ltd
Stinnsford Rd Poole
BH17 ONF

Carl Frasch
Center for Biologics Evaluation and Research
Bethesda, MD

Genosys Biotechnologies Inc
162A Cambridge Science Park
Milton Rd Cambridge

Genzyme
50 Gibson Drive Kings Hill
West Malling Kent

Gen-Probe Inc.
10210 Genetic Center Dr.
San Diego, California 92121.
USA

Gibco BRL
Inchinnan Business Park
Paisley PA4 9RF
Hybridoma Reagent Laboratory
PO Box 9975 Baltimore
MD 21224 USA

ICN Pharmaceuticals, Ltd
1 Elmwood Chineham Business Park
Basingstoke
Hants RG24 8WG

Millipore
The Boulevard Blackmore Lane
Watford Herts

Mycoplasma Experience
1, Norbury Road
Surrey, RH2 9BY

Pasteur Mérieux MSD Ltd
Clivemont Rd
Maidenhead Berks
SL6 7BU

Perkin-Elmer Applied Biosystems
Warrington
WA14 4SR

Pharmacia Biotech
Promega, Maddison, USA

Qiagen Inc.
Crawley W Sussex
RH10 2AX

Revelations 2.0
Dynatech Laboratories
Daux Road
Billingshurst
Sussex

Sigma Ltd
Fancy Rd Poole
Dorset
BH12 4QH

Statens Seruminstitut
Copenhagen, Denmark

Wyeth Lederle Vaccines
Huntercombe Lane South
Taplow Maidenhead
Berk
SL6 0PH
Immunogenetic analysis of the immune response to pneumococcal polysaccharide

Helen E. Baxendale¹, Zadie Davis², Harry N. White³, Myfanwy B. Spellerberg⁴, Freda K. Stevenson⁴ and David Goldblatt¹
¹ Immunobiology Unit, Institute of Child Health, University College London, London, GB
² Molecular Immunology Group, Tenovus Laboratory Southampton University Hospitals, Southampton, GB

Pneumococcal serotype-specific anti-capsular polysaccharide antibodies protect against invasive pneumococcal disease. Within an individual the diversity of these antibodies is limited. To evaluate the repertoire of antibodies to pneumococcus and determine whether oligoclonality is seen both between serotypes and between individuals, we sampled the B cell repertoire induced by polysaccharide and conjugate vaccine in adult volunteers. Fifteen hybridomas secreting pneumococcus-specific monoclonal antibodies were generated from five volunteers. Ten were isotype switched, six were IgG2 and four were IgA. These included two isotype switch variants of the same clone. Vh3 and Vk2 were used by 10/15 and 7/13 of the sequenced clones, respectively, with identical genes, Vh3-48 and Vk2-A17 used by a number of volunteers to a variety of serotypes. VDJ junctional characteristics and complementarity-determining region (CDR) 3 length were variable. High levels of somatic mutation in CDR1 and 2, inconsistent with a primary response, were found in 10/11 of the isotype-switched antibodies, including those induced by plain polysaccharide antigens. These data suggest that wild-type infection or nasopharyngeal carriage of Streptococcus pneumoniae in adults may induce memory and the response to subsequent immunization with plain polysaccharide or conjugate pneumococcal vaccines may have the characteristics of a secondary response.

Key words: Human / Monoclonal antibody / Streptococcus pneumoniae / Polysaccharide / Memory

1 Introduction

The molecular basis of the immune response to bacterial polysaccharides is poorly understood. Studies to determine the evolution of Ig germ-line genes and diversity in both mice and man have suggested that the responses to bacterial antigens such as phosphorylcholine and capsular polysaccharides are restricted both within and between individuals with specific Ig V genes dominating the response [1, 2].

Molecular analysis of the immune response to bacterial polysaccharides in man has been based mainly on Haemophilus influenzae type b (Hib) studies. Serological techniques have demonstrated that the immune response within an individual is oligoclonal [2, 3], gene families Vh3 and Vk2 are over-represented and, within these families, specific genes Vh3-15 (Vh26), Vh3-23 (Vk9.1) and Vk2A2 appear to dominate both within and between individuals [2, 4, 5]. While complementarity-determining region (CDR) 3 characteristics are less well defined, some investigators have demonstrated selective VDJ gene use and identical joining sites within and between individuals [2]. Preferential use of Vh3 and Vk2 gene families has also been noted for other polysaccharide antigens [6, 7]. Studies of responses to vaccines consisting of native Hib capsular polysaccharide (polyribitolribosyl phosphate, PRP) or PRP conjugated to protein have revealed that the nature of the Hib vaccine formulation can influence V region expression [9]. Analysis of Hib anti-capsular polysaccharide antibodies has revealed that antibody fine specificity, avidity and protective capacity can correlate with the expression of particular V regions [9].

Abbreviations: Hib: Haemophilus influenzae type b CDR: Complementarity-determining region CWPS: Cell wall polysaccharide R: Replacement S: Silent
Streptococcus pneumoniae is an encapsulated organism and the polysaccharide capsule is a target for protective antibodies. Due to the poor immunogenicity of native polysaccharide, pneumococcal conjugate vaccines are in accelerated development. Relatively little is known, however, of the molecular characteristics of antibodies to S. pneumoniae. The limited studies that have taken place show, by means of isoelectric focusing and L chain analysis, that the antibody response to pneumococcal polysaccharides is generally oligoclonal and within individuals is dominated by either κ or λ chains [10-12]. Sequencing of the N-terminal amino acids of a purified anti-type 14 antibody and two human IgM mAb revealed that all three antibodies used V₁κ [10, 11]. A more recent study, using phage display Ig expression from spleen lymphocytes post vaccination with a 23-valent pneumococcal polysaccharide vaccine (23V-PS), described H and L chain gene use for eight antibodies that reacted to polysaccharide vaccine. V₃κ and V₄λ dominance were demonstrated [13]. However, as these gene families dominate in the normal repertoire [14], it is unclear whether the above data represent a bias in V family or V λ gene use [15]. More detailed analysis of repertoire including a comparison of gene frequency with the normal circulating repertoire and the role of vaccine formulation in shaping the immune response to S. pneumoniae at a genetic level have not been described.

The objective of this study was to examine the molecular basis of the immune response to the 23V-PS and a new heptavalent pneumococcal-mutant diphtheria toxin (CRM₁₉₇) conjugate vaccine (7V-CRM₁₉₇) in an adult population. Fifteen human heterohybridomas secreting pneumocococcus-specific human mAb were generated. Serospecificity and antibody binding avidity were determined. V, D and J gene use, somatic mutation, canonical structure and CDR3 characteristics are described.

2 Results

2.1 Hybridomas

Four subjects (A–D) were immunized with the 7V-CRM₁₉₇ conjugate while subject E was immunized with the 23V-PPS. Fifteen stable hybridomas were derived from the five vaccinees, ten from the four conjugate recipients and five from the single recipient of the plain polysaccharide vaccine. The serotype, specificity and isotype distribution of the hybridomas is shown in Table 1. Two of the 15 clones produced IgM which reacted to all serotypes tested, preincubation with pneumococcal cell wall polysaccharide (CWPS) did not inhibit binding, and no cross-reactivity with a capsular polysaccharide derived from an unrelated organism (Hib, data not shown) was demonstrated. These two antibodies were classified as pneumococcus-specific polyreactive mAb. Nine of ten of the antibodies secreted by the hybridomas obtained from the conjugate recipients were IgG or IgA and serotype specific (Fig. 1). Of the five antibodies produced by the plain polysaccharide recipient, three were of the IgM isotype while only two were switched. Furthermore, the specificity of the antibodies derived from this vaccinee were more difficult to define with significant cross-reaction and less complete inhibition (Fig. 1). All clones came from subjects who had demonstrated high post-vaccination antibody levels of the same isotype with the exception of subject A (IgG mAb to serotype 18C) who had low post-vaccination serum levels to all serotypes and subject E (IgA mAb to serotype 9V) (Table 2). Subject D demonstrated marked elevation in IgG, A and M to serotype 4 and of the three clones characterized to date from this individual, two are isotypic variants of the same serotype 4-specific clone (Fig. 2).

2.2 V gene use

The frequency of specific V genes and gene families used by the hybridomas isolated in this study was compared with that published for a reference population in which single-cell PCR of V genes from an unselected sample of circulating IgM B cells was performed on blood from two adult volunteers [14, 15]. Two of the mAb (Db8C11 and Db7D4) are isotypic variants of the same clones and for statistical analysis of gene frequency are considered as one clone. V₃κ and V₂λ gene families dominated the repertoire and were used by 10/15 and 7/15 hybridomas, respectively (Figs. 2, 3). Statistical analysis to compare this frequency with the normal repertoire revealed that there was not significant deviation in V₃κ gene family use from the normal repertoire. Of the V₃κ genes used, two specific genes, V₃κ-48 and V₃κ-7, were each used by three of the switched hybrids. While the numbers of antibodies are small, the frequency of the use of V₃κ-48 in our monoclonal pool and specifically within the isotype-switched somatically mutated antibodies was significantly different from that which would be expected in the reference population (p = 0.025 and 0.01, respectively).

Fourteen out of fifteen of the mAb have κ or λ chain assignments and 13 of these have been sequenced. Of the light chains 13/14 were κ, the V₂λ family was used frequently and V₂κA17, V₂κA19/A3 and V₃κA27 genes were each used by three mAb. The dominant use of V₂λ is not a feature of the normal repertoire (p = 0.017) and although a bias in the use of particular V genes was
<table>
<thead>
<tr>
<th>Subject</th>
<th>mAb</th>
<th>Isotype</th>
<th>Serotype</th>
<th>$\text{V}_\text{H}$ gene</th>
<th>Homology</th>
<th>D gene</th>
<th>$\text{J}_\text{H}$ gene</th>
<th>CDR3 length</th>
<th>R/S ratio CDR1</th>
<th>R/S ratio CDR2</th>
<th>$\text{V}_\text{L}$ gene</th>
<th>Homology</th>
<th>$\text{J}_\text{L}$ gene</th>
<th>CDR3 length</th>
<th>R/S ratio CDR1</th>
<th>R/S ratio CDR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AbA5</td>
<td>G1</td>
<td>18</td>
<td>V3-48</td>
<td>97</td>
<td>N/A</td>
<td>$\text{J}_\text{H}4/5\text{b}$</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>$\text{V}_\text{e}2\text{A}17$</td>
<td>98</td>
<td>N/A</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>DMF4</td>
<td>A</td>
<td>4</td>
<td>V1-46</td>
<td>93</td>
<td>D5-24</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>$\text{V}_\text{e}3\text{A}27$</td>
<td>93</td>
<td>Jx4</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DM5</td>
<td>G2</td>
<td>6B</td>
<td>V1-3</td>
<td>92</td>
<td>N/A</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>$\text{V}_\text{e}1\text{a}1\text{I}7/10$</td>
<td>94</td>
<td>N/A</td>
<td>14</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>CbG8</td>
<td>A</td>
<td>6B</td>
<td>V3-30</td>
<td>93</td>
<td>D5-5**</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>$\text{V}_\text{e}2\text{A}19/\text{A}3$</td>
<td>97</td>
<td>Jx1</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CbB2</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>95</td>
<td>N/A</td>
<td>$\text{J}_\text{H}6\text{c}$</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>$\text{V}_\text{e}2\text{A}17$</td>
<td>96</td>
<td>Jx3</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CbE2</td>
<td>G2</td>
<td>23</td>
<td>V3-48</td>
<td>96</td>
<td>N/A</td>
<td>$\text{J}_\text{H}2$</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>$\text{V}_\text{x}$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CbH4</td>
<td>M</td>
<td>P*</td>
<td>V3-p1</td>
<td>100</td>
<td>D6-13</td>
<td>$\text{J}_\text{H}6\text{b}$</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>Db8C11</td>
<td>A</td>
<td>4</td>
<td>V3-7</td>
<td>94</td>
<td>N/A</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>$\text{V}_\text{e}2\text{A}17$</td>
<td>95</td>
<td>Jx5**</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Db7D4</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>90</td>
<td>D3-16</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>$\text{V}_\text{e}2\text{A}17$</td>
<td>92</td>
<td>N/A</td>
<td>8</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Db3G9</td>
<td>G2</td>
<td>6B</td>
<td>V3-15</td>
<td>86</td>
<td>D1-26**</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>$\text{V}_\text{e}3\text{A}27$</td>
<td>94</td>
<td>Jx3</td>
<td>12</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>6b1A7</td>
<td>M</td>
<td>P*</td>
<td>V4-39</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;17</td>
<td>0</td>
<td>0</td>
<td>$\text{V}_\text{e}3\text{A}27$</td>
<td>100</td>
<td>Jx5</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6b5B12</td>
<td>M</td>
<td>9V</td>
<td>V1-02</td>
<td>100</td>
<td>D1-7</td>
<td>$\text{J}_\text{H}6\text{b}$</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>$\text{V}_\text{e}2\text{A}19/\text{A}3$</td>
<td>100</td>
<td>Jx1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6b5D7</td>
<td>M</td>
<td>6B</td>
<td>V1-e</td>
<td>100</td>
<td>D6-6**</td>
<td>$\text{J}_\text{H}6\text{b}$</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>$\text{V}_\text{e}1\text{O}12/02$</td>
<td>100</td>
<td>Jx2</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6bC3</td>
<td>A</td>
<td>9V</td>
<td>V3-48</td>
<td>93</td>
<td>D1-1**</td>
<td>$\text{J}_\text{H}3\text{b}$</td>
<td>13</td>
<td>2</td>
<td>9</td>
<td>$\text{V}_\text{e}2\text{A}19/\text{A}3$</td>
<td>99</td>
<td>N/A</td>
<td>7</td>
<td>2.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>6b53</td>
<td>G3</td>
<td>18C</td>
<td>V3-7</td>
<td>93</td>
<td>D6-06</td>
<td>$\text{J}_\text{H}4\text{b}$</td>
<td>14</td>
<td>4</td>
<td>2.5</td>
<td>$\text{V}_\text{e}3\text{B}1+/**$</td>
<td>72</td>
<td>Jx4**</td>
<td>9</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

a) Subjects A–D received the 7V-CRM conjugate vaccine and subject E the 23 VPS vaccine. Isotype, serotype and V(D)J gene alignments and V gene % nucleotide homology to germ line are given for H and L chains using the DNAplot/Vbase programs. CDR3 amino acid length and R/S ratios in CDR1 and 2 are given. * P = polyreactive to all serotypes tested. ** Alignment to germ line poor. N/A: No alignment to germ line.
Fig. 1. Specificity of the 13 serotype-specific mAb as determined by competitive inhibition ELISA with homologous capsular polysaccharides or a combination of six heterologous pneumococcal capsular polysaccharides. Results are expressed as percentage inhibition of binding to the putative capsular polysaccharide target.

suggested, only in the isotype-switched clones was this bias significant for the single gene V_{k}2A17 ($p = 0.023$).

2.3 CDR3 and somatic mutation

The CDR3 characteristics of the hybrids were highly heterogeneous throughout and there was no inverse correlation between CDR3 length and the level of somatic hypermutation. Alignment to germ line was obtained for 8/15 of the D genes and 24/29 of the J genes. Jh4b was used by 7/14 of the H chains for which assignments could be made, however, preferential VDJ joining was not seen and CDR3 length was variable.

Of the IgG and IgA clones, 9/11 demonstrated high levels of somatic mutation and ratios of replacement to silent mutation (R/S ratios) > 3.0 in either CDR1 or CDR2 of H and/or L chain (Table 1). This included the two isotype-switched antibodies from the polysaccharide vaccine recipient. In contrast, all the IgM antibodies aligned 100% to germ line. The two clones with lower R/S ratios both used the V_{3}-48 gene. Db8C11 and Db7D4 which are isotypic variants of the same clone both demonstrated high R/S ratios in CDR1 and 2 and shared common mutations in both framework and CDR regions and identical CDR3 in both H and L chains.

2.4 Canonical classification

V chain canonical loop assignments demonstrated the dominant H1-H2 conformations: 1–3 and 1–2 characteristic of polysaccharide-binding antibodies in 13 of the 15 clones assigned (Table 3). Of those not assigned to this group, one (IgM) had the 3-1 and one (IgG) the 1-U(4) conformation. L1, L2 and L3 assignments were made for all the V_{k} genes sequenced, a database for V_{h} is not available. L chain canonical structure is considered to be less antigen restricted and most antibodies in the circulation have the 4-1-1 conformation. In our sample, L chain canonical loops were more heterogeneous. 4-1-1 was used by nine of the clones. 2-1-1, 5-1-1 and 6-1-1 were used by the remaining five.

3 Discussion

3.1 General remarks

The restricted nature of the human B cell response to bacterial polysaccharide has been the subject of ongoing investigation. It is manifest at several levels including the poor response of young children to polysaccharide antigens, the limited immunogenicity of these antigens at all ages, the absence of immunological memory after encounter with the antigen and the apparently restricted nature of the immune repertoire at a molecular level. Attempts to study diversity at the molecular level have been hampered by the technical difficulties associated with producing stable human heterohybridomas. Thus data on pneumococcus are scarce. We have been successful in generating a number of human heterohybridomas secreting antibodies to a variety of pneumococcal serotypes. The hybrids are all derived from antigen-specific PBMC taken in the day 7 post-immunization window when these cells are found at their peak in the peripheral circulation.

3.2 V gene use

While the numbers of antibodies in our study are relatively small, our data suggest a bias in Ig V gene use in the adult immune response to pneumococcus with an over-representation of V_{h}3-48 and V_{k}2A17. This bias is not serotype specific and is demonstrated by different individuals. Oligoclonality is also suggested in the response of subject D to serotype 4 in which two of the three clones produced from this individual were isogenic variants (IgG and IgA) for the same clone.

The preferential use of specific V genes has been described for a number of antigens including autoantigens in both human [16] and murine responses [17] but of particular relevance is the restriction that has been described in association with Hib capsular polysaccharide, although different specific V genes are used [4]. While the precise mechanism governing such restricted use is not entirely clear, it has been suggested [18] that the observed restriction may be explained by canonical V genes [13, 19].
Table 2. Serum concentration of IgM, IgA and IgG to five pneumococcal serotypes before (pre) and after (post) pneumococcal vaccination.

<table>
<thead>
<tr>
<th>IgM</th>
<th>A pre</th>
<th>A post</th>
<th>B pre</th>
<th>B post</th>
<th>C pre</th>
<th>C post</th>
<th>D pre</th>
<th>D post</th>
<th>E pre</th>
<th>E post</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>0.8</td>
<td>1.1</td>
<td>0.5</td>
<td>30</td>
<td>1.1</td>
</tr>
<tr>
<td>6B</td>
<td>0.5</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>1.4</td>
<td>0.9</td>
<td>7.4</td>
<td>2.5</td>
</tr>
<tr>
<td>9V</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.9</td>
<td>0.9</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>18C</td>
<td>0.7</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.2</td>
<td>1.6</td>
<td>1.6</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>23F</td>
<td>0.7</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.2</td>
<td>1.6</td>
<td>1.6</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 2. Serum concentration of IgM, IgA and IgG to five pneumococcal serotypes before (pre) and after (post) pneumococcal vaccination.

<table>
<thead>
<tr>
<th>IgA</th>
<th>4</th>
<th>&lt;0.5</th>
<th>&lt;0.5</th>
<th>&lt;0.5</th>
<th>0.8</th>
<th>0.2</th>
<th>1.1</th>
<th>&lt;0.5</th>
<th>26</th>
<th>&lt;0.5</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>0.8</td>
<td>&lt;0.5</td>
<td>0.7</td>
<td>&gt;1.5</td>
<td>&gt;1.5</td>
<td></td>
</tr>
<tr>
<td>9V</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>N/A</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td></td>
</tr>
<tr>
<td>18C</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>N/A</td>
<td>0.5</td>
<td>1.1</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23F</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>N/A</td>
<td>0.5</td>
<td>1.1</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IgG</th>
<th>4</th>
<th>0.3</th>
<th>0.6</th>
<th>0.7</th>
<th>8</th>
<th>1.4</th>
<th>17.5</th>
<th>1</th>
<th>30</th>
<th>1.1</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B</td>
<td>0.7</td>
<td>1.1</td>
<td>0.7</td>
<td>5.2</td>
<td>1</td>
<td>19.5</td>
<td>0.7</td>
<td>7</td>
<td>1.1</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>9V</td>
<td>0.5</td>
<td>1.2</td>
<td>0.5</td>
<td>0.4</td>
<td>0.6</td>
<td>2.9</td>
<td>0.6</td>
<td>1.3</td>
<td>1.1</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>18C</td>
<td>0.7</td>
<td>0.9</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
<td>4.5</td>
<td>7.3</td>
<td>19.5</td>
<td>14.5</td>
<td>1.8</td>
<td>7.3</td>
</tr>
<tr>
<td>23F</td>
<td>1.1</td>
<td>1.5</td>
<td>0.3</td>
<td>3.9</td>
<td>1.7</td>
<td>10.5</td>
<td>1.4</td>
<td>9.1</td>
<td>0.6</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

a) Isotypes and serotypes from subjects from whom hybridomas were grown are indicated in bold.

Fig. 2. V<sub>h</sub> gene amino acid alignments for the 15 pneumococcus-specific mAb. S mutations are shown in lower case and R mutations in bold.
Table 3. Canonical structure of 15 human mAb to S. pneumoniae

<table>
<thead>
<tr>
<th>Subject</th>
<th>mAb</th>
<th>Isotype</th>
<th>Serotype</th>
<th>Canonical Vh</th>
<th>Vh clan</th>
<th>Canonical VI</th>
<th>VI clan</th>
<th>Vk clan</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AbA5</td>
<td>G1</td>
<td>18C</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>DMF4</td>
<td>A</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>6-1-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DM5</td>
<td>G2</td>
<td>6B</td>
<td>1-3</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CbG8</td>
<td>A</td>
<td>6B</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CbB2</td>
<td>G2</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CbE2</td>
<td>G2</td>
<td>23F</td>
<td>1-3</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CbH4</td>
<td>M</td>
<td>P</td>
<td>1-3</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Db8C11</td>
<td>A</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>2-1-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Db7D4</td>
<td>G2</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Db3G9</td>
<td>G2</td>
<td>6B</td>
<td>1-1-1</td>
<td>11</td>
<td>6-1-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>6b1A7</td>
<td>M</td>
<td>4</td>
<td>1-3</td>
<td>1</td>
<td>3-1</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6b5B12</td>
<td>M</td>
<td>9V</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6b5D7</td>
<td>G2</td>
<td>6B</td>
<td>1-2</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6bC3</td>
<td>A</td>
<td>9V</td>
<td>1-3</td>
<td>1</td>
<td>4-1-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6b53</td>
<td>G3</td>
<td>18C</td>
<td>1-3</td>
<td>1</td>
<td>5-1-1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>


3.3 Canonical genes

The dominance of specific canonical V genes that are of high affinity and yet remain unmutated has been described for Hib [20] and these genes are considered to be the prototypic genes for optimal antigen binding. They show selective association with specific J genes and homology in the CDR3. In our study, Vh3-48 was used by three different hybridomas derived from three different individuals and each hybridoma recognized a specific canonical structure.
different capsular polysaccharide. However, each mAb used a different D and J gene and CDR3 length was highly variable (Table 1). Thus, while Vj3–48 does not share all the characteristics of canonical genes, it may contribute to an antigen-binding domain that recognizes an epitope common to several pneumococcal polysaccharides. In fact, the majority of antibodies analyzed in this study (13/15) used the H1-H2 conformation 1–2 and 1–3 which is characteristic of murine anti-polysaccharide antibodies [21] and human responses to Hib [22]. The differing serotype specificity exhibited by the three antibodies using Vj3–48 may be attributed to the L chain pairing and CDR3 [2, 4].

3.4 Mutation and memory

Plain polysaccharide vaccines are limited by their poor immunogenicity in the young and by their inability to induce immunological memory in vaccines of all ages. Conjugate vaccines overcome these limitations because the polysaccharide component of the vaccine is rendered a T-dependent antigen by conjugation to a protein. We were thus interested to compare and analyze the nature and extent of the hypermutation in the clones obtained from conjugate and plain polysaccharide vaccines, respectively. The level of random replacement mutation within individual V genes and CDR hot spots is known to vary widely [23] and R/S ratios of > 2.9 can no longer be considered to be indicative of antigen-driven selective somatic hypermutation, a feature of the memory response. However, extrapolating from detailed kinetic studies of murine splenic B cells, the level of somatic hypermutation seen in our hybridomas is inconsistent with their being derived from naive B cells. Even using a high estimation of the germinal center mutation rate (10^5 substitutions/nucleotide/cycle) [24, 25] and a high doubling time (6 h) [26], it would take over 7 days to produce the level of mutation seen in most of our isotype-switched clones had they come from a naive precursor pool. The two highly mutated class-switched clones, progeny of the same precursor and in the peripheral circulation of subject D by day 7 post-vaccination would each have taken a minimum of 9–14 days in the spleen to achieve the level of mutation demonstrated. Only one clone, AbA5, mutating and dividing at a high rate even in murine kinetics, would have achieved the level of mutation it demonstrated by day 7 and thus could possibly be derived from a naive precursor. This suggests that all the subjects had been primed prior to vaccination and the switched clones are derived from the B cell memory pool. In contrast, the IgM clones, all of which demonstrated 100% germ-line gene alignment, are indicative of a new primary response.

As none of the volunteers in this study had been previously vaccinated, immunological memory to the capsular polysaccharide must have been induced by previous encounter with S. pneumoniae or cross-reacting antigen. As capsular polysaccharides are T independent and do not induce memory, it is likely that the original encounter involved presentation of the polysaccharide naturally conjugated to protein or peptide cell wall components. In this context, the naturally conjugated capsular polysaccharides are capable of inducing a T-dependent immune response with the features of somatic hypermutation and the generation of memory. Once immunological memory is established, subsequent challenge with conjugate or plain polysaccharide is able to elicit a memory response. We have analyzed hybridomas derived from day 7 post-vaccination PBMC and in addition to the stimulation of memory cells, serological data suggest that the conjugate vaccine, unlike the plain polysaccharide, will initiate an additional primary response to the vaccine with the generation of new memory cells [27, 28].

Our data are supported by the findings of Hougs et al. [29] who have recently shown that the immune response of an adult to a single dose of Hib conjugate vaccine is dominated by cells derived from a memory pool, with the progeny of a single rearranged B cell accounting for over 50% of the clones. Analysis of the frequency of somatic mutation and the clone size suggested that this population must have been derived from a pre-existing population of B cells which had already been highly selected, mutated and clonally expanded prior to vaccination. The poor response of infants to plain polysaccharides as compared to that of adults may therefore be explained not only by the delayed ontogeny of the B cell response to plain polysaccharides, but also by the relative lack of exposure to naturally conjugated bacterial polysaccharide which results in delayed priming.

In summary, our data suggest that the day 7 post-vaccination PBMC V gene repertoire used in the adult response to pneumococcal polysaccharide and conjugate vaccines may be determined by pre-existing immune memory to wild-type pneumococcus. A bias in the use of Vh and Vk genes is suggested and although the numbers are small, this bias only becomes apparent as the immune response matures and isotype switching occurs. The dominant genes used are canonical genes known from murine studies to be selective for polysaccharide. The effect of somatic mutation within the canonical V genes on the conformation of the antigen binding groove and how this alters the antigen binding affinity must now be determined.

Further studies will be needed to compare the immune repertoire and memory induced by wild-type coloniza-
tion or infection with *S. pneumoniae* with that of naive individuals receiving different pneumococcal conjugate vaccine formulations. In addition, the mechanisms through which wild-type infection with pneumococcus can induce memory awaits evaluation.

### 4 Materials and methods

#### 4.1 Subjects and vaccines

Healthy adult volunteers were recruited and given a single dose of 23V-PS (Pneumovax II™, Pasteur Mérieux, France) containing 25 µg of each serotype, or an experimental lot of 7V-CRM197 containing 2 µg each of 4, 9V, 14, 18C, 19F, 23F and 4 µg of 6B conjugated to a mutant diphtheria toxin (kindly donated by Wyeth Lederle Vaccines and Paediatrics, NY). Blood was taken immediately prior to vaccination and on days 7 and 28 post vaccination. Lymphocytes were isolated by Ficoll density centrifugation from day 7 venesections while sera obtained at each time point were separated and stored at -80 °C.

#### 4.2 Hybridoma production

Day 7 lymphocytes were fused using PEG (PEG 4000, Gibco-BRL, NY) to the non-secreting mouse myeloma line OURI [30] and plated onto a mouse fibroblast feeder layer in 96-well tissue culture plates (Nunc, Life Technologies, Paisley, GB). The cells were cultured at 37 °C in 5% CO₂ for 2–3 weeks and then screened for pneumococcus-specific antibody production. Positive wells were subcloned twice by limiting dilution and the cell lines then expanded in standard tissue culture flasks.

#### 4.3 Antibody titer determination

Serotype-specific pneumococcal antibody titers were determined by ELISA as previously described [31] with some adaptations to make the ELISA suitable for culture supernatant screening. Nunc 96-well maxisorp plates (Life Technologies, Paisley, GB) were coated for 5 h at 37 °C with a heptavalent antigen mix prediluted in PBS comprising pneumococcal capsular polysaccharide serotypes 4, 6B, 19F at 20 µg/ml, 9V, 14 and 23F at 10 µg/ml and 18C at 2 µg/ml (obtained from American Type Tissue Collection, Rockville, MD). Plates were then washed five times with PBS/0.05% Tween (PBS-T). Cell supernatants were diluted by 50% in PBS containing 0.05% Tween (PBS-T). Cell supernatants were diluted by 50% in PBS-T with 1% BSA (BSA PBS-T), and then added directly to the coated plates and incubated for 2 h at room temperature. Plates were then washed five times and the second antibody, horseradish peroxidase (HRP)-conjugated goat anti-human polyvalent IgG (Sigma, Dorset, GB), was added at a 1/1000 dilution in PBS-T. Following a further 2-h incubation at room temperature, wells were washed again five times with PBS-T and substrate added. After 10–40 min at room temperature, 4M H₂SO₄ was added and absorbance at 490 nm was determined.

Positive wells were then rescreened on single-serotype-coated plates to determine serospecificity and isotype was determined using a panel of isotype-specific HRP-conjugated goat anti-human IgG antibodies (Sigma, Dorset, GB).

Serotype-specific IgG, A and M titers in pre- and post-vaccination sera (day 0 and day 28, respectively) were determined for each of the five serotypes for which hybridomas were made using a standard pneumococcal ELISA [31]. A standard serum, 89SF (kindly supplied by Carl Frasch, Center for Biologics Evaluation and Research) was used as the reference serum. All samples were run in duplicate. The second antibody, HRP-conjugated goat anti-human IgG, A or M (Sigma, Dorset, GB), was used at a dilution of 1/1000, 1/500 and 1/1000, respectively. IgG concentrations were calculated using ELISA software (Revelations 2.0; Dynatech, GB) from the standard curve produced by 89SF. Antibody concentration is expressed in µg/ml.

#### 4.4 Specificity

Serospecificity of the mAb was confirmed by inhibition assay. Non-specific binding to CWPS was excluded by pre-incubating supernatant diluted 50% in PBS-T-B with CWPS (Statens Seruminstitut, Copenhagen, Denmark) at a final concentration of 50 µg/ml. Cross-reactivity with other serotypes was evaluated by pre-incubating antibody at a dilution pre-determined to give an OD of 1.0 on ELISA for 30 min with 50 µg/ml (final concentration) of homologous or heterologous capsular polysaccharide and then performing the standard ELISA. Inhibition of binding of >90% by the homologous serotype and <30% by the heterologous serotype was considered to validate specificity.

Cross-reactivity of the polyreactive antibodies to another polysaccharide antigen (Hib) was evaluated by standard Hib ELISA [32].

#### 4.5 V region gene sequencing

mRNA was extracted from the hybridomas with RNAzol (Biogenesis Ltd, Bournemouth, GB) and cDNA made using the First Strand cDNA synthesis kit (Pharmacia Biotech supplied by Promega, Madison, WI) with the pd(N)e primer according to the manufacturer's instructions. cDNA was amplified in duplicate by PCR using a pooled primer mix. The V₄, V₅, and Cμ primer sequences used have been described previously [33, 34]. Cμ primer sequences were as follows: HMCGR: TTTGTTGCGCTTTGCTGTGACGC. HACR: CTCGGGAGGGAGTGTTACATCTT. HGCR: ACGGTGGGACTGTGAGTACTGGTTTGT. The PCR product was separated by agarose gel electrophoresis using ethidium bro-
mide and extracted using either the GeneClean™ protocol (BIO101, Anachem, Luton, GB) or the QIAprep Spin plasmid kit (Qiagen Inc., Crawley, GB) according to the manufacturer's instructions. Samples were then directly sequenced in forward and reverse direction using the ABI™ Prism Big Dye™ Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington, GB) using standard methods as described by the manufacturer. Each clone was sequenced a minimum of four times.

H and L chain sequences were analyzed using the DNASTAR software program (DNASTAR, Madison, WI). Nucleotide sequences were then aligned using DNAPLOT to search VBASE and given the germ-line gene nomenclature as assigned by Tomlinson et al. (http://www.mrc-cpe.cam.ac.uk).

4.6 Statistical analysis

Fisher's exact 2-tailed t-test was used to compare the gene frequency of our sample with the frequency described in normal adult populations of circulating IgM-positive B cells [14, 15]. CD5-negative IgM-positive B cells were used as the comparison group for the H chain genes [15] and IgM-positive B cells were used as the comparison group for the L chain genes [14].

Acknowledgements: We thank Dr. M. Melamed for the OURI cell line, Dr. D. Zhu for the primer sequences and Prof. R. Callard for advice during hybridoma production. H.B. is the recipient of a Wellcome Trust Research Training Fellowship. F.K.S. is supported by Tenovus UK.

References


Immunogenetics of immune response to Pneumococcus


Correspondence: David Goldblatt, Immunobiology Unit, ICH, 30 Guilford St. WC1N 1EH, London, GB
Fax: +44-171 813 8494
e-mail: D.Goldblatt@ich.ucl.ac.uk
Protocol

Preparation of human–mouse heterohybridomas against an immunising antigen

Claire F. Jessup\textsuperscript{a}, Helen Baxendale\textsuperscript{b}, David Goldblatt\textsuperscript{b}, Heddy Zola\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Child Health Research Institute, Women's and Children's Hospital, 72 King William Road, North Adelaide, SA 5006, Australia
\textsuperscript{b}Immunobiology Unit, Institute of Child Health, 30 Guilford St., London WC1N1EH, UK

Received 28 July 2000; accepted 26 August 2000

Abstract

The production of murine monoclonal antibodies against specific antigens by hybridomas is a well utilised technique. The production of hybridomas secreting specific human antibodies would have many advantages in therapeutic applications of monoclonal antibodies. The immortalised human lymphocytes themselves would also provide valuable tools in research on lymphocyte development. Preparation of human–human hybridomas has been limited by a lack of suitable fusion partners. This protocol paper describes the production of human–mouse heterohybridomas by two independent laboratories. The purpose of this protocol is to provide a basis for the development of heterohybridoma technology in laboratories with limited hybridoma experience. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Heterohybridoma; Tetanus toxoid; Pneumococcal polysaccharide

1. Background

Since their original description (Köhler and Milstein, 1975), murine hybridomas have become widely used, valuable research tools. Formed by the 'immortalization' of spleen cells from an immunised mouse, hybridomas secrete antibodies of a single specificity. The application of murine monoclonal antibodies is widespread, in many areas from biological and medical research to clinical diagnostics. Monoclonal antibodies also have potential in human therapeutic applications (reviewed in Scott and Welt (1997), Hudson (1999)). However, their usefulness is limited due to negative side effects including the recipient's immune response to the murine protein (human anti-mouse antibody response; HAMA). There are several ways to avoid or reduce HAMA, including antibody engineering to remove or replace much of the murine Ig sequence (e.g. 'humanised' antibodies (Bendig and Tarran Jones, 1996) and antibody fragments (Pietersz and McKenzie, 1995; Pliickthun and Pack, 1997)). Currently, a large proportion of peptide-based therapeutic agents undergoing clinical evaluation are antibody derivatives (Hudson, 1999).

Abbreviations: EBV, Epstein-Barr virus; FHA, filamentous haemagglutinin; HAMA, human anti-mouse antibody response; HAT, hypoxanthine, aminopterin, thymidine; HFCS, hybridoma colony stimulating factor; HT, hypoxanthine, thymidine; Ig, immunoglobulin; OMP, outer membrane protein; YACs, yeast artificial chromosomes

\textsuperscript{*}Corresponding author. Tel.: +61-8-8204-7443; fax: +61-8-8239-0267.
E-mail address: hzola@medicine.adelaide.edu.au (H. Zola).
Hybridomas are formed by fusing spleen cells from an immunised mouse to a suitable mutant myeloma partner. One commonly used method (for a detailed description see Zola (1987) and Goding (1996)) uses myeloma cells that are deficient in enzymes essential for salvage nucleic acid synthesis pathways. Following polyethylene glycol-induced fusion, unfused spleen cells cannot exist in long-term culture and eventually die. In selective hypoxanthine-, aminopterin- and thymidine-containing (HAT) media, myeloma cells that have not found a spleen cell partner also die due to the presence of aminopterin which blocks normal nucleic acid synthesis. Only myeloma–spleen cell hybrids, which can use hypoxanthine and thymidine for DNA and RNA synthesis via salvage pathways, survive. Following cloning and testing, these hybridomas provide 'immortal' cell lines which stably express monoclonal antibodies.

The use of hybridoma technology to produce human hybridomas would have many advantages. Monoclonal antibodies of human origin would elicit fewer problems in a therapeutic setting. In addition to providing a source of human monoclonal antibodies, 'immortalised' human lymphocytes themselves would provide valuable tools in research on lymphocyte development and genetic analysis. They would allow analysis of cells derived from a single clone, for example an antigen-specific B cell, rather than a heterogeneous population. One way of preparing human monoclonal antibodies is by using transgenic animals carrying human immunoglobulin (Ig) genes. In these experiments, human germline Ig loci are introduced into mice with defective endogenous Ig loci via yeast artificial chromosomes (YACs). The human Ig genes are rearranged and expressed, producing a fully human antibody repertoire (Nicholson et al., 1999). An alternative to using transgenic mice is the production of synthetic repertoires of human antibody fragments using combinatorial phage libraries (Griffiths et al., 1994). Due to the random association of heavy and light chains, this method has an advantage of allowing the production of monoclonal antibodies with unique specificities. However, this random association will not necessarily represent combinations found in vivo and hence will give a distorted view of the immune response.

Another way to produce human monoclonal antibodies is by the transformation of human lymphocytes with Epstein-Barr virus (EBV). However, lymphocytes experience a low rate of infection (Crawford, 1985) and successfully transformed cells tend to show unstable growth and secrete low levels of IgM antibody (Shay, 1985). In addition, the therapeutic use of antibodies derived from EBV-infected cells involves safety concerns (Netzer, 1983).

Alternatively, human hybridomas can be prepared by a method based on the traditional fusion approach, substituting murine spleen cells with human lymphocytes. The choice of fusion partner is of utmost importance when preparing human hybridomas. Since first being described (Olsson and Kaplan, 1980) experiments using human myelomas to make human hybridomas have met with limited success. Production of human–human hybridomas has been largely hindered by a lack of good human fusion partners (Hunter et al., 1982). Human B cell lymphoblastoid cell lines (EBV+) may be used but are characterised by the unstable production of only small amounts of antibody (Shay, 1985). Another approach is to use a human–mouse heteromyeloma as the fusion partner (Leibiger et al., 1995). However, this has not provided clear advantages in hybridoma production. The fusion of human lymphocytes to murine myeloma partners is a simple process with an efficiency higher than human–human fusions but lower than murine–murine fusions. It makes use of the existing bank of murine myeloma cell lines and allows the use of the powerful HAT selection technique. Fusions can easily be performed in laboratories familiar with traditional hybridoma techniques and without the need for specialised equipment. Although chromosomal instability is cited as a major problem associated with human–mouse heterohybrids, extensive subcloning may facilitate the isolation of stably secreting clones.

Many different human tissues including tonsil, lymph node (Thomas et al., 1999), bone marrow (Ezaki et al., 1991), spleen (Hunter et al., 1982) and synovial fluid (Krenn et al., 1999) have been used as sources of human lymphocytes for fusion. However, these are difficult to obtain and require surgery. Often these tissues become available during ad-
vanced disease states, hence lymphocytes may be biased towards certain antigens (which may or may not be desired). The recent immune history of the tissue is largely unknown. Peripheral blood represents a readily available source of human lymphocytes that can be collected by relatively non-invasive techniques. In addition, volunteers may be boosted against specific antigens to examine the elicited response.

This protocol describes the preparation of human–mouse heterohybridomas against an immunising antigen (tetanus toxoid or pneumococcal polysaccharide) by fusing human peripheral lymphocytes with a murine myeloma cell line by polyethylene glycol-mediated fusion. The protocol described is one of two methods used in the laboratories of the co-authors. Key variations, as used in the second laboratory, are described in a series of footnotes.

Both protocols yielded heterohybridomas of the desired specificities. We have not yet trialled a combination of components from both methods.

2. Type of research

1. The production of human monoclonal antibodies for potential therapeutic applications.
2. The immortalisation of human lymphocytes for further genetic and cellular analysis.

3. Time required

- Sample and myeloma cell preparation: 1 h.
- Fusion: 3 h.
- Tissue culture: 8–12 weeks to isolate positive clones.
- ELISA: 5 h (+1 overnight incubation).

Although multiple fusions could be performed throughout a days’ work, feeding, cloning and scaling up of hybrids in the weeks following will cause time constraint problems. Thus, depending on the success of the fusion, only one fusion per operator per week should be attempted.

4. Materials

4.1. Specialist equipment

- Sterile, autoclaved micropipette tips (10 μl, 100 μl, 200 μl and 1000 μl).
- Micropipettors (20 μl, 200 μl and 1000 μl).
- Disposable sterile pipettes (5 ml and 10 ml).
- Pipetting aids.
- Heparinized 10-ml tubes.
- Sterile V-bottomed tubes, screw capped.
- Syringes.
- Mixing cannulae.
- Centrifuge.
- Sterile Pasteur pipettes (glass, plugged).
- Haemocytometer.
- Tissue culture flasks (25 cm², 75 cm²).
- Sterile Petri dishes.
- Sterile 24-well and 96-well flat-bottomed tissue culture plates.
- Waterbath (37°C).
- Polysorp ELISA strips (Nalge Nunc International, Denmark).
- Maxisorp 96-well ELISA plate (Nalge Nunc).
- ELISA plate shaker.
- ELISA plate reader.
- Digital timer.
- Stir concentrator (Amicon, Massachusetts, MA, USA).

4.2. Chemicals and reagents

4.2.1. Vaccines

- Triple antigen infant vaccine (Infanrix™, SmithKline Beecham, London, UK; http://www.sb.com) containing 25 Lf Diphtheria toxoid, 10 Lf Tetanus toxoid, 25 μg Pertussis toxoid, 25 μg Pertussis filamentous haemagglutinin (FHA) and 8 μg Pertussis 69 kDa Outer Membrane Protein (OMP).
• Pneumococcal 23 valent plain polysaccharide vaccine (23 V-PS, Pneumovax II™, Pasteur Mérieux, France) containing 25 mg of each serotype.

• Pneumococcal 7 valent polysaccharide conjugate vaccine containing 2 mg each of 4, 9V, 14, 18C, 19F, 23F and 4 mg of 6B conjugated to a mutant diphtheria toxin (7V-CRM197) (kindly donated by Wyeth Lederle Vaccines and Paediatrics, NY, USA).

4.2.2. Sample and myeloma preparation

• Murine myeloma cell line P3-X63-Ag8.653 (P3653)¹ (Kearney et al., 1979).

• Sterile phosphate buffered saline (PBS).

• LYMPHOPREP™ Ficoll-hypaque separation medium (Nycomed Pharma, Oslo, Norway).

• Trypan blue viability stain (Sigma, St. Louis, MO, USA; http://www.sigma-chem.com.au).

• Mycoplasma PCR-ELISA kit (Roche Diagnostics, Indianapolis, IN, USA; http://www.roche.com).

4.2.3. Fusion

• Freshly prepared RPMI medium (with l-glutamine, 10% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) (Cell Image, North Adelaide, SA, Australia)².

• Polyethylene glycol (PEG 1500³) (Roche Diagnostics).

• HAT concentrate lyophilised (50X) (Sigma).

• HT concentrate lyophilised (50X) (Sigma).

• Hybridoma colony stimulating factor (HFCS)⁴ (Roche Diagnostics).

• 5637 culture supernatant (#HTB-9 human urinary bladder carcinoma; ATCC, Rockville, MD, USA; http://www.atcc.org) — double sterile-filtered (0.2 µm).

• Dimethyl sulfoxide (Sigma).

• Sterile water for injection.

4.2.4. ELISA

• Tetanus toxoid (2155 Lf/ml, 0.027% thiomersal) (CSL Biosciences).

• Coating buffer (15 mM Na2CO3, 30 mM NaHCO3, pH 9.6).

• Tween 20.

• Skim milk powder.

• Pooled human anti-tetanus toxoid immunoglobulin (131 IU/ml) (CSL Biosciences).


• o-Phenylenediamine (OPD) tablets (5 mg) (Sigma).

• Hydrogen peroxide (H2O2) (BDH Chemicals, Poole, Dorset).

• Phosphate citrate buffer tablets (each tablet makes 100 ml of a 0.05 M buffer solution (Sigma).

• Heptavalent antigen mix (pneumococcal capsular polysaccharides (PPS) serotypes 4, 6B, 19F at 20 mg/ml, 9V 14 and 23F at 10 mg/ml and 18°C at 2 mg/ml; ATCC).

• Goat anti-human IgG, goat anti-human IgA and goat anti-human IgM horseradish peroxidase conjugates (Sigma).

4.2.5. Immunocytochemistry

• ExtrAvidin® Peroxidase Staining Kit: Rabbit: Extra-3 (Sigma) containing: biotinylated goat anti-rabbit IgG, Peroxidase-labelled Extravidin.

• Rabbit anti-human IgG, A or M (Sigma).

• Di-aminobenzidine (DAB) (# D-8001; Sigma).

• Swine serum (DAKO, Carpinteria, CA, USA; http://www.DAKO.com).

¹Alternatively, use murine myeloma cell line: P3-X63-Ag8-OURI (termed OURI) (Thompson et al., 1991).

²Medium used by second laboratory (R16 medium) is prepared as follows: 500 ml RPMI 1640 with 25 mM Hepes and l-glutamine, 5 ml of 100X non essential amino acids, 5 ml of 100 mM sodium pyruvate, 5 ml of 200 mM l-glutamine, 10% FCS (mycoplasma free), penicillin (100 IU/ml), streptomycin (100 µg/ml), mercaptoethanol (ME) 10⁻³ M.

³PEG 4000 (GibcoBRL, NY, USA).

⁴Growth factors can instead be provided by a mouse fibroblast feeder layer obtained by peritoneal irradiation with R16, pre-plated onto 96-well plates 2 h prior to fusion at a volume of 100 µl.
5. Detailed procedure

The success of fusion is dependent on many factors. In particular, mycoplasma infection of cell lines must be eliminated. Infection may go largely undetected but can affect fusion efficiency, growth rate and immunoglobulin production. All cell lines were confirmed Mycoplasma-free by the 'Mycoplasma PCR-ELISA Kit'. Myeloma lines were tested to be HAT sensitive by sample culture in HAT-supplemented medium before use. Work must be conducted under sterile conditions, preferably in a Class 2 laminar flow workstation. Unless specified, centrifugation and incubations are conducted at room temperature.

5.1. Sample preparation

(a) Collect 10 ml^ peripheral blood in heparinized tubes from volunteers 7–10 days following immunisation or boosting with tetanus toxoid or pneumococcal polysaccharide or conjugate vaccine.
(b) Dilute blood with 10 ml PBS. To each of two V-bottomed tubes add 8 ml Ficoll-hypaque (allow to warm to room temperature before use). Gently overlay 10 ml blood/PBS mixture per tube using a 30-ml syringe and mixing cannulae. Centrifuge at 800×g for 20 min with no brake.
(c) Using a Pasteur pipette harvest lymphocytes from the interface into a fresh tube, collecting as many lymphocytes as possible without disturbing the red blood cells. Top up the tube containing the lymphocytes with PBS, centrifuge at 200×g for 5 min and discard the supernatant. Resuspend the pellet in 1 ml PBS and perform a viable cell count.

5.2. Myeloma cell preparation

(a) Myeloma cells (P3653 or OURI) should be growing well (viability >90%) in RPMI 1640 and split 1:2 the previous day. Each fusion will require one well-grown 75-cm^2 flask. Resuspend the cells by gently tapping the flask against the benchtop or by using a rubber policeman.
(b) Harvest the cells into V-bottomed tubes and centrifuge (200×g/5 min). Discard the supernatant and resuspend the cells in 1 ml PBS. Perform a viable cell count using Trypan Blue.

5.3. Fusion

(a) The night before the fusion, place two 24-well plates and one Petri dish in the incubator for pre-gassing.
(b) On the day of the fusion, place one 10-ml aliquot and one 3-ml aliquot of RPMI 1640 medium in the waterbath at 37°C. Place PEG vial at 37°C until needed.
(c) Add the appropriate amount of myeloma cell suspension to the lymphocytes to give a 1:1 ratio (from 10 ml of blood this should equal ~1×10^7 cells of each). Mix gently and centrifuge at 200×g for 5 min.
(d) Completely remove supernatant, tapping the rim on a sterile tube to remove the final drop. Flick the tube to loosen the pellet and add 1 ml of warm PEG. Immediately, using a Pasteur pipette, mix the cell pellet with the PEG, continually sucking the suspension up and down for 1 min. Immediately, begin to add the 3-ml aliquot of warm medium dropwise over 10 min with continual mixing. Add the 10-ml aliquot dropwise over 10 min with continual mixing.
(e) Centrifuge at 200×g for 5 min. Discard the supernatant and gently resuspend cells in 20 ml RPMI, passages 20 ml blood.

With continual agitation throughout the procedure, add PEG to the cell mixture drop by drop over 90 s, leave for 10 s, add 5 ml RPMI over 60 s and the residual 20 ml RPMI over the next 3 min. Wash and resuspend in 50 ml of R medium+HAT. Plate out into 96-well plates (precoated with mouse fibroblast feeder layer) at 100 µl per well.
medium. Transfer the suspension to the pre-gassed Petri dish and incubate at 37°C with 5% CO₂ for 2–3 h.

5.4. Tissue culture

(a) Dissolve HAT concentrate by adding 10 ml sterile water to the lyophilised contents and mixing well.
(b) Prepare HAT selective plating medium as follows.
   • 86 ml RPMI 1640 medium.
   • 10 ml 5637 culture supernatant.
   • 2 ml HAT (50× concentrate).
   • 2 ml HFCS.
(c) Harvest cells from the Petri dish with a Pasteur pipette into a V-bottomed tube, using extra medium to ensure all the cells are collected. Centrifuge at 200×g for 5 min.
(d) Discard the supernatant and resuspend the cells in 100 ml HAT selective media. Dispense 2-ml aliquots in two pre-gassed 24-well plates. Incubate cultures at 37°C with 5% CO₂.
(e) After 7 days, prepare HT feeding medium as follows.
   • 44 ml RPMI medium.
   • 5 ml 5637 culture supernatant.
   • 1 ml HT (50× concentrate).
   • 250 μl HFCS.

Remove 1 ml from each well without disturbing colonies and replace with 1 ml HT feeding medium. Continue feeding wells every 7 days.
(f) When medium turns yellow, which takes ~4 weeks, test supernatants by ELISA against tetanus toxoid. Re-test positives and clone positive wells by limiting dilution or by using the single cell sorting function on an automated cell sorter.

(g) Screen clones by ELISA, scale-up and cryopreserve positives (Zola, 1987). Positives should be cloned at least two times to ensure monoclonality and secretor status.

5.5. Anti-tetanus toxoid ELISA

(a) Coat ELISA strips with 100 μl tetanus toxoid (2 LF/ml) in coating buffer per well in a humidity chamber overnight at 4°C. Bash plates on benchtop to empty wells and wash three times with PBS-Tween (PBS+0.05% Tween 20). Strips can be used immediately or covered and stored at −20°C for later use.
(b) Block wells for 1 h with 275 μl skim milk (3% w/v in PBS-Tween, heated to 37°C) per well with shaking.
(c) Empty wells and add 50 μl controls or test supernatants in duplicate. Use pooled human anti-tetanus toxoid immunoglobulin at 0.01 IU/ml diluted in PBS-Tween+0.3% skim milk as positive control. We use a heterohybridoma culture supernatant that is positive for human Ig but negative on tetanus toxoid as a negative control. Incubate at room temperature for 1 h with shaking. Bash plates on benchtop to empty wells and wash five times with PBS-Tween.
(d) Add 50 μl sheep anti-human Ig-HRP (diluted 1:1000 in PBS-Tween+0.3% skim milk) per well and incubate for 1 h with shaking. Empty wells as above and wash five times with PBS-Tween.
(e) Prepare OPD substrate as follows. Dissolve one phosphate citrate buffer tablet in 100 ml sterile water. Filter sterilise (0.2 μm, Millipore) into 10-ml aliquots, which can be stored at room temperature until use. Dissolve one OPD tablet (5 mg) in 10 ml phosphate citrate buffer. Add 4 μl hydrogen peroxide (30%) to OPD solution. Mix and add 100 μl prepared substrate to each well. Incubate for 30 min with shaking.
(f) Read absorbance at 450 nm on an ELISA Plate Reader, with a reference wavelength of 630 nm.

5.6. Anti-pneumococcal polysaccharide ELISA

(a) Pneumococcal polysaccharide ELISA has been previously described (Kayhty et al., 1995). Adapt-
ions to screen hybridoma supernatant are detailed. Coat 96-well Maxisorp plates for 5 h at 37°C with heptavalent antigen mix prediluted in PBS. Wash plates five times with PBS/0.05% Tween (PBS-T).

(b) Dilute cell supernatants by 50% in PBS-T with 1% bovine serum albumin (BSA PBS-T), add directly to the coated plates and incubate for 2 h at room temperature (RT).

(c) Wash plates five times and add goat anti-human IgG-HRP at a 1/2000 dilution in BSA PBS-T.

(d) Following a further 2 h incubation at RT, wash wells five times with PBS-T and add substrate. After 10–40 min at RT, add 4 M H₂SO₄ and read absorbance at 490 nm.

(e) Rescreen positive wells on single serotype coated plates to determine serospecificity and determine isotype using a panel of isotype-specific HRP-conjugated goat anti-human Ig antibodies (use anti-IgG, A or M at a dilution of 1/1000, 1/500 and 1/1000, respectively).

(f) A standard serum, 89SF (kindly supplied by Carl Frasch, Centre for Biologies Evaluation and Research, Bethesda, MD) was used as the reference. Calculate immunoglobulin concentrations using ELISA software (Revelations 2.0; Dynatech, UK) from the standard curve produced by 89SF. Confirm serospecificity of the antibodies by inhibition assay.

- Exclude non-specific binding to cell wall polysaccharide (CWPS) by pre-incubating supernatant diluted 50% in PBS-T-B with CWPS (Statens Seruminstitut, Copenhagen, Denmark) at a final concentration of 50 µg/ml.

- Evaluate cross-reactivity with other serotypes by pre-incubating antibody at a dilution pre-determined to give an OD of 1.0 on ELISA for 30 min with 50 µg/ml (final concentration) of homologous or heterologous capsular polysaccharide and then performing the standard ELISA. Inhibition of binding of >90% by the homologous serotype and <30% by the heterologous serotype is considered to validate specificity.

- Cross reactivity of polyreactive antibodies to another polysaccharide antigen can be evaluated by standard ELISAs e.g. Haemophilus influenzae B (Phipps et al., 1990).

5.7. Immunocytochemistry

(a) Once a clonal population of target antigen specific heterohybrids is established in tissue culture flasks, assess producer status. Make a cytoslide spin of hybrid cells, fix in acetone for 1 h, air dry.

(b) Stain for isotype specific human Ig antibodies by incubation in 100 µl of rabbit anti-human IgG, A or M, prediluted in swine serum for 1 h. Wash in PBS.

(c) Incubate with a biotinylated anti-rabbit antibody for 30 min. Wash in PBS.

(d) Incubate in peroxidase labelled extravidin antisera for 1 h.

(e) Develop peroxidase activity by incubating slide in DAB solution (1 ml of 5% di-amino-benzidine in di-methylformamide made up to 100 ml with PBS plus 100 µl of 30% H₂O₂) for 10 min. Wash in PBS.

(f) Counterstain with Mayer's haematoxylin for 30 s. Wash in tap water for 2 min. Dehydrate (70% ethanol to 100% ethanol to xylene).

(g) Mount using standard mounting medium. With a light microscope, count percentage cells staining brown for antibody production. If more than 90% of cells are stained, subcloning has been adequate, if <90% are stained, further subcloning is required.

5.8. Scaling up of heterohybridoma cultures and purification of antibody

(a) Expand clonal cell populations from 75-cm² to 150-cm² tissue culture flasks.

(b) Cryopreserve aliquots of 10⁷ cells in liquid nitrogen (10% DMSO).

(c) To develop monoclonal antibody stocks, grow cells to confluence in 150-cm² flasks. Turn flasks upright and top up media every 3 days until the flasks are full.

(d) Leave flasks to stand for 2 weeks in 5% CO₂ at 37°C.

(e) Collect supernatants into 50-cm² V-bottomed
tubes and centrifuge at 200×g for 5 min to separate cell debris. (f) Concentrate antibody using a 200-ml Amicon stir concentrator with a 50 kDa filter and purify by affinity chromatography on a protein-G Superose column. (g) Dialyse purified samples in PBS overnight. Determine antibody titre using the standard ELISA as described above and store samples at −80°C for further use.

6. Results

6.1. Production of anti-tetanus toxoid heterohybridomas

From 11 fusions prepared in the first laboratory using 1–10 ml blood from donors immunised against tetanus toxoid, hybridoma growth appeared in an average of 54% of wells after 3 weeks in 24-well plates (Table 1). Two fusions prepared using 2 ml blood from infants resulted in hybrid growth in 100% of wells. One fusion using 10 ml adult blood resulted in growth in only five of 48 wells. One fusion using the murine myeloma partner SP2/0 resulted in no viable heterohybridoma colonies, hence subsequent fusions were prepared using P3653 as the fusion partner. Most wells that tested positive for anti-TT Ig by ELISA were cloned once and cryopreserved immediately. Of 18 clones that were scaled up, at least two stopped secreting specific antibody after 3 months in continual culture. Samples used for fusion were taken from patients from day 7 to day 60 post-boost. One fusion, using infant blood at day 45 post-boost produced the highest number of positive wells (13/48). Clones from one of these wells produced antibody that was cross reactive with PRP polysaccharide by ELISA.

6.2. Production of anti-pneumococcal heterohybridomas

From five fusions prepared in the second laboratory using 50 ml blood from adult donors boosted against pneumococcal polysaccharide, 15 stable heterohybridoma clones secreting specific antibody were produced (Table 2). Secretion continued in excess of 6 months continual culture by clones produced from pneumococcal conjugate vaccine recipients (subjects A–D) but was lost over that period in 4/5 clones produced from the pneumococcal plain polysaccharide vaccine. Producing status of greater than 90% of a clone producing IgG to pneumococcal serotype 18C is demonstrated by immunocytochemistry in Fig. 1.

6.3. Growth characteristics of heterohybridomas

Growth of heterohybridoma colonies was variable and slower than murine hybridoma colonies prepared in the same laboratory, with some remaining small

Table 1
Success of heterohybridoma fusions using lymphocytes from donors immunised against tetanus toxoid

<table>
<thead>
<tr>
<th>Label</th>
<th>Source</th>
<th>Vol. of blood</th>
<th>Fusion partner</th>
<th>Ratio (myeloma:PBL)</th>
<th>Day post boost</th>
<th>Growth Human Ig</th>
<th>Anti-TT Ig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>Tonsil</td>
<td></td>
<td>p3653</td>
<td>1:2</td>
<td>–</td>
<td>13/24</td>
<td>6/10</td>
</tr>
<tr>
<td>MF21</td>
<td>Adult</td>
<td>10 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d14</td>
<td>14/48</td>
<td>8/14</td>
</tr>
<tr>
<td>MF21b</td>
<td>Adult</td>
<td>10 ml</td>
<td>SP2/0</td>
<td>1:1</td>
<td>d14</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>CW21</td>
<td>Adult</td>
<td>10 ml</td>
<td>p3653</td>
<td>1:10</td>
<td>d21</td>
<td>19/48</td>
<td>8/25</td>
</tr>
<tr>
<td>SJd14</td>
<td>Adult</td>
<td>10 ml</td>
<td>p3653</td>
<td>1:10</td>
<td>d14</td>
<td>5/48</td>
<td>1/5</td>
</tr>
<tr>
<td>JD7.5 mo</td>
<td>Infant</td>
<td>2 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d45</td>
<td>48/48</td>
<td>13/48</td>
</tr>
<tr>
<td>KK6.5 mo</td>
<td>Infant</td>
<td>2 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d14</td>
<td>48/48</td>
<td>3/48</td>
</tr>
<tr>
<td>MS7mo</td>
<td>Infant</td>
<td>1 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d30</td>
<td>34/48</td>
<td>8/34</td>
</tr>
<tr>
<td>BG6mo</td>
<td>Infant</td>
<td>1 ml</td>
<td>p3653</td>
<td>2:1</td>
<td>d60</td>
<td>9/12</td>
<td>3/9</td>
</tr>
<tr>
<td>VC7mo</td>
<td>Infant</td>
<td>1 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d30</td>
<td>11/24</td>
<td>1/11</td>
</tr>
<tr>
<td>JM7mo*</td>
<td>Infant</td>
<td>1 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d30</td>
<td>6/24</td>
<td>–</td>
</tr>
<tr>
<td>XGd8</td>
<td>Adult</td>
<td>5 ml</td>
<td>p3653</td>
<td>1:1</td>
<td>d8</td>
<td>11/24</td>
<td>0/11</td>
</tr>
</tbody>
</table>

* No HFCS added to media.
Table 2
Genotypic analysis of anti-pneumococcal antibody-producing heterohybridoma clones

<table>
<thead>
<tr>
<th>Subject</th>
<th>Mab</th>
<th>Isotype</th>
<th>Serotype</th>
<th>V\textsubscript{H} gene</th>
<th>D gene</th>
<th>J\textsubscript{I} gene</th>
<th>V\textsubscript{L} gene</th>
<th>J\textsubscript{I} gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AbA5</td>
<td>G1</td>
<td>18</td>
<td>V3-48</td>
<td>N/A</td>
<td>J\textsubscript{H}4/5b</td>
<td>Vk2A17</td>
<td>N/A</td>
</tr>
<tr>
<td>B</td>
<td>DMF4</td>
<td>A</td>
<td>4</td>
<td>V1-46</td>
<td>D5-24</td>
<td>J\textsubscript{H}4b</td>
<td>Vk3A27</td>
<td>Jk4</td>
</tr>
<tr>
<td></td>
<td>DM5</td>
<td>G2</td>
<td>6B</td>
<td>V1-3</td>
<td>N/A</td>
<td>J\textsubscript{H}4b</td>
<td>VI\textsubscript{11v17/1b}</td>
<td>N/A</td>
</tr>
<tr>
<td>C</td>
<td>CbG8</td>
<td>A</td>
<td>6B</td>
<td>V3-30</td>
<td>D5-5\textsuperscript{a}</td>
<td>J\textsubscript{H}4b</td>
<td>Vk2A19/A3</td>
<td>Jk1</td>
</tr>
<tr>
<td></td>
<td>CbB2</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>N/A</td>
<td>J\textsubscript{H}6c</td>
<td>Vk2A17</td>
<td>Jk3</td>
</tr>
<tr>
<td></td>
<td>CbE2</td>
<td>G2</td>
<td>23</td>
<td>V3-48</td>
<td>N/A</td>
<td>J\textsubscript{K}2</td>
<td>NK</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CbH4</td>
<td>M</td>
<td>P\textsuperscript{a}</td>
<td>V3-p1</td>
<td>D6-13</td>
<td>J\textsubscript{H}6b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>Db8C11</td>
<td>A</td>
<td>4</td>
<td>V3-7</td>
<td>N/A</td>
<td>J\textsubscript{H}4b</td>
<td>Vk2A17</td>
<td>Jk5\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>Db7D4</td>
<td>G2</td>
<td>4</td>
<td>V3-7</td>
<td>D3-16</td>
<td>J\textsubscript{H}4b</td>
<td>Vk2A17</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Db3G9</td>
<td>G2</td>
<td>6B</td>
<td>V3-15</td>
<td>D1-26\textsuperscript{a}</td>
<td>J\textsubscript{H}4b</td>
<td>Vk3A27</td>
<td>Jk3</td>
</tr>
<tr>
<td>E</td>
<td>6h1A7</td>
<td>M</td>
<td>9V</td>
<td>V4-39</td>
<td>N/A</td>
<td>N/A</td>
<td>Vk3A27</td>
<td>Jk5</td>
</tr>
<tr>
<td></td>
<td>6h5B12</td>
<td>M</td>
<td>9V</td>
<td>V1-02</td>
<td>D1-7</td>
<td>J\textsubscript{H}6b</td>
<td>Vk2A19/A3</td>
<td>Jk1</td>
</tr>
<tr>
<td></td>
<td>6h5D7</td>
<td>M</td>
<td>6B</td>
<td>V1-c</td>
<td>D6-6\textsuperscript{b}</td>
<td>J\textsubscript{H}6b</td>
<td>V1012/02</td>
<td>Jk2</td>
</tr>
<tr>
<td></td>
<td>6hC3</td>
<td>A</td>
<td>9V</td>
<td>V3-48</td>
<td>D1-1\textsuperscript{b}</td>
<td>J\textsubscript{H}3b</td>
<td>Vk2A19/A3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>6h53</td>
<td>G3</td>
<td>18C</td>
<td>V3-7</td>
<td>D6-06</td>
<td>J\textsubscript{H}4b</td>
<td>V3B1 + \textsuperscript{c}</td>
<td>Jk4</td>
</tr>
</tbody>
</table>

\* P, polyreactive with all pneumococcal serotypes tested.
\textsuperscript{a} Alignment to germline poor.

even at 4 weeks. Growth rates were improved when both HFCS and 5637 supernatant were present in the medium, and the presence of these factors appeared to be particularly important during cloning stages. However, the second laboratory found a mouse fibroblast feeder layer provided sufficient support for heterohybridoma production. The cloning efficiency of the heterohybridomas was less than that of murine hybridomas by both limiting dilution and automated cell sorting and varied greatly (0–72% growth in wells plated with single cells, median 33%). Microscopically, heterohybrid cells appeared larger and more irregular than murine hybridomas prepared in the same laboratory. During early stages, even cells within the same colony appeared to have different shapes. Analysis of the DNA content in two heterohybridoma fusion wells by staining with propidium iodide (Darzykiewicz and Juan, 1997) demonstrated that hybrids contained ~2 times as much DNA as the parental P3653 murine myeloma (Fig. 2). Whereas P3653 showed sharp peaks relating to DNA levels in cells at $G_1 (1 \times)$ and $G_2/M (2 \times)$ phases, the peaks of heterohybrids were broader, indicating variable amounts of DNA (ranging from $2 \times$ to $4 \times$) at each phase. By flow cytometry, human CD19 and CD45-RO were not detectable on heterohybridomas tested. Human IL-6R was detected on at least one clone, while surface murine immunoglobulin was not detected on any heterohybridomas.

Fig. 1. Detection of IgG-producing cells of an anti-pneumococcal (serotype 18C) heterohybridoma clone by immunohistochemistry.
In six wells containing uncloned hybrids, some or all of the cells stained positive for human CD44 (Fig. 3).

7. Cautions and caveats

HFCS is a commercial medium supplement designed to replace feeder cells in murine hybridoma preparation. However, we found that HFCS contains human immunoglobulins. Hence, if wells are to be tested for human Ig production, supernatants should be replaced with fresh medium without HFCS 24 h prior to testing.

Mycoplasma infection can go undetected in murine cell lines, with no obvious impact on myeloma proliferation rate or the success of pure murine hybridoma production. However, human–mouse heterohybridoma cell lines are less robust and it is imperative that fusion partners are confirmed mycoplasm negative by DNA PCR and/or growth in mycoplasma selective media (Uphoff et al., 1992). Mycoplasma growth may be suppressed by standard tissue culture antibiotics so prior to supernatant sampling, cell lines must be grown in antibiotic-free media for three passages and then in continuous culture for 72 h. A number of mycoplasma treatment regimes have been described (Uphoff et al., 1992) in which a variety of antibiotics are used in rotation. These methods are time consuming and even the most effective regimes do not assure 100% mycoplasm elimination. It is therefore strongly advised that if the fusion partner is contaminated, that cell line is discarded and a clean cell line obtained.

Due to the lower cloning efficiency of heterohybridomas, it is suggested that the target number of cells in each well be increased during cloning (e.g. instead of aiming for 1, 3 and 5 cells per well, aim for 1, 10 and 50).

OPD is a suspected carcinogen so care must be taken when handling tablets and the resulting solution. Wear gloves and glasses when pipetting the substrate.

8. Discussion

Human–mouse heterohybridomas were successfully produced against tetanus toxoid protein and pneumococcal polysaccharide using both of the methods detailed above. The original aim of the authors was to prepare heterohybridomas against immunising antigens in order to analyse gene usage and the presence of mutations in human antigen-specific B cells. These techniques allowed detailed genetic analysis of 15 anti-pneumococcal antibody-producing clones of which two (Db8C11 and
Db7D4) were isotype switched subclones of the same precursor (Table 2; Baxendale et al., 2000). The temporal course in acquisition of diversity by somatic hypermutation and isotype class switching may be assessed if isotypic variants of the same clone are obtained and supernatant analysis facilitates correlation of immunoglobulin gene use and somatic mutation with antibody function. Heterohybrids provide advantages over other approaches, such as single-cell RT-PCR of antigen-specific cells, by providing a large clonal population of genetically identical cells derived from a single antigen-specific precursor. Such information is more valuable than correlates drawn from analyses of heterogenous populations. Another advantage of the heterohybridoma approach is that supernatants from a single fusion may be screened against many antigens. This may be especially valuable when analysing blood from infants in early life (following multiple vaccinations) or when a combination vaccine is given (e.g. DTP).

Despite numerous differences between the two approaches, each technique was successful in producing heterohybridomas against the specific, intended antigen. One difference was the amount of starting material used, which was determined by the nature of the immunising antigen. The success rate in heterohybridoma production will vary depending on the nature of the immunising antigen and the immune history of the vaccine recipient. Tetanus toxoid, a protein antigen, is a potent immunogen and elicits a primary response in infancy and a strong secondary immune response in the healthy adult. Pneumococcal polysaccharide is a weak immunogen, is not immunogenic in infancy and does not induce memory. Adults may however have been primed through wild type infection via the nasal mucosa. Heilmann et al. (1987) have performed detailed kinetic analysis of the immune response to pneumococcal polysaccharide. The dominant circulating isotype is IgA. Pneumococcal IgG producing B cells peak in the circulation on day 7–8 post vaccination. The absolute numbers of pneumococcal specific IgG producing B cells varies 5–6-fold depending on the pneumococcal serotype and is in the region of 600/10^6 mononuclear cells (0.06%). Tetanus toxoid IgG producing B cells at their peak constitute 0.3% of circulating B cells (Oshiba and Gelfand, 1996). Factors such as these will clearly affect the time selected to bleed post vaccination, the fusion success rate and the dominant isotype in hybrids produced. Prior to heterohybridoma production for a new immunising antigen a detailed kinetic analysis of the response must be made to improve the chance of success. Obviously, human subjects cannot be immunised without ethical cause. This limits the scope of our approach to antigens currently used for immunisation or where lymphocytes can be obtained from patients with known disease. For many purposes, including our own, these are the antigens of interest. However, for other applications, methods such as in vitro immunisation of human lymphocytes hold potential in circumventing this problem (Kawahara et al., 1992).

Due to the low level of circulating antigen-specific cells, even in volunteers boosted with protein antigens such as tetanus toxoid, one approach to improve success is to stimulate the lymphocytes in vitro prior to fusion. This can be done using antigen (e.g. tetanus toxoid (Butler et al., 1983)) or polyclonal mitogens (e.g. pokeweed mitogen (Teng et al., 1983) or LPS (Yoshinari et al., 1996)), but in each case may direct specificities away from the initial in vivo response. Originally, HB's plan was to transform human lymphocytes with EBV prior to fusion to increase the frequency of fusion. However, EBV infection of B cells depends on the expression of CD21 which is lost by proliferating or terminally differentiated cells. Thus, as was observed in our experiments, EBV preferentially transforms mature, resting B cells over activated or plasma cells. Hence, although in vitro stimulation prior to fusion may increase cell numbers or fusion efficiency, it may bias fusion events away from the desired cells (antigen-specific antibody secreting B cells).

There was a marked increase in fusion efficiency when using blood from infants to prepare heterohybridomas. It seems either the cells themselves possess a fusion or growth advantage or the factors they secrete may assist formation and proliferation of otherwise unstable heterohybrids. Certainly, there is evidence that immature lymphocytes may provide important factors. Westerwoudt et al. (1983) cite human cord serum as having a strong growth-promoting activity on hybridomas while the use of human cord blood lymphocytes as feeder cells for human hybridomas has also been reported (Kurpisz et al., 1987).
The choice of fusion partner was another difference. P3-X63-Ag8.653 (P3653) myeloma is a non-secreting HAT-sensitive murine line derived from the IgG1-producing P3-X63-Ag8 (Kearney et al., 1979). It has a high fusion efficiency and is widely used to prepare murine hybridomas. OURI is also derived from P3-X63-Ag8 and was named for its high resistance to ouabain (OUabain ResIstant). Oubain is toxic to EBV-transformed human B-cells (EBVTB) and was used to selectively kill unfused EBVTBs in the production of heterohybridomas during preliminary fusion experiments. Although ouabain resistance was not necessary with direct B cell–myeloma fusions, the line was still used in the second laboratory, as it remains an exceptionally good fusion partner. There are some suggestions that PEG of a higher molecular weight (e.g. 4000) may result in a lower toxicity to the cells during fusion (Shirahata et al., 1998), while others suggest this variable is of minor importance (Goding, 1996). Our results indicate that variables such as the type of PEG used, the myeloma:lymphocyte ratio and the exact fusion procedure do not significantly affect hybridoma yield. Despite these findings, systematic exploration of variables such as PEG concentration (30–50%), addition of DMSO to the fusion mix, pH or temperature may result in increases in heterohybridoma fusion efficiency. Clearly, high fusion efficiencies are of utmost importance when aiming to fuse cells present at such low frequencies.

An emerging technique to dramatically increase the efficiency and reproducibility of fusion is electrofusion, where cells are fused by tightly controlled electronic pulses (Foung et al., 1990; Yoshinari et al., 1995; Zimmerman et al., 1990). This technique claims high fusion efficiencies and can be used with fewer starting cells. In addition, methods analysing the DNA content in fusing cells or dual-colour labelling would allow immediate assessment of the fusion process. In combination, these techniques could be used to determine conditions required for optimal fusion of human and murine cells. The use of 96-well plates instead of the traditional 24-well plates could be beneficial when trying to isolate specific heterohybridomas. Although it makes for more work in screening, the chance of secretors being overgrown by non-secretors is decreased. Since the ratio of secreting and non-secreting clones may be much lower with heterohybridomas than murine hybridomas, this is an especially important point.

As found by other investigators (Butler et al., 1983; Lane et al., 1982) growth rates and cloning efficiencies of the heterohybrids were low when compared to murine hybridomas. The addition of growth factors to the medium improved growth rates and cloning efficiency of both murine and human-mouse hybridomas in this laboratory. Although HFCS is designed with murine hybridomas in mind, its inclusion also supported heterohybridomas, presumably due to the presence of human IL-6 (which also works on murine cells). In the second laboratory, heterohybrids were successfully maintained by using a mouse fibroblast feeder layer alone. However, research to find more specialised and potent growth factors is warranted. Even a slight increase in growth rate would greatly shorten the amount of time required from fusion to isolation of final clone, considering the multiple cloning and expansion steps required. The addition of supernatant from a human tumour line (5637) further assisted growth of heterohybridomas, perhaps by providing further factors of human origin. Similarly, Zhu et al. (1993) found the use of conditioned media from various human fibroblast and lymphoblastoid lines improved heterohybridoma growth, cloning efficiency and antibody secretion.

One problem is the chromosomal instability experienced by heterohybridomas which may have been reflected by the irregular morphology shown within heterohybridoma colonies. Heterohybrids preferentially lose human chromosomes (Kozbor and Croce, 1985) hence the human Ig genes may be quickly lost in culture. This accords with our observation and others (Schlom et al., 1980) that in at least some clones less and less antibody was detected in culture over time. However, this chromosomal loss is not random and chromosomes 14 and 22 (containing heavy and λ chains, respectively) tend to be retained while chromosome 2 (containing the κ chain) is lost more easily (Croce et al., 1980; Erikson et al., 1981). This would explain the majority of reports where antibodies produced nearly exclusively contain the λ chain (Krenn et al., 1999). There have, however, been numerous reports of heterohybrids producing antibodies (Lane et al., 1982; Schlam et
al., 1980) containing the $\kappa$ chain. In our experiments and others (Butler et al., 1983; Thielmans et al., 1984) extensive subcloning and immunohistochemical analysis facilitated the isolation of heterohybridomas stably secreting high levels of Ig. Yoshinari et al. (1995) investigated a range of cell division inhibitors, anti-cancer drugs and agents which lengthen cell doubling times and found that ouabain and cytochalasin B stimulated and prolonged the production of $\kappa$ chain antibody-producing heterohybrids. While such agents may improve chromosomal stability, their inclusion may further hamper the already slow growth of heterohybridomas. Perhaps the answer is a complex heterohybridoma media supplement containing human cytokines, growth factors (including those derived from infant B cells) and selective agents designed to facilitate the retention of human chromosomes.

Although numerous reports exist outlining the immortalisation of human lymphocytes, most use PBL stimulated prior to fusion (Butler et al., 1983) or cells from malignant disease states (e.g. multiple myeloma (Thielmans et al., 1984)). We have reported the successful production of antigen-specific human–mouse heterohybridomas from unstimulated peripheral blood lymphocytes, and used these hybrids to analyse human Ig gene sequences. In agreement with other researchers (Sikora and Wright, 1981) human–mouse heterohybridomas were formed with an efficiency less than that of murine–murine hybridomas, but presumably greater than that of human–human hybridomas which tend to form with very low frequency (Kozbor and Croce, 1985). Once formed, human–human hybridomas are relatively stable but notoriously secrete low levels of antibody (Thielmans et al., 1984). Human–mouse heterohybridomas may initially secrete higher levels of immunoglobulin, but face distinct problems due to loss of human chromosomes. However, their ease of preparation and increased fusion efficiency made them ideal for our purposes. With future improvement of human fusion partners and development of optimal fusion conditions human–human hybridomas may become a viable option for the long term production of human Ig for therapeutics. Until that time, human–mouse heterohybridoma production represents a simple, effective alternative and a valuable technique for sampling the immune response to provide further genetic and phenotypic analysis.

9. Quick procedures

9.1. Sample collection

(a) Collect blood.
(b) Dilute 1:1 with PBS. Separate lymphocytes over Ficoll at 800 $\times$ g for 20 min.
(c) Harvest lymphocytes and wash with PBS. Perform cell count.

9.2. Myeloma preparation

(a) Resuspend cultured cells.
(b) Harvest flask(s) at 200 $\times$ g for 5 min and resuspend in PBS. Perform cell count.

9.3. Fusion

(a) Pregas Petri dish and plates in incubator overnight.
(b) Prewarm 3-ml and 10-ml aliquots of medium and PEG vial to 37°C.
(c) Mix myeloma cells and lymphocytes (1:1) and pellet.
(d) Add 1 ml PEG to pellet, mixing for 1 min. Add 3 ml medium dropwise over 10 min. Add 10 ml medium dropwise over 10 min. Pellet cells.
(e) Resuspend cells in 20 ml medium, incubate in Petri dish at 37°C for 2–3 h.

9.4. Tissue culture

(a) Dissolve lyophilised HAT concentrate.
(b) Prepare HAT selective medium.
(c) Harvest cells from Petri dish and pellet.
(d) Plate cells in 2-ml aliquots in HAT medium in 24-well plates. Incubate at 37°C in 5% CO$_2$.
(e) After 7 days, feed wells with 1 ml HT medium. Repeat every 7 days.
(f) Screen yellow supernatants by ELISA. Retest and clone positives.
(g) Scale up and cryopreserve positive clones.
9.5. Anti-tetanus toxoid ELISA

(a) Coat wells with 100 μl tetanus toxoid (2 Lf/ml) overnight at 4°C. Wash three times with PBS-T.
(b) Block with 275 μl skim milk (3%) for 1 h with shaking.
(c) Add 50 μl heterohybridoma supernatant per well in duplicate for 1 h with shaking. Wash five times with PBS-T.
(d) Add 50 μl anti-human Ig-HRP (1:1000 in PBS-T+0.03% skim milk) per well for 1 h with shaking. Wash five times with PBS-T.
(e) Prepare OPD substrate and add 100 μl per well for 30 min with shaking.
(f) Read absorbance at 450 nm.

9.6. Anti-pneumococcal polysaccharide ELISA

(a) Coat wells with heptavalent antigen mix in PBS for 5 h at 37°C. Wash five times with PBS-T.
(b) Dilute heterohybridoma supernatants (1:1) in BSA PBS-T and add to wells for 2 h at room temperature.
(c) Wash five times with PBS-T. Add anti-human IgG-HRP (1:2000 in BSA PBS-T) for 2 h at room temperature.
(d) Wash five times with PBS-T and add substrate for 10–40 min at room temperature. Add 4 M H₂SO₄ and read absorbance at 490 nm.

9.7. Immunocytochemistry

(a) Prepare a cytopsin of heterohybridoma cells. Fix in acetone for 1 h.
(b) Stain with 100 μl rabbit anti-human IgG, A or M in swine serum for 1 h. Wash in PBS.
(c) Incubate with biotinylated anti-rabbit antibody for 30 min. Wash in PBS.
(d) Incubate with peroxidase labelled extravidin antiserum for 1 h.
(e) Incubate with DAB solution for 10 min. Wash in PBS.
(f) Counterstain with haematoxylin for 30 s. Wash in tap water for 2 min. Dehydrate (70% ethanol to 100% ethanol to xylene).
(g) Mount and observe by light microscopy.

9.8. Scaling up of cultures and purification of antibody

(a) Expand clones to 150-cm² tissue culture flasks.
(b) Once confluence is reached, cryopreserve aliquots.
(c) Turn flasks of confluent cells upright and add media every 3 days until full.
(d) Leave flasks in incubator for 2 weeks.
(e) Collect supernatants and pellet cellular debris.
(f) Concentrate antibody in stir concentrator (50 kDa filter) and purify on a protein-G Superose column.
(g) Dialyse against PBS overnight and store at −80°C.

10. Essential literature references

Baxendale et al. (2000).
Kayhty et al. (1995).
Phipps et al. (1990).

References


