Antigen Specific B Cells in the Immune Response to *Haemophilus influenzae* type b PRP Conjugate Vaccine

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SUMMARY

Haemophilus influenzae is a pathogenic gram negative bacterium, which exists in encapsulated and non encapsulated forms. The serotype b of the encapsulated form can cause invasive infections such as epiglottitis, meningitis, septic arthritis, and septicemia in humans.

The protective immune response to *Haemophilus influenzae* type b (Hib) results from antibodies developed against the polyribosylribitol phosphate (PRP) capsular polysaccharide of the bacterium. Polysaccharide antigens are less immunogenic than protein antigens. In early childhood the immune response to polysaccharides is even more restricted compared to adults, due to the immaturity of the immune system. Children under two years are more susceptible to invasive Hib infections, as they are unable to mount an adequate antibody response against the PRP polysaccharide. To prevent invasive Hib infections in early childhood, infants in most developed countries are immunised against Hib with PRP conjugate vaccines as part of the routine immunisation schedule. Effective Hib vaccines have been developed by conjugating PRP with a carrier protein to elicit a strong response which has some characteristics of T-dependent antigen responses.

However, the details of the development of the antibody response to Hib conjugate vaccines are not fully understood. The aim of this study was to develop potential methods to identify, enumerate and characterise PRP specific B cells in young children and in adults following immunisation with Hib conjugate vaccines and compare with antigen specific B cell responding to a pure protein antigen, tetanus toxoid (TT).

Immunofluorescence staining followed by flow cytometry was performed on peripheral blood lymphocytes (PBL) from Hib conjugate vaccine and TT immunised adults. PRP binding specific B cells could not be detected in PBL of immunised adults by immunofluorescence staining and flow cytometry, although TT binding specific B cells were identified, suggesting that PRP binding specific B cells may be present in very small numbers in peripheral blood or may be relatively confined to lymphoid tissues.

As an alternative immunofluorescence staining procedure, murine anti-PRP monoclonal antibodies were developed for use in indirect immunofluorescence assays in an attempt to detect PRP specific B cells by flow cytometry. The anti-PRP monoclonal antibodies were of IgM class and had functional activity against Hib. However, the monoclonal antibodies proved not to be useful for isolating PRP binding specific B cells due to binding to lymphocytes through non PRP molecules.

To test the hypothesis that PRP specific B cells may be relatively confined to secondary lymphoid tissue, flow cytometry and enzyme linked immuno spot (ELISPOT) assays were developed and performed on PRP-T immunised (PRP conjugated to tetanus toxoid) mouse spleen and peripheral blood cells. Significant numbers of anti-PRP and anti-TT antibody secreting cells were identified by ELISPOT assays in spleen cells of mice after booster immunisation. The percentage of anti-TT antibody secreting cells identified was nearly twice the percentage of PRP antibody secreting cells. Very few anti-TT antibody secreting cells were identified in peripheral blood of immunised mice and the number of anti-PRP antibody secreting cells was extremely low in peripheral blood samples from these mice. In contrast to the ELISPOT results, PRP and TT binding specific B cells were not able to be identified by flow cytometry using spleen cells from immunised and boosted mice.

ELISPOT assays were performed on tonsil cells of children less than four years of age, who were immunised with Hib conjugate vaccines and TT vaccines as part of routine immunisation schedules, to identify anti-PRP antibody secreting cells. Anti-PRP and anti-TT antibody secreting cells were identified by ELISPOT assays in tonsil cells. The percentage of TT antibody secreting tonsil cells was two and a half times greater than that of PRP antibody secreting tonsil cells.

These results demonstrate that compared to TT specific B cells, PRP specific B cells are either not present in peripheral blood or circulate in very low numbers after immunisation with Hib conjugate vaccine. Furthermore, the generation of anti-PRP antibody secreting cells may be low in comparison to the generation of anti-TT antibody secreting cells, and the cells that are present may be relatively restricted to lymphoid tissues. In conjunction with previous studies, these findings further support the concept that Hib conjugate vaccines does not ensure a full TD response in spite of being coupled to a carrier protein. Since isolation of PRP antigen specific B cells using the techniques has proven to be a challenging task an alternative approach is proposed for characterization of B cells involved in antibody response to Hib conjugate vaccines.

DECLARATION

I declare that this thesis does not incorporate without acknowledgement any material previously accepted or submitted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Kodituwakku, A. P., Flesch, I. E. A, Zola, H. & Roberton, D. M. Divergence in the generation of antibody secreting cells to polysaccharide and carrier protein molecules of Hib conjugate vaccine (manuscript in preparation).

Development of antibody response to *Haemophilus influenzae* type b PRP conjugate vaccine in young children. Australasian Society for Immunology (ASI) Annual Scientific Meeting, Adelaide, 2002.

* see appendix

Abbreviations and standard units

Ag:	Antigen
ASC:	Antibody secreting cell
BCR:	B cell receptor
Bi:	Biotin
BSA:	Bovine serum albumin
DNP:	Dinitrophenyl
DT:	Diphtheria toxoid
ELISA:	Enzyme linked immunosorbant assay
ELISPOT:	Enzyme linked immunospot
FCS:	Fetal calf serum
FGG:	Fowl IgG
FITC:	Fluorescein isothiocyanate
FLU:	Fluorescein
HAS:	Human serum albumin
HEL:	Hen egg white lysozyme
HGG:	Human gammaglobulin
Hib:	*Haemophilus Influenzae* type b
HPH:	Helix pomatia haemocyanin
HRBC:	Horse red blood cells
Ig:	Immunoglobulin
IL:	Interleukin
IFN:	Interferon
KLH:	Keyhole limpet haemocyanin
MACS:	Magnetic activated cell sorting
MFI:	Mean fluorescence intensity
MNC:	Mononuclear cell
NK:	Natural killer
NP:	Nitriphenyl acetyl
OA:	Ova albumin
OD:	Optical density
PALS:	Periarteriolar lymphatic sheath

PBL:	Peripheral blood lymphocytes
PBMC:	Peripheral blood mononuclear cells
PE:	Phycoerythrin
PE-Cy5:	Cychrome
PLA_2:	Phospholipase A2
PRP:	Polyribosylribitol phosphate
RNP:	Ribonucleoprotein
RF:	Rheumatoid factor
SAPE:	Streptavidin-PE
SD:	Standard deviation
SEM:	Standard error of the mean
SLE:	Systemic lupus erythematosus
SN:	Supernatant
SRBC:	Sheep red blood cells
TCR:	T cell receptor
TD:	Thymus-dependent
TGG:	Turkey gamma globulin
TI:	Thymus-independent
TNP:	Trinitrophenyl
TT:	Tetanus toxoid

ml:	Millilitre
µg:	Microgram
µl:	Microlitre
g:	Gram
IU:	International unit
L:	Litre
Lf:	Limits of flocculation
M:	Molar

CHAPTER 1

INTRODUCTION

1.1 Biology of *Haemophilus influenzae* type b

Haemophilus influenzae is a pathogenic gram negative bacterium associated with human disease (Turk, 1984). It exists in encapsulated and non encapsulated forms. Invasive *H. influenzae* infection is often caused by encapsulated strains of the bacterium. The encapsulated strains are surrounded by a high molecular weight negatively charged polysaccharide capsule consisting of repeating units of polyribosylribitol phosphate (PRP) (Fig. 1.1) (Crisel *et al.*, 1975). According to the antigenic specificity of the polysaccharide capsule, the encapsulated strains are further subdivided into six serotypes, designated a to f (Pittman, 1931). The capsular serotype b strain of the organism is responsible for more than 95% of all invasive *H. influenzae* infections (Mason *et al.*, 1982).

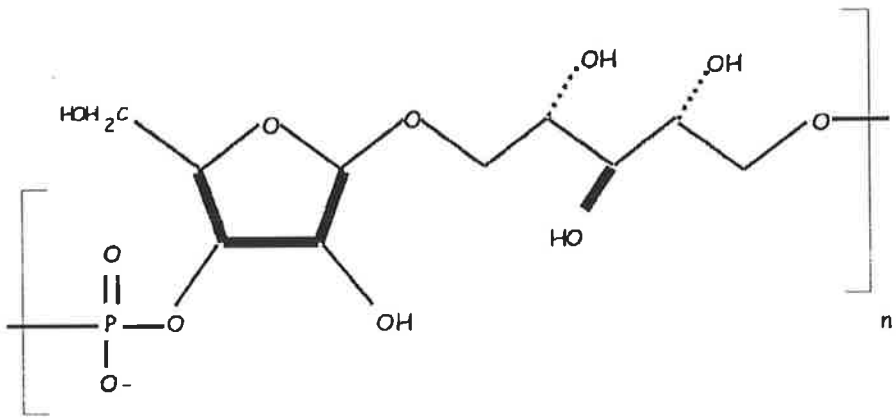


Fig. 1.1 Structure of a *Haemophilus influenzae* type b capsular PRP unit; 3-β-D-ribose (1→1) ribitol-5-phosphate (Lindberg, 1999).

1.2 Pathogenicity and clinical manifestations of invasive *Haemophilus influenzae* type b disease

Haemophilus influenzae type b (Hib) is transmitted from person to person either by direct contact or by inhalation of droplets of respiratory tract secretions containing the organism (Turk, 1984; Murphy *et al.*, 1989). Once the bacteria come in to contact with the upper respiratory tract, they may either be eradicated by host defense mechanisms or they may colonise the mucosa. The colonising bacteria may persist without manifesting any symptoms (Turk, 1984). The bacteria adhere to epithelial cells of nasopharynx by fimbriae and undergo in situ multiplication (Weber *et al.*, 1991; van Ham *et al.*, 1994). However, on occasions, the bacteria may enter the blood stream by penetrating the upper respiratory mucosal epithelium and then disseminate to distant body sites such as the meninges of the brain, the lungs, the joints or the soft tissues (Turk, 1984). Alternatively, the colonisation can spread within the respiratory tract.

In the general population, the nasopharyngeal colonisation rate for Hib is usually up to about 5% (Michaels *et al.*, 1976; Ward *et al.*, 1978; Howard *et al.*, 1988), but may reach higher percentages among contacts of patients with invasive Hib disease (Ward *et al.*, 1978; Shapiro and Wald, 1980; Barbour *et al.*, 1995) and high risk groups such as day care attendees (Murphy *et al.*, 1993; Stephenson *et al.*, 1985), health care workers (Shapiro and Wald, 1980) or certain ethnic populations (Bijlmer *et al.*, 1989; Gratten *et al.*, 1994).

Meningitis is the most common serious clinical manifestation of invasive Hib infection (Kayhty *et al.*, 1984). It can result in residual neurological damage including deafness, mental retardation, seizure disorders or paralysis in up to 45% of

survivors (Cochi *et al.*, 1985). The mortality rate of Hib meningitis, in young children, ranges from about 2% to 15% (MacDonald *et al.*, 1984).

Other important clinical manifestations of invasive Hib infection include bacteraemic pneumonia, epiglottitis, septicaemia, cellulitis and osteoarticular infections such as septic arthritis and osteomyelitis (Fig1.2) (Dajani *et al.*, 1979; Kayhty *et al.*, 1984).

Usually, invasive Hib infections occur in children less than 5 years of age (Kayhty *et al.*, 1984; Cochi *et al.*, 1985; Kristensen *et al.*, 1990). The majority of infections present during the first two years of life, with meningitis and epiglottitis being the two most severe Hib infections, resulting in serious morbidity and death (Gilbert, 1987; Clements *et al.*, 1993). Meningitis occurs earlier than epiglottitis, with a peak incidence around the first year of life (Takala *et al.*, 1989; Bijlmer, 1991).

Invasive Hib disease is rare in adults, except in some individuals with underlying chronic illnesses or immunosuppression (Takala *et al.*, 1990; Farley *et al.*, 1992).

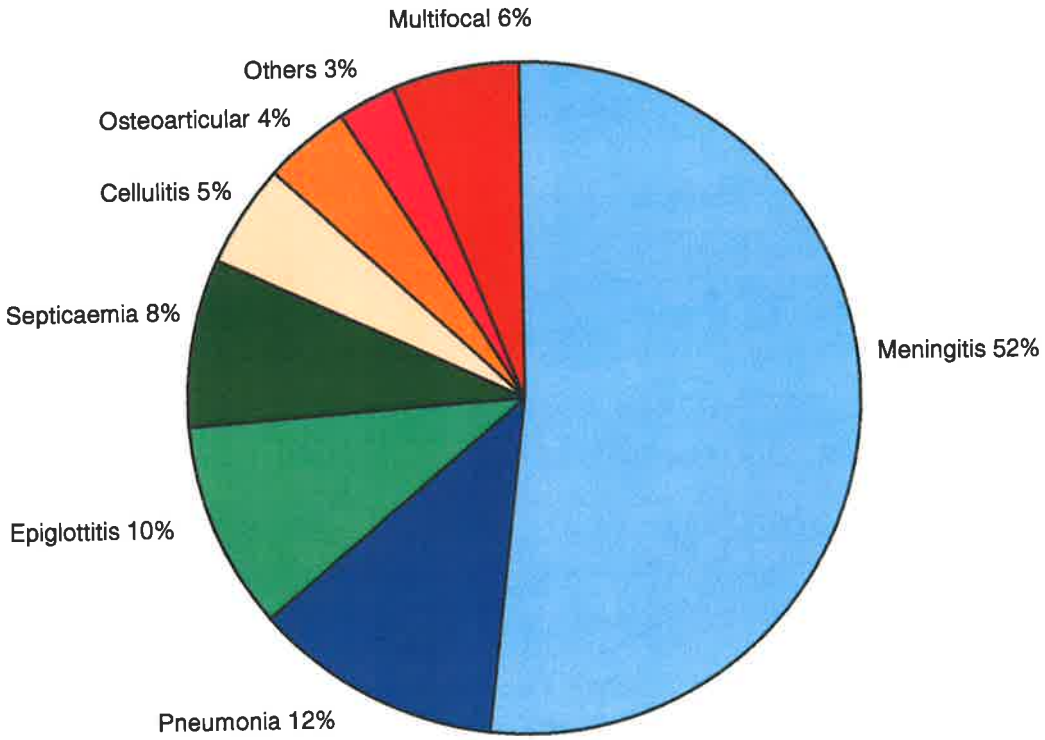


Fig. 1.2 Worldwide spectrum of invasive Hib disease. Data from Peltola, 2000.

1.3 Epidemiology of invasive Hib disease

The epidemiology of invasive Hib disease varies considerably among countries. In the European countries, Hib infection is frequently seen during early summer and autumn (Takala *et al.*, 1989), whereas in Australia it peaks during autumn and winter (Gilbert *et al.*, 1990). The incidence of invasive Hib infection has been well described in the United States and in most of the European countries.

Before the development of efficacious Hib vaccines, invasive Hib disease was the most common serious bacterial infection among young children in most developed countries (Gilbert *et al.*, 1990). In the United States, the annual incidence of invasive Hib disease was approximately 100 cases per 100,000 children less than 5 years old (Cochi *et al.*, 1985). Very high case rates have been found in certain native populations in the US, such as White Mountain Apache Indians and Alaskan Eskimos (Losonsky *et al.*, 1984; Ward *et al.*, 1986). In the United Kingdom (Tudor-Williams *et al.*, 1989) and in Scandinavian countries (Claesson, 1993), the corresponding figures were about 34 per 100,000 and 50 to 65 per 100,000 respectively. Following a thorough analysis of data, Peltola has estimated that the overall worldwide incidence of invasive Hib disease was around 70 per 100,000 per year in children below 5 years of age (Peltola, 2000). Approximately 20% of these patients died, mainly of Hib meningitis. The annual incidence of invasive Hib disease in adults has been estimated at less than 1 per 100,000 (Takala *et al.*, 1990; Farley *et al.*, 1992).

Invasive Hib infection has been an important disease in Australia especially among Australian Aboriginal children (Hanna, 1990). Prior to the introduction of effective routine Hib immunization in 1993, the annual incidence of Hib infection among Australian non Aboriginal children was 53 per 100,000 under five years of age, whereas the figure was 460 per 100,000 for Aboriginal children, showing a marked susceptibility of indigenous Australian children to invasive Hib infection (Harris *et al.*, 1994). The incidence of invasive Hib infection among Central Australian Aboriginal children was nearly two fold higher than that of other Aboriginal children (Hanna, 1990). Furthermore, the onset of Hib disease in Aboriginal children was at a much younger age with a median at 6 months, compared to non Aboriginal children (Hanna, 1990). More than 80% of Hib infections in Aboriginal children occurred

before 18 months of age, whereas only about 50% of non Aboriginal cases are seen in this age group.

In Australia, Hib meningitis accounted for about 40-50% of all invasive Hib disease with a peak incidence in the second year of life, and was responsible for nearly 70% of cases of childhood bacterial meningitis (Hanna and Wild, 1991; McGregor *et al.*, 1992; Harris *et al.*, 1994). The annual incidence of Hib meningitis was five fold higher in Aboriginal children than in non Aboriginal children with a peak incidence around 5 months of age. The overall case fatality rate of Hib meningitis was approximately 5% (Harris *et al.*, 1994). Up to 15% of survivors developed permanent neurological damage.

Hib epiglotittis was more common in Australian children than has been reported in other developed countries, with a case rate of around 30-40% (McGregor *et al.*, 1992; Harris *et al.*, 1994). It occurred later than Hib meningitis and was rare among Australian Aboriginal children (Gilbert, 1991; Harris *et al.*, 1994). All other clinical manifestations of invasive Hib disease among Australian children amounted to about 20% (McGregor *et al.*, 1992).

Although it has been suggested that invasive Hib disease is not common in Asia (Lau *et al.*, 1995; Wang and Lin, 1996; Yang *et al.*, 1996), recent surveys have shown a significant incidence in some Asian countries, similar to that in Europe (Salisbury, 1998; Limcangco *et al.*, 2000; Levine and Wenger, 2002).

1.4 Immune response to *Hib*

Immunity to invasive Hib infection is strongly associated with the antibody response to capsular polysaccharide (Schneerson *et al.*, 1971; Anderson *et al.*, 1972). Capsular polysaccharide antibodies are directed against the repeated linear epitopes of PRP units (Pillai *et al.*, 1991).

Newborn infants are protected from invasive Hib disease by transplacental acquisition of maternal anti-PRP capsular polysaccharide antibodies during pregnancy and through breast feeding (Amstey *et al.*, 1985). The antibody concentration gradually declines during the first three months after birth. Until about 18 months of age infants mount poor antibody responses to the PRP capsular polysaccharide of Hib bacteria (Smith *et al.*, 1973; Peltola *et al.*, 1977a; Amstey *et al.*, 1985). The reasons for this inability of infants in contrast to older children and adults are still poorly understood.

Protective anti-PRP antibody levels are usually achieved by five years of age without any evidence of documented infection (Robbins *et al.*, 1973). These antibodies to PRP polysaccharide are believed to be induced by upper respiratory tract colonisation with Hib and probably through exposure to cross reactive bacteria such as apathogenic *Escherichia coli* K100 in the gut (Schneerson and Robbins, 1975; Insel and Anderson, 1982; Ulanova *et al.*, 1996).

Immune protection from invasive Hib disease is achieved by opsonic and bactericidal activities. Hib bacteria can activate both the alternative and classical pathways of the complement system of the host (Anderson *et al.*, 1972; Quinn *et al.*, 1977). In the presence of activated complement components, anti-PRP antibodies promote phagocytosis of Hib by polymorphonuclear cells through opsonisation as well as

inducing complement mediated killing of the bacteria (Steele *et al.*, 1984; Musher *et al.*, 1986; Amir *et al.*, 1990b).

1.5 Polysaccharide antigens

Polysaccharides are important antigenic determinants of several clinically significant human pathogenic bacteria including Hib. They make up the capsules of *Haemophilus influenzae* type b, *Streptococcus pneumoniae,* and *Neisseria meningitidis* (Barrett, 1985).

These polysaccharide antigens appear not to be processed and re-expressed with major histocompatibility complex (MHC) class II molecules by antigen specific B cells, and therefore the antigens are not recognised by T helper cells (Harding *et al.*, 1991; Ishioka *et al.*, 1992). As a result, the antibody response to polysaccharides is elicited in the absence of MHC restricted specific T cell regulation. Therefore, polysaccharides are classified as thymus-independent antigens (TI).

On the other hand, antibody responses to protein antigens involve specific cooperation between B and T cells. Protein antigens are processed inside antigen specific B cells and the peptide fragments are transported back to the B cell surface in association with MHC class II molecules, thus allowing the antigen specific B cells to interact with T helper cells and activating them (Lanzavecchia and Bove, 1985; Sarobe *et al.*, 1991; Marsh *et al.*, 1992). The activated T cells stimulate the B cells through direct cognate interactions, CD40-CD40 ligand contact and by releasing T cell derived cytokines (Parker, 1993; Kawabe *et al.*, 1994). This led to the classification of protein antigens as thymus-dependent (TD) antigens.

Polysaccharide antigens are further defined as type 2 TI antigens as they fail to elicit antibody responses in CBA/N mice that carry the X-linked immune B cell defect (*xid*) (Table 1.1) (Amsbaugh *et al.*, 1972; Mosier *et al.*, 1976). In contrast, TI type 1 antigens such as bacterial cell wall lipopolysaccharides initiate responses in *xid* mice.

Table 1.1

Some examples of T cell independent and dependent antigens (Stein, 1992; Bondada *et al.*, 2000).

T cell independent	
Type 1	**Type 2**
Bacterial cell wall components	Bacterial capsular polysaccharides
Brucella abortus	Dextran
Lipopolysaccharides	Polyvinyl pyrrolidone
Dextran sulfate	Haptenated ficoll
T cell dependent	
Bacterial proteins such as toxoids,	
Viruses, Parasites	
Proteins such as albumin, globulin, haemocyanin	
Sheep erythrocytes	

1.6 Antibody response to polysaccharide antigens

Polysaccharide antigens elicit a TI antibody response that exhibits several characteristic features compared to the TD response of protein antigens (Table 1.2). Anti-polysaccharide antibodies develop late in ontogeny (Gold *et al.*, 1977; Peltola *et al.*, 1977a), are isotype restricted (Insel *et al.*, 1985) and are of lower affinity with no affinity maturation (Cowan *et al.*, 1978; Barrett and Ayoub, 1986). Furthermore, there is no effective immunological memory development and the secondary immune response resembles the primary response (Kayhty *et al.*, 1984). In contrast, antibody responses to protein antigens are present at birth, have no such isotype restriction, result in the formation of memory cells and undergo affinity maturation during subsequent exposures to the antigens (Stein *et al.*, 1983; Manser, 1990).

Table1.2

Comparison of antibody responses to thymus dependent and independent antigens (Stein, 1992).

Feature	Thymus-dependent	Thymus-independent
Ontogeny	Present at birth	Type 1, early Type 2, 3-18 months after birth
Affinity	High: maturation with repeated exposure	Low: no maturation
Memory	Yes	Little or no
Class, subclass	Heterogeneous	Usually restricted

1.6.1 Late ontogeny

The most important feature of the antibody response to polysaccharide antigens compared to proteins in terms of bacterial infections in infancy is the poor responsiveness during early childhood. The anti-polysaccharide response develops late in ontogeny, and children until about 2 years of age are generally unable to produce antibodies to polysaccharide antigens (Rosen *et al.*, 1983; Kayhty *et al.*, 1984; Leinonen *et al.*, 1986). A fully mature responsiveness to polysaccharide antigens is acquired by 5 years of age. As a result, infants and young children are highly susceptible to infections with encapsulated bacteria (Gray and Dillon, 1986).

In the mouse, the ability to respond to polysaccharide antigens depends on the presence of a late appearing Lyb5+ B cell subset of B2 lymphocytes (Smith *et al.*, 1985; Barrett and Ayoub, 1986; Mond *et al.*, 1995; Chernishova *et al.*, 1998). However in humans the precise equivalent subset of B cells is not yet identified. Therefore, whether the delayed responsiveness of polysaccharides in humans is related to a late appearing B cell subpopulation is not known.

1.6.2 Isotype restriction

IgM, IgG and IgA are the major classes of antibodies in all humoral immune responses. In the antibody response to TD antigens, IgG is the dominant class of antibodies and the IgG to IgM ratio is further increased during the secondary response (Stevens *et al.*, 1983; Barrett, 1985; Granstrom *et al.*, 1988). IgA antibodies are produced in small amounts (Granstrom *et al.*, 1988; Barington *et al.*, 1994). In contrast, the response to polysaccharides produces both IgG and IgM classes with a greater proportion of IgM antibodies and with very little change in the ratio during

subsequent antigen challenge (Shyamala *et al.*, 1988; Lortan *et al.*, 1993). IgA isotype is also produced in small to moderate quantities (Shyamala *et al.*, 1988; Lortan *et al.*, 1993; Carson *et al.*, 1995).

IgG1 antibodies are the predominant IgG subclass antibodies generated in response to protein antigens (Slack *et al.*, 1980; Stevens *et al.*, 1983; Lagergard *et al.*, 1992). IgG4 is produced in moderate amounts, while IgG2 and IgG3 antibodies are expressed in smaller quantities (Slack *et al.*, 1980; Seppala *et al.*, 1988).

The subclass composition of IgG antibodies to polysaccharides is mainly restricted to IgG2 (Hammarstrom *et al.*, 1985; Barrett and Ayoub, 1986; Shackelford *et al.*, 1988; Lagergard *et al.*, 1992). IgG1, IgG3 and IgG4 antibodies are produced in lesser quantities with IgG4 being the least. Expression of the IgG2 subclass in children has a positive correlation with age in antibody responses and reaches adult levels around 10 years of age (Morell *et al.*, 1972; Shackelford *et al.*, 1985). In children, IgG2 antibodies are produced in lesser or in similar proportions to IgG1, in response to polysaccharide antigens (Kayhty *et al.*, 1988; Shackelford *et al.*, 1988).

IgA antibodies to polysaccharides include both IgA1 and IgA2 subclasses in substantial proportions (Grimfors *et al.*, 1989; Ladjeva *et al.*, 1989; Carson *et al.*, 1995) whereas in response to protein antigens IgA antibodies are predominantly of the IgA1 subclass (Ladjeva *et al.*, 1989; Barington *et al.*, 1994; Engstrom *et al.*, 1995).

1.6.3 Low affinity

Antibody affinity indicates the relative binding strength between an antibody binding site and a complementary antigenic determinant. The functional affinity or avidity is the collective average strength of the binding interactions between a population of antibodies and a complex antigen (Griswold, 1987). The avidity of anti-bacterial antibodies is of considerable importance in the immunity to bacterial infections as they positively correlate with complement mediated bactericidal activity (Amir *et al.*, 1990a; Delvig *et al.*, 1994; Granoff *et al.*, 1998).

The affinities of antibodies against polysaccharide antigens are very much lower than those of antibodies to protein antigens (Schalch *et al.*, 1979; Stein *et al.*, 1982; Persson *et al.*, 1988; Konradsen, 1995). Furthermore, there is little or no evidence of affinity maturation in secondary antibody responses to polysaccharides. The absence of affinity maturation is attributed to limited somatic hypermutations of the variable region of the immunoglobulin gene (Stein, 1992). The secondary responses to protein antigens are accompanied by affinity maturation resulting in the generation of antibodies with very much higher affinities than those of the primary response by a combination of somatic hypermutaion of germ line genes and an antigen driven selective expansion of high affinity antibody secreting B cell clones (Stein *et al.*, 1982; Brown *et al.*, 1984; Zhang *et al.*, 1988; Junker and Tilley, 1994).

1.6.4 Lack of effective memory response

Memory B cells mount a rapid and an enhanced secondary antibody response during subsequent antigen exposure. Thymus-independent immune responses to polysaccharide antigens are not associated with effective memory cell development

rechallenge with the polysaccharide is not increased or is increased only to a small degree compared to the primary response. In contrast, TD responses are accompanied by effective memory cell development resulting in secondary antibody responses of higher magnitude and affinity (Stein *et al.*, 1982).

1.6.5 Initiation of anti-polysaccharide response

Polysaccharides consist of multiple repeating antigenic epitopes that can bind to several surface antigen receptors of a specific B cell (Feldmann and Easten, 1971). The extensive cross linking of the antigen receptors provides the initial signal in B cell activation in the absence of specific T cell help (Dintzis *et al.*, 1983). A second activation signal is generated by co-ligation of complement receptor 2 (CD21) on the B cells by polysaccharide-C3d complexes, formed by complement activation via the alternative pathway (Griffioen *et al.*, 1991). These signals induce B cells to proliferate and differentiate into antibody secreting plasma cells.

1.6.6 Marginal zone B cell involvement

Marginal zone B cells in the outer region of the periarteriolar lymphatic sheath (PALS) region of the spleen are mainly associated with antibody responses to polysaccharide antigens (Amlot *et al.*, 1985; Timens *et al.*, 1989). These cells are located in the slow flowing sinusoidal system allowing close contact with blood borne antigens (Kraal, 1992). They have a characteristic phenotype of IgM^{high}, $IgD^{low/-}$, $CD21^{high}$, $CD23^{low/-}$ (Gray *et al.*, 1982; Gray *et al.*, 1984; Oliver *et al.*, 1997). In contrast, recirculating follicular B cells are IgM ^{low}, IgD ^{high}, CD21 ^{int}, CD23 ^{int}

(Oliver *et al.*, 1997; Loder *et al.*, 1999). These B cells are involved in the antibody response to protein antigens.

Marginal zone macrophages present polysaccharide antigens to marginal zone B cells (Humphrey, 1985). Polysaccharide antigens bind to surface antigen receptors of specific B cells. Highly expressed CD21 molecules on the B cells facilitate the binding of polysaccharide antigens opsonised by complement fragment C3d to mount a rapid antibody response (Peset Llopis *et al.*, 1996). This rapid response partially compensates for the lack of high affinity B cells in the antibody response to polysaccharide antigens.

The strategic location of the marginal zone B cells and their unique activation and differentiation potential allows them to respond more rapidly than other B cells in the spleen, particularly to polysaccharide antigens (Oliver *et al.*, 1999). However, murine studies have shown that recirculating follicular and germinal centre B cells can differentiate into the marginal zone B cell phenotype and participate efficiently in the anti-polysaccharide antibody response (Dammers *et al.*, 1999; Vinuesa *et al.*, 2003).

The ability to produce fully competent antibody responses to polysaccharide antigens is acquired gradually during childhood (Kayhty *et al.*, 1984). Some capsular polysaccharides and their various serotypes generate significant antibody responses earlier than others. Antigens such as group A meningococcal polysaccharide and serotype 3 pneumoccocal polysaccharide elicit significant antibody responses in infants as early as 6 months of age (Peltola *et al.*, 1977b; Cowan *et al.*, 1978), whereas the PRP polysaccharide antigen is not immunogenic until about 18 months (Peltola *et al.*, 1984). Furthermore, the response to serotype 19 pneumococcal polysaccharide is poor in children under the age of 5 years (Douglas *et al.*, 1983).

This delayed development of antibody responses to polysaccharides could be due to the late appearance of antigen specific marginal zone B cells to different antigens at different time points. Studies have shown that the phenotypic expression and functional capacity of some marginal zone B cells are similar to those of the B1 subpopulation of B cells (Fagarasan and Honjo, 2000; Martin *et al.*, 2001). This suggests that marginal zone B cells may arise from B1 cells. Furthermore, animal studies have demonstrated that B1 cells can mount adaptive immune responses to some TI-2 antigens such as NP-Ficoll (Tanigaki *et al.*, 2002). However, the absence of marginal zone B cells in these animals increased their susceptibility to infections with blood borne encapsulated bacteria. These findings reflect the limited ability of B1 cells to mount antibody responses to T1-2 antigens and emphasize the importance of marginal zone B cells in responses to such antigens. This argument is further supported by the unresponsiveness of infants to polysaccharides despite having a large population of B1 cells during early childhood (Bhat *et al.*, 1992).

The rapid responsiveness of splenic marginal zone B cells to polysaccharides is unique to these cells (Oliver *et al.*, 1999). Following activation by polysaccharide antigens, marginal zone B cells proliferate and form foci of antibody secreting plasma cells (Liu, 1997). However, a small number of proliferating B cells may migrate to follicular regions and differentiate to plasma cells without undergoing a germinal centre reaction.

The B cell response to protein antigens is mainly initiated in T cell zones in the PALS of the spleen and equivalent T cell zones in other secondary lymphoid organs, with specific interactions between B and T cells (Liu *et al.*, 1991; Kelsoe and Zheng, 1993). Splenic marginal zone B cells also respond to protein antigens and after

encountering the antigen, these B cells migrate to T cell zones and obtain specific T cell cooperation for activation (MacLennan and Liu, 1991). Some of the activated B cells in the PALS produce short lived plasma cells secreting the first burst of IgM antibodies of a primary protein antibody response (Liu *et al.*, 1991; McHeyzer-Williams *et al.*, 1992). Others migrate to follicular regions and initiate the germinal centre reaction resulting in further proliferation and differentiation into either memory cells or antibody secreting plasmablasts. Plasmablasts transform into long lived plasma cells outside the germinal centre (MacLennan and Liu, 1991). B cell clones activated by protein antigens persist for several months in contrast to the B cell clones activated by polysaccharides (Lane *et al.*, 1986).

1.6.7 Germinal centre formation

Germinal centre formation is a characteristic feature of the antibody response to TD antigens (Kraal *et al.*, 1986; Liu *et al.*, 1991). All important events of the TD response such as clonal selection, affinity maturation, class switching, memory cell development and plasmablast generation occur inside the germinal centre (Kraal *et al.*, 1982; Kroese *et al.*, 1990; Berek and Ziegner, 1993). Germinal centre B cells have a characteristic differentiating feature from other B cell populations by binding with peanut agglutinin (PNA) (Rose *et al.*, 1980).

TI antigens do not usually elicit germinal centre formation in their antibody responses. However, there have been reports that bacterial polysaccharide $\alpha(1\rightarrow6)$ dextran and other TI antigens such as NP-Ficoll induce minimal germinal centre formation with non-specific T cell help (Wang *et al.*, 1994; Sverremark and Fernandez, 1998a; Toellner *et al.*, 2002). It has been suggested that a distinct subset

of B cells is involved in the germinal centre responses to such antigens (Billian *et al.*, 1996). However, the T cells present in such germinal centres do not provide full costimulatory signals to antigen specific B cells for effective class switching, affinity maturation and memory development compared to the germinal centre responses to TD antigens. The reasons for germinal centre formation following such TI antigen challenge and their functions in the response to these antigens are not yet known.

1.6.8 T cell and accessory cell involvement

Although polysaccharides are classified as TI antigens, studies have shown that factors such as interleukin (IL)-2, IL-4, IL-5, IL-6 and interferon gamma (INFγ), derived from T cells are required to elicit an anti- polysaccharide antibody response (Murray *et al.*, 1985; Ambrosino *et al.*, 1990; Pecanha *et al.*, 1991; Macedo-Soares *et al.*, 1996). However, it is clear that classical cognate MHC class II restricted pathway is not involved in this stimulatory activity (Cosgrove *et al.*, 1991). Furthermore, the anti-polysaccharide antibody response also requires costimulation from accessory cells such as macrophages, dendritic cells and natural killer cells (Snapper and Mond, 1996; Buchanan *et al.*, 1998; Chelvarajan *et al.*, 1998).

1.6.9 Unresponsiveness to polysaccharide antigens in early childhood

Several hypotheses can be postulated for the unresponsiveness of polysaccharides in early childhood. Delayed maturation of infant B cells has been suggested as the foremost reason for this unresponsiveness (Schelonka and Infante, 1998; Muthukkumar *et al.*, 2000).

A functional spleen with a marginal zone containing mature B cells is essential in mounting anti-polysaccharide responses (Timens *et al.*, 1989; Peset Llopis *et al.*, 1996). All cellular components in the human infant spleen mature to an adult immuno-phenotype and morphology by about 5 months of age except for marginal zone B cells (Timens *et al.*, 1989). Infant marginal zone B cells have a strong coexpression of surface IgM and IgD antigen receptors in contrast to IgM^{high} and $IgD^{low/-}$ cells in adults (Timens *et al.*, 1989). This strong coexpression is present on almost all marginal zone B cells in the first year of life and then gradually changes to an adult pattern of expression by about 4 years of age (Timens *et al.*, 1989).

CD21, a receptor for complement fragment C3d, is absent or in some cases expressed only weakly on infant B cells including marginal zone B cells until about the age of 2 years (Timens *et al.*, 1989; Griffioen *et al.*, 1992). Polysaccharide antigens activate the alternative pathway of complement and generate C3d products that form polysaccharide-C3d complexes (Griffioen *et al.*, 1991). Through binding of C3d to CD21 polysaccharide antigens promote marginal zone antigen specific B cell activation (Timens *et al.*, 1989).

Extensive cross linking of surface IgM antigen receptors on immature infant B cells induces a negative effect on B cell activation in the absence of T cell help, suggesting a contribution to the unresponsiveness to polysaccharides during early childhood (Chang *et al.*, 1991). Biochemical analyses have revealed that infant B cells have a deficit, relative to adults in expression of protein tyrosine kinases, resulting in a reduced induction of intracellular proteins for cell cycle progression in response to surface IgM antigen receptor cross linking (Wechsler and Monroe, 1995; Horikawa *et al.*, 1999).

Since T cell derived factors are necessary for antibody responses to polysaccharide antigens, immature T cells producing low levels of IL-4, IL-6 and INFγ during early childhood could also contribute to the limited ability of infants to elicit an antibody response to polysaccharides (Lewis *et al.*, 1991; Adkins *et al.*, 1994). Furthermore, the stimulatory signals provided by accessory cells for polysaccharide responses could also be ineffective due to delayed maturation of these cells during infancy (Chelvarajan *et al.*, 1998; Muthukkumar *et al.*, 2000).

However, the exact underlying mechanisms of the infant unresponsiveness to polysaccharide antigens are still not clearly understood.

1.7 Antibody response to PRP polysaccharide antigen

The antibody response to PRP antigen exhibits the characteristic features of a polysaccharide antigen response discussed before. PRP antigen is not immunogenic in children less than 18 months of age (Peltola *et al.*, 1977a; Peltola *et al.*, 1984; Granoff and Cates, 1985). The ability to produce antibody to PRP polysaccharide antigen does not reach adult levels till about 5 years of age (Smith *et al.*, 1973; Granoff and Munson, 1986).

The subset of B cells involved in the PRP antibody response has not been described. However, Barrett *et al* has demonstrated that anti-pneumococcal antibody secreting cells are generated from CD5 negative B cells (B2 subpopulation) (Barrett *et al.*, 1992). The appearance of these B cells in early childhood correlated with the ontogeny of the antibody response to pneumoccocal polysaccharide antigen in children. Furthermore, patients with selective anti-polysaccharide antibody

deficiency (SPAD), including a diminished antibody response to PRP capsular polysaccharide, have an over abundance of CD5 positive B cells and a markedly reduced CD5 negative B cell count compared with normal control subjects (Antall *et al.*, 1999).

1.7.1 Anti-PRP antibody isotypes

IgG, IgM and IgA classes of antibodies are produced in similar proportions in response to PRP capsular polysaccharide antigen (Dahlberg, 1981; Kaplan *et al.*, 1983; Lagergard *et al.*, 1984). Anti-PRP IgG antibodies are restricted mainly to IgG1 and IgG2 subclasses with similar proportions of both subclasses in some adults while others show either IgG1 or IgG2 predominance (Makela *et al.*, 1987; Shackelford *et al.*, 1987; Claesson *et al.*, 1988; Herrmann *et al.*, 1992). IgG3 and IgG4 anti-PRP antibodies are generated in smaller proportions, with IgG4 being the least (Makela *et al.*, 1987; Herrmann *et al.*, 1992). IgG1 is the predominant subclass in young children (Claesson *et al.*, 1988; Jelonek *et al.*, 1993), reflecting the positive correlation of IgG2 expression with age (Morell *et al.*, 1972; Shackelford *et al.*, 1985). Anti-PRP IgG1 and IgG2 antibodies express a frequently predominant cross reactive idiotype (CRI) (Lucas and Granoff, 1990).

Anti-PRP antibodies exert both opsonic and bactericidal activities in the presence of complement (Steele *et al.*, 1984; Musher *et al.*, 1986; Amir *et al.*, 1990a). IgG anti-PRP antibodies demonstrate both bactericidal and opsonic activities, whereas the IgM antibodies are more bactericidal and less opsonic than IgG antibodies (Schreiber *et al.*, 1986). IgG1 anti-PRP antibodies are more efficient in complement dependent

opsonisation and bactericidal activity than IgG2 anti-PRP antibodies (Amir *et al.*, 1990b).

1.7.2 Anti-PRP antibody repertoire

The anti-PRP polysaccharide antibody response is oligoclonal with limited variable region diversity (Insel *et al.*, 1985). The variable region repertoire of anti-PRP IgG antibodies has been extensively analysed. Heavy chains (VH) of the antibodies are encoded by three to four gene segments from the V_HIII family (reviewed in (Lucas *et al.*, 1998)). In contrast, light chain (VL) usage is more complex, with as many as 12 distinct segments from a number of V_κ and V_λ families. However, a single particular gene segment known as A2 from the V_κII family encodes the VL region for most anti-PRP antibodies (Scott *et al.*, 1989b; Lucas and Granoff, 1990). These antibodies are referred to as A2 antibodies and have a cross reactive idiotype. The antibodies expressing non A2 gene usage are called non A2.

Scott *et al* found that A2 gene undergoes only very few or no somatic mutations, while other VL gene segments undergoes some somatic mutations (Scott *et al.*, 1989a; Scott *et al.*, 1991). However, surprisingly antibodies with A2 gene usage are of higher avidity than non A2 antibodies (Nahm *et al.*, 1995). This is probably, because they contain a 10 amino acid light chain complementarity-determining region 3 (CDR-3), with an arginine residue at position 95a (Adderson *et al.*, 1993a). CDR-3 with arginine or lysine at position 95a results in stronger binding to PRP polysaccharide compared to other residues at that position (Lucas *et al.*, 1998).

1.7.3 Anti-PRP antibody affinity

Anti-PRP antibodies are of low affinity (Hetherington, 1988; Griswold *et al.*, 1989). However, Adderson *et al* detected a high rate of somatic mutation in the immunoglobulin genes in B cells in the antibody response to PRP antigen (Adderson *et al.*, 1998a). In spite of this, there has been only minimal or no evidence of affinity maturation during subsequent exposure to the antigen, suggesting a limited antigen driven selection of coding changes in response to PRP polysaccharide (Griswold *et al.*, 1989; Adderson *et al.*, 1998a; Makela *et al.*, 2003). This explanation is validated by the fact that affinity of anti-PRP antibodies in adults is low in spite of recurrent natural exposure to PRP polysaccharide throughout adult life (Hetherington, 1988; Griswold *et al.*, 1989).

1.7.4 T cell help

Although PRP polysaccharide is classified as a TI antigen, *in vitro* studies have indicated that direct physical interaction between B and T cells enhances the anti-PRP antibody response more than the stimulatory activity provided by T cell derived cytokines per se (Breukels *et al.*, 1999). However, T cell contact with PRP specific B cells cannot be via the classical MHC class II restricted helper pathway, as there is no MHC class II involvement in the antibody response to polysaccharides

1.8 *Haemophilus influenzae* type b vaccines

1.8.1 Pure PRP polysaccharide vaccine

The initial Hib vaccine was developed using pure PRP polysaccharide antigen (Rodrigues *et al.*, 1971). Efficacy of the PRP vaccine was studied in a double blind field trial involving 100,000 infants and children in Finland (Peltola *et al.*, 1977a). The protective antibody response was highly age dependent. The vaccine was poorly immunogenic in children below 18 months of age and no protection was seen in this age group, the population most susceptible to invasive Hib infections (Peltola *et al.*, 1977a). The vaccine induced uncertain protection in children aged between 18 to 23 months (Peltola *et al.*, 1984; Granoff and Cates, 1985), while children older than 24 months were protected with a 90% efficacy (Peltola *et al.*, 1984). Furthermore, the antibody response to pure PRP vaccine was of low affinity and was not associated with effective immunological memory development (Anderson *et al.*, 1977; Kayhty *et al.*, 1984).

Serious sequelae, the high risk of mortality from invasive Hib disease and increasing antibiotic resistance of the Hib bacterium (Mason *et al.*, 1982; Meyrovitch *et al.*, 1984; Gairi *et al.*, 1986) have led to the development of more effective Hib vaccines.

1.8.2 PRP conjugate vaccines

Goebel and Avery in 1929 described that physical coupling of a polysaccharide to a carrier protein leads to enhance immunogenicity of the polysaccharide (Goebel and Avery, 1929). Taking this into account, Schneerson *et al* and Anderson demonstrated that covalently linking PRP to a carrier protein converts the TI response of PRP

antigen to a TD response with antibody generation in infants and a booster effect during subsequent exposures (Schneerson *et al.*, 1980; Anderson, 1983).

This finding led to the development of PRP conjugate vaccines over the next few years to overcome the limitation of unresponsiveness of PRP in early childhood. Four different types of PRP conjugate vaccines were produced with various carrier proteins coupled to PRP antigen (Schneerson *et al.*, 1980; Decker *et al.*, 1992; Santosham, 1993) (Table 1.3). The diptheria toxoid (D), outer membrane protein complex of Neisseria meningitis (OMPC), a non-toxic mutant diptheria toxin (CRM), and tetanus toxoid (T) have been used as carrier proteins. These PRP conjugate vaccines have been more effective than the original plain PRP vaccine (Eskola *et al.*, 1993; Murphy *et al.*, 1993).

Table 1.3

PRP conjugate vaccines. Data from Decker and Edwards, 1998; NHMRC, 2000.

Abbreviation (Trade name) *Manufacturer*	**PRP/0.5 ml dose**	**Carrier protein**	**Year of licensure (USFDA*)**
PRP-D		Diptheria toxoid	1987
(ProHIBIT®)	25 μg	18 μg	
Pasteur Merieux Connaught			
PRP-CRM		Mutant diptheria toxin CRM_{197}	1988
(HibTITER®)	10 μg	25 μg	
Wyeth-Lederle			
PRP-OMP		*Neisseria meningitidis* outer membrane protein	1989
(PedvaxHIB® liquid)	7.5 μg	125 μg	
(PedvaxHiB® lyophilized)	15 μg	250 μg	
Merck, Sharpe & Dohme			
PRP-T		Tetanus toxoid	1993
(ActHib®)	10 μg	18-30 μg	
Pasteur Merieux Connaught			
(Hiberix®)	10 μg	30 μg	
Smith Kline Beecham			

*USFDA- United States Food and Drug Administration

The enhanced immunogenicity of PRP conjugate vaccines is due to specific interactions that occur between the PRP specific B cells and T helper cells (Fig.1.3) (Guttormsen *et al.*, 1999; Kamboj *et al.*, 2001). The PRP component binds to antigen receptors of PRP specific B cells (BCR), which then internalise the PRP-protein conjugate vaccine antigen. The protein is processed inside the B cell and peptide fragments are then presented to specific CD4 positive T helper cells through T cell receptors (TCR) in association with MHC class II molecules mounting a carrier

specific stimulation. The T helper cells are also activated by B7 costimulatory molecules of the PRP specific B cells, resulting in IL-2 secretion. The activated T helper cells further stimulate the specific B cell via the CD40-CD40L pathway as well as by secreting IL-4.

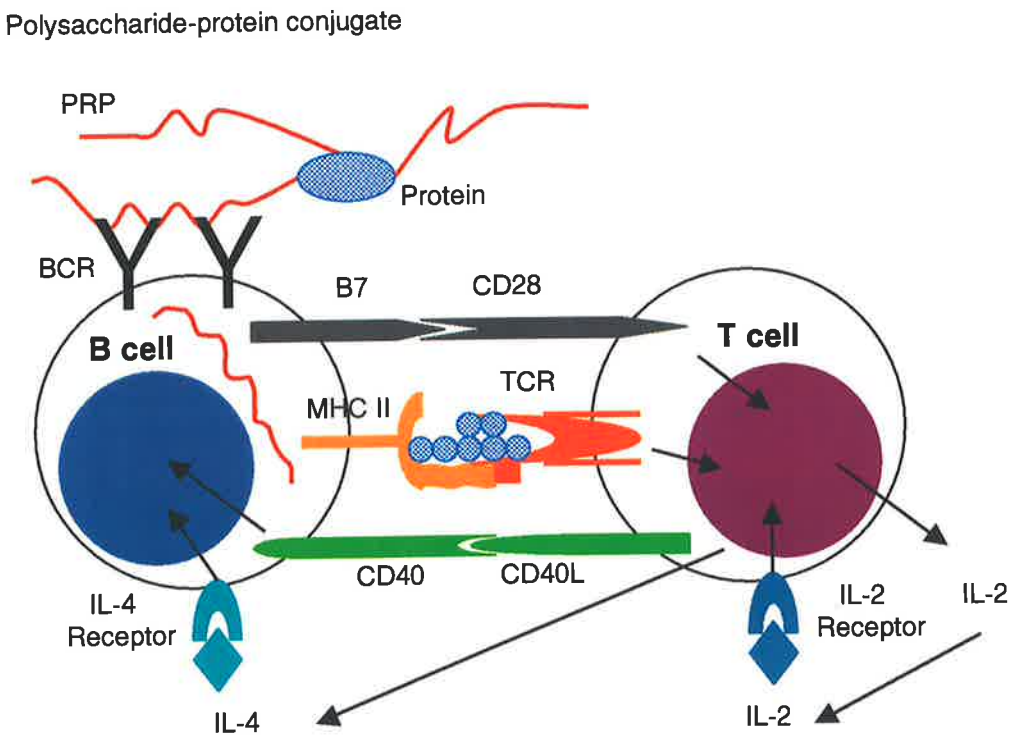


Fig.1.3 Schematic diagram illustrating T cell help to PRP specific B cells following stimulation with a conjugate vaccine antigen. Modified from Guttormsen *et al.*, 1999.

1.9 Safety and efficacy of Hib conjugate vaccines

Several studies have been performed to describe the safety and efficacy of Hib conjugate vaccines.

In general, adverse reactions to all four PRP conjugate vaccines are few and minor, and are similar to those with plain PRP polysaccharide vaccine (Booy *et al.*, 1992; Decker *et al.*, 1992; Holmes *et al.*, 1993). Local reactions of pain, redness and swelling are less with PRP-D and PRP-CRM vaccines than with PRP-T and PRP-OMP vaccines (Decker *et al.*, 1992). Systemic reactions seen with all four PRP conjugate vaccines do not differ from those of other childhood immunisation vaccines (Decker *et al.*, 1992; Vadheim *et al.*, 1993).

The first efficacy trial of a PRP conjugate vaccine was performed in Finland in 1985 (Eskola *et al.*, 1987). Infants were immunised with PRP-D at 3, 4, and 6 months of age followed with a booster dose at 14-18 months of age. The efficacy of PRP-D vaccine after three primary doses was 83% (95% confidence interval (CI) 26–96%). A further analysis was performed by Eskola and colleagues after enrolling over 100,000 children in the study (Eskola *et al.*, 1990). The protective efficacy of PRP-D increased to 94% (95% CI 83–98%) following a booster dose in children less than 2 years of age. However, in a subsequent trial in the US, the efficacy of PRP-D in Alaskan native infants immunised at 2, 4 and 6 months of age was 35% (95% CI -57–73%) (Ward *et al.*, 1990). An increased risk of Hib infection occurring at younger ages and poor immunogenicity of the vaccine during early infancy may have limited the efficacy of PRP-D vaccine in Alaskan infants (Ward, 1991).

The efficacy of PRP-D and PRP-CRM vaccines in young children after immunising with two primary doses of either vaccine at 4 and 6 months of age and with a booster dose at 14-18 months of age was compared in a later study in Finland (Peltola *et al.*, 1994). The protective rates for the PRP-D and PRP-CRM vaccines were 87% (95% CI 69-96%) and 95% (95% CI 76-99%) after two primary doses respectively. No cases of invasive Hib disease occurred in children up to 2 years of age after the booster immunisation with either vaccine. It was suggested that both vaccines are protective, however, a booster dose in the second year of life is necessary for better protection with PRP-D vaccine.

In a separate clinical trial in the US, when infants were administered PRP-CRM at 2, 4 and 6 months of age, the vaccine efficacy was 100% (95% CI 47-100%) after two doses (Black *et al.*, 1991). However, in another similar trial in the US, the efficacy of the PRP-CRM vaccine was 73% (95% CI 47-88%) after 1 dose, 90% (95% CI 68-98%) after two doses and 93% (95% CI 70-99%) after three doses (Vadheim *et al.*, 1994).

A double blind placebo trial was performed to assess the effectiveness of the PRP-OMP vaccine among Navajo infants in the US (Santosham *et al.*, 1991). During a two year period approximately 2000 infants were immunised with the vaccine at 2 and 4 months of age. At 18 months of age the point estimate of efficacy of PRP-OMP was 95% (95% CI 72-99%). No cases of invasive Hib disease occurred before the age of 15 months in the immunised group.

In two controlled efficacy trials with PRP-T vaccine in the UK and the US, none of the infants developed invasive Hib disease, resulting in a vaccine efficacy of 100% (95% CI 80-100%) after completion of a three dose primary course (Vadheim *et al.*,

1993; Booy *et al.*, 1994). However, during a follow up period of a further one year in the UK study, the efficacy was reduced to 95% (95% CI 74-100%), as one case of invasive Hib disease occurred in the vaccine group and 18 cases among the controls (Booy *et al.*, 1994).

In summary, Hib conjugate vaccines have high efficacy. PRP-D is the least immunogenic of the Hib conjugate vaccines in infancy. Therefore, a booster dose in the second year of life for this vaccine is beneficial.

1.10 Hib conjugate vaccination schedules

PRP conjugate vaccines have been included in routine childhood immunisation schedules in developed countries in recent years to prevent Hib invasive disease in young children.

In 1991, the US introduced Hib conjugate vaccines to their routine vaccination schedule, recommending all children be immunised with either PRP-OMP at 2, 4 and 12 months of age or PRP-CRM at 2, 4, and 6 months followed with a booster dose at 15 months of age (Granoff *et al.*, 1993b; Singleton *et al.*, 1994). In 1993, PRP-T was also included as one of the Hib vaccines in the US immunisation program at similar intervals to PRP-CRM vaccine (King and Hadler, 1994).

In Finland, from 1988, two doses of either PRP-D or PRP-CRM were given at 4 and 6 months for primary immunisation followed with a booster dose at 14-18 months. There was then a switch to PTP-T in 1990 (Peltola *et al.*, 1992). Finally in 1994,

PRP-CRM was reintroduced for all infants at the same time points as before (Peltola *et al.*, 1999).

Since 1992, three doses of PRP-T have been administered to infants in the UK at 2, 3 and 4 months of age, with no booster immunisation given during the second year of life (Booy *et al.*, 1992).

Routine childhood Hib immunisation was introduced to the Australian standard vaccination schedule in 1993 (Table 1.4) (Burgess *et al.*, 1994; NHMRC, 1994). The epidemiology of Hib disease in Australian Aboriginal children and in non Aboriginal children was taken into account when formulating the vaccine regimes (Hanna, 1990). PRP-OMP was the choice of vaccine for Australian Aboriginal children, as it stimulated a brisk antibody response after the first dose in infants 2 months of age (Hanna, 1990; Burgess *et al.*, 1994). PRP-CRM was chosen for non Aboriginal children, as Hib disease was not common under the age of 6 months in this population. PRP-OMP was were given at 2, 4 and 12 months, while PRP-CRM was administered at 2, 4, 6 months and was followed with a booster dose at 18 months of age. A study conducted by Guthridge and colleagues later found that Australian non-Aboriginal infants also elicit a strong protective response to PRP-OMP when immunised at 2, 4 and 12 months (Guthridge *et al.*, 2000). In May 2000, the Australian standard vaccination schedule for Hib was changed over to PRP-OMP for all Australian children, reducing the complexity of the schedule (NHMRC, 2000).

Although invasive Hib disease is an important cause of morbidity and mortality in children in developing countries in Asia (Peltola, 1998; Limcangco *et al.*, 2000; Lolekha *et al.*, 2000; Shah, 2003), until now, Hib vaccination has not been added to routine immunisation schedules in most of these countries, largely because of the cost

of the conjugate vaccines and the perceived lesser importance of invasive Hib infection.

Table 1.4

Australian standard vaccination schedule for *H.influenzae* type b (NHMRC, 1994; NHMRC, 2000).

Vaccine	Age (months)
Aboriginal children:	
PRP-OMP	2, 4 and booster at 12
Non-Aboriginal children:	
PRP-CRM	2, 4, 6 and booster at 18
All children *	
PRP-OMP	2, 4 and booster at 12

*Children born after 1st may 2000

1.11 Impact of Hib conjugate vaccination

Widespread use of Hib conjugate vaccines with high coverage has resulted in a rapid decrease in Hib invasive disease. In the US, the incidence of invasive Hib disease among children under 5 years of age had declined by nearly 99% by 1997 (Meissner and Pickering, 2002). Furthermore, by 1996, the incidence had reached almost zero in Finland (Peltola *et al.*, 1999). In the UK, the incidence had reduced by 98% by 1998 (Heath *et al.*, 2000).

The routine childhood immunisation protocol used for Hib in Australia has been very effective in reducing the incidence of invasive Hib disease (Fig.1.4). In Australia, after three years of the Hib vaccination program with coverage of nearly 80%, the incidence in children less than 5 years of age was reduced by approximately 90% in 1996 (Herceg, 1997; Hull *et al.*, 1999). The corresponding Hib vaccine coverage figures in the US and UK were nearly 95% (Hull *et al.*, 1999). By 2000 in Australia, the incidence of invasive Hib disease in children had declined by 96% to an annual rate of 1.7 per 100,000 (Horby *et al.*, 2003).

Furthermore, PRP conjugate vaccines also reduce the oropharyngeal carriage rates of Hib in young children (Takala *et al.*, 1991; Murphy *et al.*, 1993; Barbour *et al.*, 1995). Nevertheless, some populations such as Alaskan Natives and Australian Aborigines with high incidences of Hib disease continue to have significant Hib carriage rates in spite of immunization with PRP conjugate vaccines (Galil *et al.*, 1999; Guthridge *et al.*, 2000). A recent survey has shown that a serum anti-PRP antibody concentration of greater than 5 μg/ml was needed for prevention of colonization, a much higher concentration than needed for protection against invasive Hib disease (Fernandez *et al.*, 2000).

The large decrease in the incidence of invasive Hib disease may not be solely due to the protection of immunised individuals. It is possible that the reduced number of Hib carriers among the immunised population may have resulted in a decreased exposure of non immunised individuals to Hib bacteria. Furthermore, a reduction of invasive Hib disease has also been seen in older age groups, even those not eligible for routine Hib immunisation, probably due to this effect of herd immunity (Horby *et al.*, 2003).

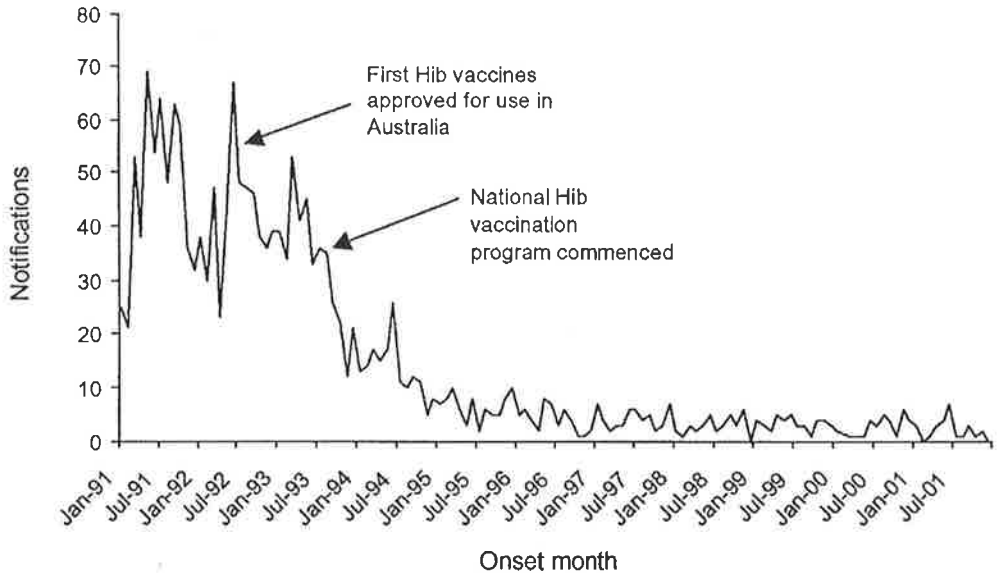


Fig.1.4 Notification of invasive Hib disease in Australia, 1991-2002 (Burgess, 2003).

1.12 Antibody response to Hib conjugate vaccines

PRP conjugate vaccines elicit a TD antibody response, overcoming the unresponsiveness to the pure PRP antigen vaccine in early childhood (Seppala *et al.*, 1988; Decker *et al.*, 1992). The magnitude of the antibody response to PRP conjugate vaccines is greater than the response to pure PRP polysaccharide vaccine (Makela *et al.*, 1987; Jelonek *et al.*, 1993).

The four conjugate vaccines differ in PRP polysaccharide size, carrier protein, and covalent linkage (Decker and Edwards, 1998). These differences result in different abilities to evoke anti-PRP antibody responses in infants (Decker *et al.*, 1992; Granoff *et al.*, 1992; Schlesinger and Granoff, 1992; Granoff *et al.*, 1993a; Granoff *et al.*, 1993c; Lucas and Granoff, 1995) (Fig. 1.5).

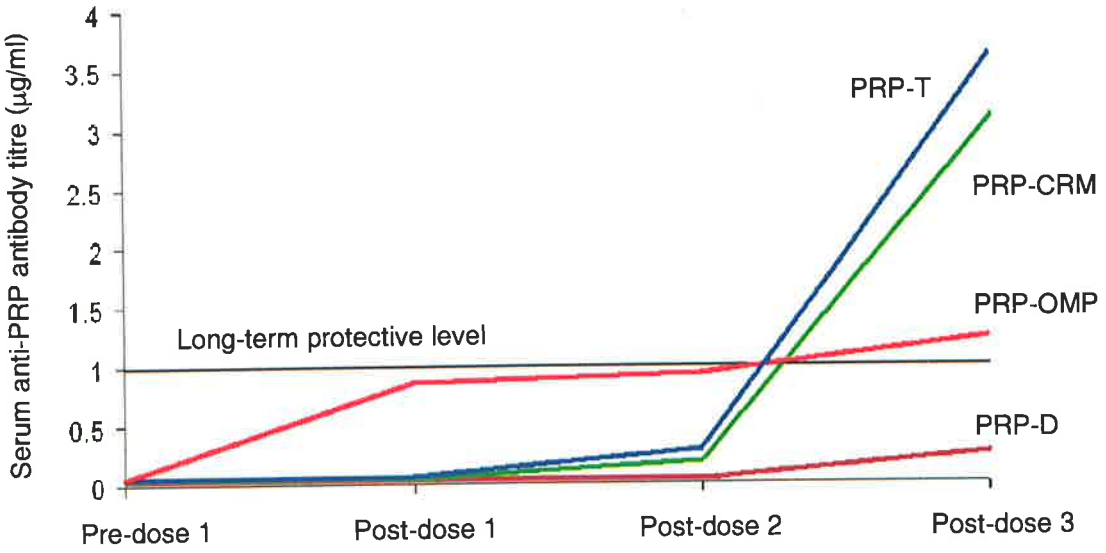


Fig. 1.5 Comparison of antibody responses to primary immunisation at 2, 4 and 6 months with each of the four conjugate Hib vaccines. Data from Decker and Edwards, 1998.

PRP-D conjugate vaccine is poorly immunogenic in infants under 6 months of age (Eskola *et al.*, 1987). A protective antibody level is elicited in infants only after three doses, yet the antibody concentration is very much less than the concentrations generated by the other three conjugate vaccines. (Decker *et al.*, 1992). However, PRP-D immunisation in the second year of life generates higher level of protective antibodies (Jelonek *et al.*, 1993).

PRP-OMP induces a protective antibody response in infants following a single injection at 2 months of age (Einhorn *et al.*, 1986; Decker *et al.*, 1992; Granoff *et al.*, 1992), whereas PRP-T and PRP-CRM vaccines require two doses to stimulate

significant antibody production during the first year of life (Decker *et al.*, 1992; Granoff *et al.*, 1992; Greenberg *et al.*, 1994). Adjuvant characteristics of OMP could be the reason for the early response to PRP-OMP vaccine (Ambrosino *et al.*, 1992a). However, PRP-OMP elicits only modest increases in antibodies on subsequent immunizations at 4 and 6 months (Decker *et al.*, 1992; Granoff *et al.*, 1992).

In a study conducted by Granoff *et al*, more than 90% of infants had antibody concentrations of greater than 1 μg/ml, after three injections of either PRP-OMP, PRP-CRM or PRP-T vaccine at 2, 4 and 6 months of age (Granoff *et al.*, 1992). However, another comparative trial reported that serum antibody concentrations greater than 1 μg/ml were achieved in 55%, 75% and 83% of infants immunised with three doses of either PRP-OMP or PRP-CRM or PRP-T (Decker *et al.*, 1992). Only 30% of infants achieved antibody levels more than 1 μg/ml with three doses of PRP-D vaccine. In both these trials, antibody response to PRP-T vaccine was the highest.

In summary, all four Hib conjugate vaccines produce protective antibody responses during early childhood and the concentrations are significantly higher than against pure PRP polysaccharide vaccine (Jelonek *et al.*, 1993). PRP-CRM, PRP-OMP and PRP-T vaccines induce higher concentrations of protective antibodies than PRP-D vaccine during first year of life.

1.12.1 Anti-PRP antibody isotypes

PRP conjugate vaccines generate IgG, IgM and IgA anti-PRP antibodies (Seppala *et al.*, 1988; Granoff *et al.*, 1993a). The IgG antibody response levels are greatest, while IgM and IgA antibodies are produced in moderate amounts. However, in some

individuals probably with previous recurrent exposure to PRP capsular polysaccharide or to cross reactive antigens, the PRP conjugate vaccines elicit an IgA anti-PRP antibody response (Hougs *et al.*, 1993; Barington *et al.*, 1994).

PRP-T vaccine produces the highest concentration of IgG antibodies followed by PRP-CRM, PRP-OMP and then PRP-D (Granoff *et al.*, 1993a; Jelonek *et al.*, 1993). PRP-D vaccine induces a higher proportion of IgM antibodies than the other three vaccines. IgA antibody proportions are not much different for each PRP conjugate vaccine.

IgG1 and IgG2 antibodies are the main IgG subclasses induced in response to PRP conjugate vaccines, with IgG1 being predominant (Shackelford *et al.*, 1987; Seppala *et al.*, 1988; Kroon *et al.*, 1997; Breukels *et al.*, 2002). In some individuals IgG2 is the predominant IgG subclass in response to PRP-D vaccine (Makela *et al.*, 1987; Seppala *et al.*, 1988).

In young children, anti-PRP IgG antibodies to all four conjugate vaccines are mainly IgG1 (Kayhty *et al.*, 1988; Holmes *et al.*, 1991; Ambrosino *et al.*, 1992b; Jelonek *et al.*, 1993). IgG2 antibodies are induced in late infancy but the concentrations are significantly smaller than those of IgG1 and IgM. Booster doses in the second year significantly increase IgG2 antibodies (Ambrosino *et al.*, 1992b). This indicates delayed maturation of IgG2 production in children (Shackelford *et al.*, 1985).

In response to PRP conjugate vaccines, IgA antibodies include both IgA1 and IgA2 subclasses in substantial proportions (Barington *et al.*, 1994).

1.12.2 Memory response

The antibody responses to conjugate vaccines are accompanied by effective memory cell development leading to generation of long term IgG antibodies following subsequent exposure to PRP antigen (Ambrosino *et al.*, 1992b, Granoff, 1993 #1281; Kurikka *et al.*, 1995). The enhanced response is elicited rapidly even in the absence of measurable antibodies in blood at the time of antigen challenge (Zepp *et al.*, 1997).

The magnitude of the memory response to subsequent challenge with pure PRP polysaccharide in infants primed with PRP-T is about half of that of infants primed with other PRP conjugate vaccines (Granoff *et al.*, 1993a). However, secondary antibody responses to all PRP conjugate vaccines are very much above the protective levels (Granoff *et al.*, 1993a).

Studies comparing the priming capacity of all four PRP conjugate vaccines have reported that two primary doses followed with a booster dose at 14 months of either PRP conjugate vaccine or pure PRP vaccine induce high antibody concentrations with strong IgG responses, and predict the possibility of full protection for children up to 36 months of age (Kayhty *et al.*, 1992; Kurikka *et al.*, 1995). PRP-T vaccine induces the strongest antibody response, while the response of PRP-D is the lowest.

A recent study has shown that protective anti-PRP antibody levels elicited in response to PRP conjugate vaccines could persist for at least 8 to 9 years in children (Makela *et al.*, 2003). This long term persistence of the antibodies may depend on anamnestic responses to recurrent exposure to Hib bacteria and to cross reactive antigens.

1.12.3 Anti-PRP antibody affinity

Antibodies generated by all PRP conjugate vaccines except PRP-D have higher affinity than those elicited by pure PRP antigen. (Hetherington, 1988; Griswold *et al.*, 1989; Schlesinger and Granoff, 1992; Agbarakwe *et al.*, 1995; Makela *et al.*, 2003). However, it is much lower than that of antibody against protein antigens (Brown *et al.*, 1984; Junker and Tilley, 1994; Aboud *et al.*, 2000; Breukels *et al.*, 2002).

The affinity of anti-PRP antibodies depends on the type of conjugate vaccine formulation. Primary antibody responses to PRP-OMP and PRP-D conjugate vaccines are of lower affinity compared with those elicited by the other two PRP conjugate vaccines (Hetherington and Rutkowski, 1990; Schlesinger and Granoff, 1992; Granoff and Lucas, 1995). PRP-CRM induces antibodies of two to three fold higher affinity than vaccination with PRP-OMP (Schlesinger and Granoff, 1992; Chung *et al.*, 1995; Lucas and Granoff, 1995). The affinity of anti-PRP antibodies elicited by PTP-T is intermediate. As expected, the affinities of antibodies generated by PRP conjugate vaccines in early childhood are significantly lower than those of adults (Hetherington, 1988; Hetherington and Rutkowski, 1990).

Analyses of VH sequences of the anti-PRP antibody repertoire in adults after immunisation with conjugate vaccines have found that the frequencies of somatic mutations are surprisingly comparable or even less than those seen in response to PRP polysaccharide (Adderson *et al.*, 1993b; Adderson *et al.*, 1998a). Furthermore, Barington *et al* have reported that the total number of mutations in the VL region in response to PRP conjugate vaccines is less than that seen in other unrelated antibody responses (Barington *et al.*, 1996). Even in the TD form of anti-PRP responses, the

affinity maturation is minimal probably due to restricted antigen driven selection of coding changes (Adderson *et al.*, 1998a; Campbell *et al.*, 2002).

Anti-PRP antibody affinity is positively correlated with bactericidal activity in serum (Hetherington and Lepow, 1992; Agbarakwe *et al.*, 1995). Therefore, it is an important determinant of host defense and should be considered as vital in evaluating antibody responses to immunization.

1.12.4 Anti-PRP antibody repertoire

The genetic diversity of the variable region of the antibody repertoire to PRP conjugate antigen is similar to that elicited by pure PRP polysaccharide antigen with few exceptions (Lucas and Granoff, 1990; Adderson *et al.*, 1991; Adderson *et al.*, 1993b).

An analysis performed by Chung and colleagues demonstrated that antibodies to PRP conjugate vaccines had more VκIII gene selection in their light chains than those elicited by PRP polysaccharide vaccine (Chung *et al.*, 1993). A more recent study has shown that the variable region sequences of anti-PRP antibodies to PRP conjugate vaccine in infants are similar to those found in adults (Lucas *et al.*, 2003).

Furthermore, differences in the VL gene selection in response to different types of PRP conjugate vaccines are also seen. PRP antibodies with A2 gene expression are detected in about one third of infants immunised with PRP-OMP vaccine (Granoff *et al.*, 1993c). In contrast, the A2 expression in response to PRP-T or PRP-CRM in infants is more than 60%.

The anti-PRP antibody repertoire in children in response to PRP-CRM vaccine is more diverse than that in adults (Adderson *et al.*, 1998b). For most of the antibodies, the VH region is encoded by at least 6 different gene segments from V_HIII family in young children, whereas only two of these segments have been identified in adults. However, the antibodies that express a VH repertoire similar to that of adults bind PRP antigen with higher avidity than those with a more diverse repertoire.

The combination of gene usage for VL also is influenced by age. Following immunization with a single dose of PRP-OMP vaccine, V_κIII usage is not seen in 2 month old infants, but can be detected at 30% and 70% in 18 months old children and in adults respectively (Lucas *et al.*, 1993). On the other hand, V_λVII antibodies are frequently present in 2 month old infants (43%) but not seen during adult life.

There is no such age-correlated pattern for ant-PRP polysaccharide antibodies encoded by the A2 gene, although the frequency is less at 2 months of age (Lucas *et al.*, 1993; Lucas and Reason, 1998). Furthermore, it has been shown that the expression of A2 antibodies in elderly populations is similar to that of younger adults (Lucas and Reason, 1998).

Very recent research has shown that infants immunised with PRP-OMP or PRP-CRM express A2 light chains with atypical residues at position 95 in addition to arginine, that have not been detected in previous studies of adults (Lucas *et al.*, 2003).

All analyses of the anti-PRP antibody repertoire have been performed after immunization with either PRP polysaccharide or PRP conjugate antigens. There is not much information available on the diversity of 'naturally' occurring antibodies to Hib. A recent study has suggested that the usage of VH region genes following

exposure to the Hib organism may be very similar to that of vaccinated individuals (Beck *et al.*, 2000).

1.12.5 Priming with carrier protein

The use of PRP conjugate vaccines has another advantage over polysaccharide vaccine. Much experimental data demonstrated that anti-PRP antibody responses to PRP conjugate vaccines are enhanced if the individual is primed by prior immunisation with the carrier protein (Barington *et al.*, 1991; Kayhty *et al.*, 1991; Castillo de Febres *et al.*, 1994; Kurikka, 1996). It is thought that the enhancement is likely due to increased stimulation of PRP specific B cells from carrier primed T helper cells. Furthermore, Nohynek and colleagues later discovered that infants with higher levels of maternally acquired anti-TT antibodies respond better to PRP-T vaccine than infants with lower levels of maternal ant-TT antibodies (Nohynek *et al.*, 1999). This is attributed to the enhanced formation of antigen-antibody complexes and efficient presentation to antigen presenting cells.

1.12.6 Combining with other vaccines

Several studies have been performed by combining PRP-T conjugate vaccine with other routine primary vaccines such as diptheria-tetanus-acellular pertussis (DTaP) vaccine (Pichichero *et al.*, 1997; Lee *et al.*, 1999), DTaP-inactivated polio vaccine (Kanra *et al.*, 1999; Lin *et al.*, 2003) or DTaP-hepatitis B vaccine (Pichichero *et al.*, 1999; Greenberg *et al.*, 2000), in order to simplify the administration by reducing the number of injections especially during infancy. Although the combined vaccines induced protective anti-PRP antibodies, the concentrations achieved after a primary

course of immunisation were lower than the levels achieved following a similar number of separate PRP-T conjugate immunisations (Pichichero *et al.*, 1997; Greenberg *et al.*, 2000). However, there was little difference in the booster responses with pure PRP or PRP conjugate vaccines following the completion of a primary course of combined PRP or separate PRP conjugate immunisation. Furthermore, it has been shown that combined PRP vaccines induce anti-PRP IgG antibodies with higher avidity (Goldblatt *et al.*, 1998; Pichichero *et al.*, 1999). Therefore, the inclusion of PRP conjugate vaccine combined with other primary vaccines into childhood immunisation schedules has several potential advantages.

1.12.7 Protective anti-PRP antibody levels

In studies following immunisation with Hib vaccines, antibody concentrations of more than 0.15 μg/ml were thought to be adequate for short term protection, and antibody concentrations greater than 1.0 μg/ml were suggested as providing protection over the following years against Hib disease (Makela *et al.*, 1977; Kayhty *et al.*, 1983). However, these parameters have been set by studying the response to PRP polysaccharide vaccine. The protective anti-PRP antibody concentrations following immunisation with PRP conjugate vaccines may be different due to priming with memory development and production of higher affinity antibodies.

Little is known about the cellular and molecular basis for the antibody response to PRP conjugate antigens despite their widespread use in immunization against Hib. A study performed in mice using TI-2 antigen dextran and a TD form of dextran conjugated to a carrier protein has suggested that both forms of antigen activate the same type of B cells (Sverremark and Fernandez, 1998b). If this is a generalised

occurrence, it can be postulated that even after conversion of PRP polysaccharide antigen to PRP conjugate polysaccharide antigen, the same B cells that are involved in the antibody response to the polysaccharide antigen are used. Therefore, it is possible that antigen specific B cells used in the antibody response to true TD antigens are different from the antigen specific B cells involved with PRP conjugate polysaccharide antigens. Further understanding of the anti-PRP antibody response to PRP conjugate antigen at the cellular level is required.

1.13 Antigen specific B cells

B cells are a key constituent of the immune system. B cells, which initially develop in fetal liver and subsequently in bone marrow, are the precursors of antibody secreting plasma cells in the acquired immune response.

Mature B cells possess both IgM and IgD immunoglobulin molecules on the cell surface as receptors for antigen binding (Barnikol and Hilschmann, 1982; Yuan and Witte, 1988). Each B cell expresses these antigen receptors (BCR) with a single antigen-binding specificity. This results from mechanisms, which inhibit further immunoglobulin gene rearrangements once a cell has undergone a productive re-arrangement (Melchers *et al.*, 2000). Although the immune system can produce antibody responses to a vast number of antigens, only a few thousand B cells express receptors specific for any given antigenic epitope (Delves and Roitt, 2000).

Antigen specific B cells capture antigens with their BCR (Drake *et al.*, 1989). The binding of the antigen to BCR triggers B cell activation. The antigens are internalised by receptor mediated endocytosis.

Protein antigens are processed by antigen specific B cells and presented in association with MHC class II molecules to T helper cells resulting in further B cell activation through specific cognate interactions (Lanzavecchia and Bove, 1985; Sarobe *et al.*, 1991; Marsh *et al.*, 1992). Activated antigen specific B cells proliferate and some differentiate into short lived plasma cells and others migrate to follicular regions initiating germinal centre reactions by interacting with follicular dendritic cells (FDC) (Jacob *et al.*, 1991a; Liu *et al.*, 1991).

In the germinal centre, antigen specific B cells proliferate rapidly, increasing the number of B cells involved in the antibody response (clonal proliferation) (Kroese *et al.*, 1990). Genes in the B cells encoding the immunoglobulin variable region undergo somatic hypermutation followed by selection, leading to increased binding strength between antibody and antigen (Jacob *et al.*, 1991b; Berek and Ziegner, 1993). This process is known as affinity maturation. Immunoglobulin genes also undergo further rearrangements to produce other classes of antibodies (e.g. IgG, IgA, IgE) of the same antigen specificity during the immune response (isotype switching) (Kraal *et al.*, 1982).

B cells that secrete high affinity antibodies are positively selected by competition for the binding of antigens trapped in immune complexes on follicular dendritic cells (FDC) (Kroese *et al.*, 1990; Kosco-Vilbois and Scheidegger, 1995). Antigen specific T helper cells provide further costimulation to these B cells, which differentiate into either antibody secreting plasmablasts or memory cells. B cells with low affinity for antigen die by apoptosis. Plasmablasts leave the germinal centre and develop into plasma cells. Some of these plasma cells migrate to bone marrow and continue to secrete antigen specific antibodies for longer durations (Merville *et al.*, 1996).

Memory cells have a longer life span, generally have higher affinity for antigen and have a lower threshold of activation (Schittek and Rajewsky, 1990; McHeyzer-Williams *et al.*, 1992). They either reside at antigen draining sites in secondary lymphoid tissues or join the recirculating pool of lymphocytes (Liu *et al.*, 1988). A subpopulation of memory B cells also home to the bone marrow (Paramithiotis and Cooper, 1997). Memory B cells mount a quantitatively and qualitatively superior secondary antibody response during subsequent exposure to the same antigen (Liu *et al.*, 1988; Kroese *et al.*, 1990)

In response to polysaccharide antigens, internalised polysaccharides are not processed and presented to T helper cells for further B cell activation through cognate interactions (Stein, 1985; Harding *et al.*, 1991). These B cells, activated directly by the polysaccharide antigens, proliferate and differentiate into antibody producing plasma cells with out germinal centre formation. However, non specific T cell and accessory cell help is obtained in mounting the antibody response (Ambrosino *et al.*, 1990; Pecanha *et al.*, 1991; Macedo-Soares *et al.*, 1996; Snapper and Mond, 1996).

1.13.1 Isolation of antigen specific B cells

The isolation of antigen specific B cells is perhaps the most challenging application of cell separation techniques, because the frequency of cells specific for any particular antigen is usually less than 1% (Oshiba *et al.*, 1994; Irsch *et al.*, 1995), and because it is difficult to find a second marker to assess purity.

During the past 30 years, various methods have been developed to isolate antigen specific B cells in order to study B lymphocyte function. Because of their relatively small numbers, most of the techniques for antigen specific B cell isolation have used

antigen priming to increase the number of specific B cells. Three principal techniques have been used to isolate antigen specific B cells based on BCR expression. These are: (1) capture on an antigen coated solid matrix; (2) rosetting with antigen coated red blood cells or magnetic particles, (3) staining with fluorescent antigen and isolation by flow cytometric cell sorting. Although these techniques have been progressively developed to achieve the best possible outcome, each has particular limitations and disadvantages.

A satisfactory antigen specific B cell isolation technique should result in good yield and high purity. The yield is defined as the proportion of antigen specific cells recovered after purification from the initial mixture. Purity is the percentage of cells in the final product that bind antigen specifically. Assessment of purity requires the use of markers or techniques that differ from those used to effect the separation, to avoid circulatory logic (Zola *et al.*, 1985). Some important publications that have reported isolation of antigen specific B cells are reviewed below and are summarised in Tables 1.5A-C.

1.13.1.1 Capture on an antigen coated solid matrix (Table 1.5 A)

The initial attempts to isolate antigen specific cells date back to the late 1960's. In 1968, Wigzell and Andersson isolated antigen specific cells by passing immunised mouse lymph node cells through antigen coated glass and plastic bead columns in an effort to study antigen specific immunoglobulin receptors (Wigzell and Anderson, 1968). The immunising antigens were either human serum albumin (HSA), bovine serum albumin (BSA) or ovalbumin (OA). The columns were effective in binding antigen specific cells. Yields of isolated antigen specific cells were 60-95%.

Haemolytic plaque assays were used to determine the specificities of isolated cell populations. However, the data presented in the publication do not allow determination of purity. Elution of the bound cells by mechanical agitation resulted in reduced cell viability and an enrichment factor of 2.5 fold, suggesting that the isolated populations contained significant numbers of non antigen specific cells. Furthermore, control columns, coated with syngeneic serum only, also bound large numbers of cells.

Truffa-Bachi and Wofsy used antigen coated acrylamide beads to reduce the non-specific binding of cells when attempting to purify antigen specific B cells (Truffa-Bachi and Wofsy, 1970). They described the use of antigen coated acrylamide bead columns to isolate antigen specific cells from spleen cells of mice immunised with phenyl-β-lactoside (*lac*) hapten. The specificity of the separated B cells was tested by detecting plaque forming cells. Only 70–90% of the anti-*lac* specific cells bound to the antigen coated acrylamide beads. This was thought to be due to low affinity for phenyl-β-lactoside antigen of the anti-*lac* antibody expressed on the surface of the cells, or the surface antibodies being stripped away as the cells passed over the beads. In the presence of *lac*-hapten nearly all anti-*lac* plaque forming cells were recovered in the filtrate. Non-specific binding of cells to beads was overcome to only a limited extent using this method. Again, this procedure was effective for removing cells with a particular specificity, but not for recovering antigen specific cells at high purity.

In 1971, Edelman *et al* published a method to isolate antigen specific cells using nylon fibres (Edelman *et al.*, 1971). In this method, Dnp_{38}-BSA, $tosyl_{30}$-BSA and BSA antigens were covalently coupled to transparent nylon fibres. Spleen cells collected from mice immunised with Dnp_{38}-bovine γG immunoglobulin, $tosyl_{30}$-BSA

and unconjugated BSA were incubated with the antigen coupled fibres. The adsorbed antigen specific cells were removed from the fibres either mechanically, by plucking the taut fibres, or chemically, by incubating with competitive inhibitors. The viability of the cells that were removed from the fibres was 80-90%. Although antigen coupled fibres bound many more immunised spleen cells than unimmunised spleen cells, there was significant non specific binding, as shown by the number of cells remaining on the fibres after addition of soluble antigen to inhibit the antigen specific binding. The antigen specific purity of the eluted cell population was estimated to be 63-88%, in terms of cross reactivity with a different hapten, but the data do not allow assessment of the absolute purity of the antigen specific cells.

Non specific binding of cells to the immunoadsorbent and removal of bound cells without affecting cell viability and function are the two major obstacles in isolating antigen specific cells using solid phase immunoadsorbents (Haas *et al.*, 1974). Haas and his colleagues described the use of antigen coated gelatin as an immunoadsorbent for cell fractionation in an attempt to reduce these limitations. Dinitrophenyl (DNP) and fowl IgG (FGG) immunised mouse spleen cells were isolated on antigen coated gelatin surfaces. The bound cells were recovered with relatively little loss of viability by melting the gelatin at 37°C. The specificities of the bound cell populations were 53% and 35% for DNP and FGG respectively in terms of cross reactivity. Furthermore, only four fold enrichment of antibody secreting cells was seen in the isolated cell population.

In 1984, Pike and Nossal tested the capacity of four fluorescein (FLU) haptenated T-independent antigens [*E. coli* lipopolysaccharide (LPS), *Brucella abortus,* Ficoll and polymerized flagellin] to cause activation, proliferation and differentiation of B cells

into antibody secreting cells (Pike and Nossal, 1984). In this experiment, FLU specific B cells were isolated from mouse spleen cells by incubating the mouse spleen cells in petri dishes coated with FLU-gelatine and removing the bound cells by melting the gelatine. FLU specific B cells were cultured at limiting dilution and antibody production was assessed by haemolytic anti-FLU plaque-forming assay. Only up to 12% and 8% of isolated B cells produced anti FLU plaques when stimulated with FLU-LPS and FLU-Ficoll respectively. The yield and enrichment were not reported.

Hapten, 2,4,6-trinitrophenyl (TNP) and FITC specific B cells were isolated from primed mouse spleen cells using panning with haptenated gelatin, in order to assess antigen presentation (Liano and Abbas, 1987). The isolated B cells were cultured *in vitro* and antibody secretion was measured by anti TNP and FITC plaque forming assays. If plaque formation following *in vitro* culture is considered to reflect the purity of the isolated B cells, the purity of the TNP specific B cell population was only about 8%.

Steenbakkers *et al* in 1993 isolated HIV specific B cells from spleen cells of mice immunised with HIV antigens by panning on antigen coated culture plates (Steenbakkers *et al.*, 1993). The isolated antigen specific B cells were fused with a myeloma cell line and cultured at limiting dilutions to produce antibody secreting hybridomas. By measuring antibody production, the yield and purity of the isolated HIV specific B cells were assessed as 5% and 24% respectively.

De Wildt and colleagues reported the isolation of single B cells for the production of monoclonal antibody fragments (de Wildt *et al.*, 1997). They isolated U1 ribonucleoprotein (U1 RNP) auto-antigen specific B cells from peripheral blood of

patients with systemic lupus erythematosus (SLE) by incubating the cells in U1 RNP coated culture wells and removing the bound cells with trypsin. The harvested cells were labelled with B cell markers and were sorted as single B cells into microcultures, which were used for proliferation assays for immunoglobulin gene cloning. When the supernatants of the B cell cultures were tested for specific antibody secretion, only 0.5-1.5% of B cell cultures were positive for anti U1 RNP specific antibodies. This reflects the low purity of the isolated antigen specific B cell population or a low proportion of antigen binding B cells differentiating into antibody secreting cells.

Many other research groups have used immnoadsorbent techniques to isolate antigen specific B cells. However, in most of these studies, the specificity of the isolated cells was either not recorded or has not been measured using independent specificity testing methods (Alderson *et al.*, 1987; Lang *et al.*, 1990; Persson *et al.*, 1991; Steenbakkers *et al.*, 1994; Schilizzi *et al.*, 1998).

1.13.1.2 Rosetting techniques (Table 1.5 B)

1.13.1.2.1 Rosetting with antigen coated red blood cells

The rosetting technique for isolation of antigen specific cells was first reported by Brody in 1970 (Brody, 1970). The basic principle of this technique is to fractionate antigen specific lymphoid cells by incubating cells with antigen coated red blood cells to form rosettes. The rosettes can then be separated from non-rosetted cells by sedimentation on a density gradient. In the initial experiments, unprimed mouse spleen and bone marrow cells were incubated with sheep red blood cells (SRBC), and rosettes were separated using a zonal sedimentation technique. Purity of the isolated antigen specific B cells was not determined. This study demonstrated synergy

between antigen binding cells and the cells that did not directly bind the antigen, but was not effective as a purification method, because non rosetting cells were not separated effectively from rosettes in the sedimentation method used.

In 1973, Wilson described a velocity sedimentation procedure to isolate SRBC specific B cells by rosette formation (Wilson, 1973). Spleen cells from immunised mice were incubated with SRBC to form rosettes. The rosettes were purified by velocity sedimentation, and rosette enriched and rosette depleted cell populations were injected in various combinations into irradiated mice. After 7 days, haemolytic plaques assays were performed on spleen cells of these mice to determine the generation of anti-SRBC antibody secreting cells. The purity of the isolated SRBC specific B cells was not determined. However, it was estimated that enrichment of SRBC cells in the rosette forming population was about 12 fold.

Walker *et al* in 1977 reported a negative selection method for purifying antigen-specific cells by rosetting and removing non-antigen specific cells from cell populations (Walker *et al.*, 1977). Turkey gamma globulin (TGG) immunised mouse B cells were initially incubated with large amounts of TGG to trigger endocytosis of the surface receptor Ig of antigen specific B cells by capping with the antigen. The non-antigen specific B cells were removed from the antigen specific B cell population by rosetting with red cells coated with anti mouse $F(ab')_2$ Ig. Following *in vitro* culture for 5-6 days, only 0.1% of the isolated B cells produced anti TGG plaques. This suggests a very low purity or a very low proportion of antigen specific cells transforming into antibody secreting cells in culture. However, the antigen specific cells were enriched 64 - 132 fold. The potential advantage of this indirect method is

the ability to purify antigen specific cells without binding to another cell population for rosette formation.

The rosetting technique was used to fractionate thymus independent antigen binding B cells from unprimed mouse spleen cells (Snow *et al.*, 1983). TNP specific B cells were isolated by using trinitrophenylated-horse red blood cells (TNP-HRBC) to form antigen specific rosettes. Several purification steps were performed to increase the purity of the rosette forming cells. At the end of five short centrifugation steps, 40% of cells were rosette forming cells. After centrifugation through a Percoll gradient the proportion of cells forming rosettes increased to as much as 70%. The isolated cells were cultured for 18 hours and stained with TNP-FITC to assess antigen binding ability. About 40% of the cultured cells stained with TNP-FITC, and the cultured cells did not bind unrelated antigens. In the presence of free antigen, 95% of the cultured cells did not stain with TNP-FITC. This study was important in that it used an independent test for purity with inhibition of binding by free antigen to confirm specificity. Purity was about 40%, a major improvement on earlier studies.

In 1986, Myers *et al* described a modified rosetting technique to isolate antigen binding virgin and memory B cells (Myers *et al.*, 1986). The method reported by Snow *et al* in 1983 was modified further, using different buffering systems, changes to the centrifugation steps and use of red cells with different haptenation densities to separately isolate virgin and memory B cells. However, the purity was assessed in terms of ability to form rosettes. After the final purification step, the mean purity of TNP antigen binding virgin B cells from unprimed mouse spleen cells was 71%. The mean purity of TNP antigen binding memory B cells from immunised mouse spleen cells was nearly 60%. Following overnight culture of isolated B cells, rerosetted B

cell percentages decreased to 56% for virgin cells and 30% for memory cells. A direct correlation between the haptenation density of red cells and the purification and functional activity of B cells was demonstratetd. It was suggested that the shorter incubation period with the antigen and lower hapten densities prevented B cell activation.

1.13.1.2.2 Rosetting with antigen coated magnetic particles

More recent studies have reported the use of antigen coated immunomagnetic beads to isolate specific B cells from human peripheral blood. In 1988, Egeland *et al* isolated rheumatoid factor (RF) positive B cells from patients with rheumatoid arthritis and from healthy blood donors by incubating peripheral blood mononuclear cells (PBMC) with HSA and anti HSA IgG coupled supraparamagnetic particles to form RF specific B cell rosettes (Egeland *et al.*, 1988). Non rosetting cells were separated by placing the mixture in a magnetic field. Although the numbers of RF specific B cells in peripheral blood are small, a high yield of RF positive cells was achieved with 10^3-10^4 fold enrichment. When RF positive cells were transformed by Epstein-Barr virus, more than 92% of isolated cells produced RF antibodies in ELISA plaque assays.

Tetanus toxoid (TT) binding B cells were isolated from blood from immunised donors by incubating cells with TT coated immunomagnetic beads and detecting rosette formation under a microscope (Oshiba *et al.*, 1994). The results indicated that 0.34% of B cells bound with TT antigen. This mean percentage increased to 2% following booster immunisation. Preincubating the cells with free antigen inhibited 90% of rosette formation.

Irsch *et al* in 1995 developed a new method to isolate low frequency antigen specific B cells by high gradient magnetic activated cell sorting (MACS) (Irsch *et al.*, 1995). Peripheral blood mononuclear cells from patients with severe allergy to bee venom or *Parietaria officinalis* were incubated either with digoxigenin conjugated phospholipase A2 (PLA_2) or *Paro*I (PLA_2 and *Paro*I are major allergens of bee venom and *Parietaria officinalis* respectively). The cells were then labeled with anti-digoxigenin antibodies coupled to supraparamagnetic microbeads (MACS) and phycoerythrin (PE) conjugated anti-digoxigenin antibodies. Allergen binding cells were separated in magnetic columns and analysed by multiparameter flow cytometry. According to flow cytometric analyses, the frequencies of PLA_2 and *Paro*I binding cells were 1.2% and 0.3% in the blood of allergic donors. These values increased to 66% and 39% respectively, following MACS enrichment. The purities of the allergen binding cell populations were reported as 75%. Allergen binding cells consisted of B cells, plasma cells and allergen specific IgE antibodies bound to basophilic granulocytes.

In 1999, Leyendeckers and his colleagues combined two step immunomagnetic enrichment (MACS) with multiparameter flow cytometry to isolate and characterise TT specific memory B cells from blood from immunised human adult donors (Leyendeckers *et al.*, 1999). Immunomagnetically preselected CD19 positive B cells were incubated with TT conjugated microbeads to isolate TT binding B cells. These B cells were enumerated and further characterised by multiparameter flow cytometric cell sorting. On average, 10% of the enriched TT binding B cells were positive when stained with TT conjugated PE. Results for inhibitory assays were not presented, but were stated to show specificity of the TT binding B cells. An indirect assessment of yield suggested that 98% of antigen specific cells in the sample were recovered. They

obtained up to a 20,000 fold enrichment of TT specific B cells. Furthermore, Leyendeckers *et al* also showed similar results for isolation of PLA_2 specific B cells from blood from patients with severe allergy to wasp venom.

In another experiment, TT specific B cells were isolated from *in vitro* immunised adult PBMC by Zafiropoulos *et al* in order to study induction of somatic mutations (Zafiropoulos *et al.*, 2000). *In vitro* immunised PBMC were incubated with biotinylated TT, and subsequently antigen binging cells were isolated by streptavidin coated paramagnetic beads (MACS). On flow cytometric analysis, 85% of these isolated cells were TT binding CD19 positive B cells. This could be considered as the true purity of the isolated TT specific B cells.

Immunomagnetic rosetting methods have been widely used to separate antigen specific B cells, resulting in satisfactory yields and purities of over 50% (Woodard *et al.*, 1995; Irsch *et al.*, 1999). However, in most instances, confirmatory tests have not been performed to assess the purities of the isolated specific cell populations (Lenzner *et al.*, 1998; Petrarca *et al.*, 1999; Lucas *et al.*, 2001; Kramer, 2002; Srivastava *et al.*, 2002; Zhou *et al.*, 2002).

1.13.1.3 Staining with fluoresceinated antigen and isolation by flow cytometry (Table 1.5 C)

The cell sorting technique to isolate antigen specific cells was first described by Julius *et al* in 1972 (Julius *et al.*, 1972). Mouse spleen cells primed for keyhole limpet hemocyanin (KLH), HSA or human gammaglobulin (HGG) were labelled either by a direct or indirect immunofluorescence method, and were then separated using flow cytometric cell sorting to isolate antigen binding cells. Sorted cell fractions contained 40-52% fluorescein conjugated KLH antigen binding cells. This was a 400-500 fold

enrichment of antigen binding cells compared to the original cell populations. Antigen binding cell percentages for HSA and HGG antigens in the sorted populations were 55-65%. However, the enrichment factors for these two antigens were only 42-62 fold, as there were greater numbers of fluorescent cells in the starting populations. No independent test of purity, or inhibition by unlabelled antigen, was described, leaving open the possibility of undetected non specific binding.

Greenstein and his colleagues in 1980 demonstrated the use of flow sorting techniques to isolate TNP binding B cells in studies of functional B cell heterogeneity (Greenstein *et al.*, 1980). In this experiment, unprimed mouse spleen B cells were stained with fluoresceinated trinitrophenylated bovine serum albumin (FL-TNP-BSA) and fluorescence positive cells were sorted. Fluorescence positive cells formed about 6% of the purified B cell population. When fractionated cell populations were examined by fluorescence microscopy, 90% of the positively sorted cells were fluorescence positive whereas the negatively sorted cells were 5% fluorescence positive. Nearly 0.5% of the unstained control population was also fluorescence positive. This could be due to cell auto-fluorescence, and needs to be considered as a background fluorescence level. Performing inhibitory staining assays by pre-incubating B cells with TNP-BSA showed that there was nearly 9% non-specific fluorescence staining. Therefore, the purity of the TNP-BSA specific B cells in the sorted population could be estimated to be approximately 82%.

In 1987, Hayakawa *et al* reported the isolation of phycoerythrin (PE) binding memory B cells from immunised mouse spleen cells by cell sorting (Hayakawa *et al.*, 1987). Three months after immunisation, 0.02-0.05% of the spleen memory B cells were positive for PE binding. Although a PE binding memory cell subpopulation was not

detected in unprimed control spleen cells, non specific binding cannot be ruled out. Inhibitory assays were not performed to determine the purity.

Immunofluorescence and cell sorting was used to isolate ovalbumin (OA) and helix pomatia haemocyanin (HPH) specific splenic B cells from immunised mice (Hoven *et al.*, 1989). The yields and purities of the isolated ovalbumin and HPH binding B cell populations were 1%, 3.5% (yield) and 42%, 84% (purity) respectively. About 20-50% of the isolated antigen binding B cells resulted in antibody production in ELISPOT assay. This was explained as possibly being due to the antigen binding cell population consisting of lymphoblasts and plasma cells in addition to antigen specific memory B cells. It was noted that thorough washing of the cell suspension prior to fluorescein labeling was necessary to prevent non specific binding, since antibodies present in the cell suspension against the vaccine antigen bound to almost all B cells through Fc receptors and formed immune complexes with the antigen.

In 1992, Lalor *et al* used multiparameter flow cytometric cell sorting to isolate antigen specific memory B cells from 4-hydroxy-3-nitriphenyl acetyl (NP) immunised mouse spleen cells to functionally characterise NP specific B cell clonal involvement (Lalor *et al.*, 1992). When IgG^+ IgM^- NP binding B cells were sorted into limiting dilution cultures, only 2.5% of input cells secreted anti NP IgG antibodies.

Dextran specific B cells were isolated from spleen cells from immunised mice to examine the involvement co-signaling pathways in the antibody response (Specht *et al.*, 1999). Spleen cells were stained with the B cell marker B220 and FITC conjugated dextran and subsequently were analysed by flow sorting. Approximately 17% of cells were dextran binding B cells. When cells were preincubated with excess

amounts of unconjugated dextran, the antigen binding B cell population was reduced to background levels of about 5%. This demonstrated the specificity of the antigen binding B cells. The purity of the sorted dextran specific B cells could be calculated as 70%. The yield and enrichment was not recorded.

In order to quantitatively study memory B cell development upon antigen rechallenge and maintenance of the memory B cell compartment, McHeyzer-Williams isolated NP specific memory B cells, based on B220 and CD138 expression from immunised mouse spleen cells by flow cytometry (McHeyzer-Williams *et al.*, 2000). The purity of the isolated memory B cell population was not reported. However, in their experiments, only 55% of the isolated NP specific CD138 positive cell subpopulation (plasma cells) secreted anti NP antibodies in ELISPOT assays. If the results of the ELISPOT assays are considered to reflect the purity of the isolated NP specific plasma cells, the purity of the isolated cells appears low. The yield and enrichment factors were not reported.

A single epitope multiple staining immunofluorescence technique was developed to detect very small numbers of antigen specific B cells by Townsend *et al* (Townsend *et al.*, 2001). Spleen cells from mice transgenic for hen egg white lysozyme (HEL) specific immunoglobulin were stained with FITC conjugated HEL and biotin conjugated HEL, and were then analysed by flow cytometry. Double positive B cells were sorted as antigen specific cells. This reduced the chances of detection of non-specific antigen binding leading to inclusion of false positive cells which causes high background. However, independent confirmatory tests were not performed to determine the purity of the isolated population. The sensitivity of this method was

estimated as one antigen specific B cell in 10 million splenocytes. This method could be considered as extremely sensitive and specific, and is worthy of wider application.

Despite the very high cost of the instrument, flow cytometry has been a popular technique for isolating antigen specific B cells. However, most publications have not reported independent assessment of the purities of the selected populations (Schittek and Rajewsky, 1990; Smith *et al.*, 1997; Vinuesa *et al.*, 2001; Youngman *et al.*, 2002).

Table 1.5 A

Capture on an antigen coated solid matrix

Authors	Year	AgSC separation technique	Antigens	Species	Cell population	Comments
Wigzell *et al*	1968	Immunoadsorbent with antigen coated glass, plastic beads	HSA, BSA, OA	Mouse	Immunised lymph node cells	Yield 60-95%, Enrichment 2.5 fold Viability 85%, Non-specific binding
Truffa-Bachi *et al*	1970	Immunoadsorbent with antigen coated acrylamide beads	*lac* hapten	Mouse	Immunised spleen cells	Yield 70-90%, Non-specific binding
Edelman *et al*	1971	Immunoadsorbent with antigen coated nylon fibres	Dnp_{38}, $Tosyl_{30}$, BSA	Mouse	Immunised spleen cells	Purity 63-88%*, Viability 80-90% Non-specific binding up to 33%
Haas *et al*	1974	Panning with antigen coated gelatin	DNP, FGG	Mouse	Immunised spleen cells	Purity 35-53%*, Enrichment 4 fold Viability 95-100%
Pike *et al*	1984	Panning with antigen coated gelatin	FLU	Mouse	Unprimed spleen cells	Purity 12%, 8%
Liano *et al*	1987	Panning with antigen coated gelatin	TNP	Mouse	Immunised spleen cells	Purity 8%
Steenbakkers *et al*	1993	Panning with antigen coated plates	HIV antigen	Mouse	Immunised spleen cells	Yield 5%, Purity 24%
De Wildt *et al*	1997	Panning with antigen coated plates	U1 RNP	Human	SLE patient PBL	Purity 0.5-1.5%

*Not determined by confirmatory testing. AgSC: antigen specific cells

Table 1.5 B

Rosetting techniques

Authors	Year	AgSC separation technique	Antigens	Species	Cell population	Comments
Brody	1970	Rosetting with SRBC	Not used	Mouse	Unprimed spleen, marrow cells	Yield < 0.1%, Non-specific binding
Walker *et al*	1977	Rosetting non-ASC (Neg. selection)	TGG	Mouse	Immunised B cells	Purity 0.1%, Enrichment 64-132 fold
Snow *et al*	1983	Rosetting with Ag coated HRBC	TNP	Mouse	Unprimed spleen cells	Yield 70%, Purity 40%
Myers *et al*	1986	Rosetting with Ag coated HRBC	TNP	Mouse	Primed / unprimed spleen cells	Purity Virgin 71%*, Memory 60%* After culture purity 56%* and 30%*
Egeland *et al*	1988	Immunomagnetic rosetting	RF	Human	RA patient PBMC	Purity 92%, Enrichment 10^3-10^4 fold
Oshiba *et al*	1994	Immunomagnetic rosetting	TT	Human	Immunised B cells	Yield 2%, Purity 90%,
Irsch *et al*	1995	MACS and flow cytometric analysis	PLA_2 *Paro*I	Human	Allergic patient PBMC	Purity up to 75%, Enrichment PLA_2 55 fold, *Paro*I 120 fold
Leyendeckers *et al*	1999	MACS and flow cytometric analysis	TT	Human	Immunised B cells	Yield 98%, Purity 10% Enrichment 20,000 fold
Zafiropoulos *et al*	2000	MACS and flow cytometric analysis	TT	Human	Immunised PBMC	Purity 85%

*Not determined by confirmatory testing. AgSC: antigen specific cells.

Table 1.5 C

Staining with fluoresceinated antigen and isolation by flow cytometry

Authors	Year	AgSC separation technique	Antigens	Species	Cell population	Comments
Julius *et al*	1972	Flow cytometric cell sorting	KLH HAS, HGG	Mouse	Immunised spleen cells	Purity KLH 40-52%* HAS, HGG 55-65%*
Greenstein *et al*	1980	Flow cytometric cell sorting	TNP	Mouse	Unprimed spleen B cells	Purity 82% Enrichment 15 fold
Hayakawa *et al*	1987	Flow cytometric cell sorting	PE	Mouse	Immunised spleen B cells	PE binding up to 0.05%
Hoven *et al*	1989	Flow cytometric cell sorting	HPH, OA	Mouse	Immunised spleen B cells	Purity HPH 84%,* OA 42%* 20-50% AgSC secreted Ig
Lalor *et al*	1992	Flow cytometric cell sorting IgG+IgM- NP+ B cells	NP	Mouse	Immunised spleen cells	Purity 2.5%
Specht *et al*	1999	Flow cytometric cell sorting	Dextran	Mouse	Spleen cells	Purity 70%
McHeyzer-Williams *et al*	2000	Flow cytometric cell sorting	NP	Mouse	Immunised spleen cells	Yeild, Purity not recorded 55% plasma cells secreted Ig
Townsend *et al*	2001	Flow cytometric cell sorting by single epitope multiple staining	HEL	Mouse	Transgenic mouse spleen cells	Highly sensitive Yield, purity not reported

*Not determined by confirmatory testing. AgSc: antigen specific B cells.

The three principal techniques of isolation of antigen specific B cells discussed above have their own advantages and disadvantages. Methods using cell capture on an antigen coated solid matrix have separated antigen specific B cells with purities ranging from 0.5% to 88% (Edelman *et al.*, 1971; de Wildt *et al.*, 1997). Rosetting techniques have provided purities from 0.1% to 90% (Walker *et al.*, 1977; Egeland *et al.*, 1988). The purities achieved by staining with fluorescent antigen and isolation with flow cytometric cell sorting have been 2.5% to nearly 85% (Hoven *et al.*, 1989; Lalor *et al.*, 1992). This wide discrepancy of purities within each technique may be due to the isolation of B cell populations specific for different antigens and the use of differing methods for assessing specificity of the isolated B cells.

The lowest purities have been recorded when the specificity was tested by plaque forming assays from cultures of isolated B cells (Walker *et al.*, 1977; Liano and Abbas, 1987; Lalor *et al.*, 1992; de Wildt *et al.*, 1997). This may not reflect the true purity of the isolated population however, since only a proportion of antigen specific B cells may differentiate into antibody secreting cells in culture. The small proportions of antibody secreting cells enumerated in plaque assays have been shown to increase in some experimental situations by use of combinations of cytokines and other stimulatory factors in the cell cultures (Pecanha *et al.*, 1991). Furthermore, different subpopulations of B cells have differing abilities to generate antibody secreting cells. For example, the marginal zone B cells in the spleen have been reported to proliferate and differentiate into plasma cells more rapidly than circulating B cells (Oliver *et al.*, 1999). The ability of marginal zone B cells to generate antibody secreting cells in response to TI antigen stimuli also is greater than that of follicular B cells (Oliver *et al.*, 1997; Oliver *et al.*, 1999). Therefore, when comparing and

analysing methods for isolation of antigen specific B cells in terms of yield and purity, it is important to consider the cell populations and the type of antigen used in the isolation.

Purity and enrichment are the two key factors that determine the efficiency of any isolation technique. The other important features of a good purification method are the maintenance of cell viability and function, reproducibility and general applicability. The degree of purity required will depend on the intended use of the cells. Some of the methods discussed were designed primarily to remove cells reacting with a particular antigen, and were successful for that purpose. Other studies required enriched antigen specific populations for functional studies. However, recovery of pure antigen specific cells has proved to be difficult. When the properties of antigen specific cells are the subject of the study, it is necessary to determine the purity of the isolated population. It is therefore essential to test for purity by a process independent of the purification method. Inhibition of binding of the labelled antigen by unlabelled antigen is a useful control in fluorescence based assays. Specific functional assays, such as plaque assays on cultured antigen specific B cells, can ensure specificity, but may underestimate purity, since some antigen specific B cells may not differentiate into antibody secreting cells.

The main limitation in isolating antigen specific B cells in the techniques reviewed above has been the non-specific binding of cells, which in turn decreases the purity of the isolated population. There is no single method which provides a high yield of antigen specific B cells with very high purity.

The method that, in the analysis of the literature, shows the most promising isolation is purification by magnetic beads followed by flow cytometry (Irsch *et al.*, 1995; Leyendeckers *et al.*, 1999; Zafiropoulos *et al.*, 2000). Examination of the analytical flow cytometric patterns of these studies suggests that if magnetic separation was followed by flow sorting a high purity could be achieved. Use of flow cytometry in isolation also provides an important tool to study and analyse the properties and characteristics of the isolated cells.

1.14 Rationale of this project

As discussed in this Chapter, conjugation of PRP polysaccharide to a carrier protein changes the nature of the immune response to PRP antigen from TI to TD. This leads to the development of an antibody response in early infancy, dominance of IgG antibody production with a predominance of the IgG1 subclass, and development of immunological memory resulting in an enhanced secondary response. However, compared to a protein antigen response, PRP conjugate vaccines elicit a substantial proportion of IgG2 and considerable amounts of IgA2 antibodies. Furthermore, the antibody response to PRP conjugate antigen originates from a few virgin B cells (Barington *et al.*, 1996), limited repertoire diversity, low affinity and minimal somatic hypermutations with no significant affinity maturation.

These features indicate that the response to PRP conjugate vaccine antigens still contains some characteristics of the antibody response to pure (unconjugated) PRP antigen. Even though the antibody response to PRP conjugate antigens is improved, it still falls short of a full TD response. Although T cells activated by carrier protein

and cytokines secreted by them play an important role in stimulating PRP specific B cells, it is suggested that the B cells may not be as responsive to T cell help as protein antigen specific B cells (Mond *et al.*, 1995). However, the reasons for the "partial" TD response to PRP conjugate antigen are not clearly understood. It is possible that antigen specific B cell subpopulations are involved in the antibody responses to PRP conjugate antigens and protein antigens.

This research study was designed to develop techniques which might lead to improved methods of determination of B cell subset involvement in the antibody response to PRP conjugate antigen during early childhood and in adults following immunization with Hib conjugate vaccines.

1.14.1 Aims

1. Develop potential methods to identify, enumerate and characterise PRP specific B cells in response to Hib conjugate vaccine in young children and in adults and compare these with antigen specific B cells responding to a protein antigen.

2. Determine whether B cell involvement leading to the antibody response to PRP conjugate antigen is restricted to any distinct B cell subpopulation compared to that of a protein antigen.

To achieve these aims, there was a need to develop techniques to isolate PRP specific B cells with high purity and good yield. Thereafter, that isolation technique could be used to identify the phenotypes of PRP conjugate antigen specific B cells and pure protein antigen specific B cells to determine whether distinct B cell subpopulations

are involved in the antibody responses to a TD form of PRP antigen and to a pure TD antigen. B cell markers such as CD5 (B1/B2 subpopulation), CD19, CD20, CD21 (complement C3 binding), CD22, CD23, CD27 (memory B cell), CD43, CD45 (recirculating follicular B cells), HLD-DR, PNA (GC B cell) and BCR expression markers such as anti IgM, anti IgD and anti IgG could be used for phenotyping the antigen specific B cells.

CHAPTER 2

MATERIALS AND METHODS

General materials and methods are described here. More specific details are given in each chapter. All chemicals and reagents used were of analytical standard.

2.1 Buffers and solutions

Buffers and solutions were filter sterilised using 0.2 μm filters whenever required.

2.1.1 Phosphate buffered saline (PBS)

pH 7.2-7.6; Osmolality 281-297

NaCl 160 g, KCl 4 g,

Na_2HPO_4 23 g, KH_2HPO_4 4 g,

Anhydrous salts were dissolved in 20 litres of milli-Q water and stored at room temperature. For cell functional studies PBS preparations were sterilised by autoclaving.

2.1.2 PBS-Tween

PBS-Tween was prepared by adding 1 ml of Tween-20 to 2 litres of PBS (0.05% PBS-Tween).

2.1.3 PBS-Azide

PBS-Azide was prepared by adding 1 ml of 20% sodium azide to 2 litres of PBS (0.05% PBS-Azide).

2.1.4 PBS-BSA

1% PBS-BSA was prepared by dissolving 1 g of bovine serum albumin (BSA) in 100 ml of PBS.

3% PBS-BSA was prepared by dissolving 3 g of BSA in 100 ml of PBS.

2.1.5 Carbonate buffer (0.05 M)

Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g

Anhydrous salts were dissolved in 1 litre of milli-Q water and the pH was adjusted to 9.6

2.1.6 Casein solution

Casein solution was prepared by dissolving 10 g of sodium casein in 1 litre of PBS-Tween (1% Casein).

2.1.7 Conjugate buffer

Conjugate buffer was prepared by dissolving 1 g of BSA in 1 litre of PBS-Tween.

2.1.8 Tris buffer

Tris base 6 g, NaCl 2.9 g, $MgCl_2$ 0.5 g

Anhydrous salts were dissolved in 500 ml milli-Q water and the pH was adjusted to 9.5

2.1.9 Red cell lysis solution

pH 7.2-7.4 NH_4Cl 8.26 g, $NaHCO_3$ 1.0 g, EDTA 0.037 g

A 10× stock solution was prepared by dissolving the anhydrous salts in 100 ml of milli-Q water. The solution was diluted 1/10 in distilled water prior to use.

2.1.10 RPMI 1640

RPMI 1640 sterile culture medium (GIBCO™ Invitrogen Corporation) was purchased from Life Technologies, Victoria, Australia.

2.1.11 Fetal calf serum (FCS)

Sterile FCS was purchased from Commonwealth Serum Laboratories (CSL), Victoria, Australia and was heat inactivated for 30 minutes in a 56° C water bath. Aliquots of 50 ml were stored at -20° C.

2.1.12 Penicillin Streptomycin Glutamine (PSG)

10,000 U/ml penicillin, 10 mg/ml streptomycin, 200 mM L-glutamine

Benzyl Penicillin (~1600 U/mg) 3.125 g, Streptomycin sulphate 5 g

L-glutamine 14.615 g

Dissolved in 500 ml of milli-Q water and filter sterilised using 0.2 μm filter and then 5 ml aliquots were stored at -20° C.

2.1.13 RF 10

RF 10 sterile cell culture medium was prepared by adding 50 ml of heat inactivated FCS and 5 ml of PSG to 500 ml of RPMI 1640.

2.1.14 HAT

Media supplement (50×) lyophilised powder from Sigma Chemical Co., Missouri, USA. 5×10^{-3} M hypozanthine, 2×10^{-5} M aminopterin and 8×10^{-4} M thymidine.

Reconstituted with 10 ml of water for injection and stored at 2-8° C.

2.1.15 HT

Media supplement (50×) lyophilised powder from Sigma Chemical Co., Missouri, USA. 5×10^{-3} M hypozanthine and 8×10^{-4} M thymidine.

Reconstituted with 10 ml of water for injection and stored at 2-8° C.

2.2 Reagents

2.2.1 USFDA standard anti-Hib antibody serum

Standard human anti-Hib antibody serum (lot 1983) was purchased from the FDA, USA. It contains 60.9 μg/ml of anti-PRP IgG antibody and 70 μg/ml of total anti-PRP antibody.

2.2.2 Quality control serum for anti-PRP ELISA

Quality control high antibody serum was collected from an adult volunteer working in the Department of Paediatrics, University of Adelaide, on day 14 post immunisation with PRP-OMP vaccine (PedvaxHib® Merck Sharp & Dohme, New South Wales, Australia).

Quality control low antibody serum was made from pooling serum samples from five unimmunised adult volunteers in the Department of Paediatrics, University of Adelaide.

Quality control negative antibody serum was a long term stored sample collected from an unimmunised infant with Hib epiglottitis at treated previously at the Women's and Children's Hospital. This infant did not demonstrate detectable anti-PRP antibody levels by ELISA during the acute illness.

2.2.3 Reagents for immunofluorescence staining

All reagents were titrated to determine the concentration for optimal specific staining prior to use in inmmunofluorescence experiments (Table 2.1).

Table 2.1

Reagents for immunofluorescence staining

Reagent	Source	Catalogue No.	Remarks
Anti-human antibodies			
CD19-PE-Cy5 (CyChrome)	Pharmingen, CA, USA	30658X	Mouse IgG1, κ
Anti-mouse antibodies			
CD45R/B220-FITC	Pharmingen, CA, USA	01124A	Rat IgG2a, κ
Immunoglobulin-FITC $F(ab')_2$ (Gamma and light chains)	AMRAD biotech, VIC, Australia	985051020	Host: Sheep
Streptavidin-PE	Sigma Chemical Co., MO, USA	S-3402	

2.2.4 Reagents for ELISA

Second step antibody reagents were titrated to determine the concentration for optimal specific staining prior to use in ELISA experiments. All reagents are listed in Table 2.2.

Table 2.2

ELISA reagents

Name	Source	Catlogue No.	Remarks
Anti-human IgG immunoglobulin peroxidase conjugated affinity isolated (Gamma chain specific)	AMRAD Biotech, VIC, Australia	982033020	Host: Sheep
Anti-mouse immunoglubulin peroxidase conjugated affinity isolated (Gamma and light chain specific)	AMRAD Biotech, VIC, Australia	985033020	Host: Sheep
ABTS	ROCHE Diagnostics, IN, USA	BM 102946	

2.2.5 Mouse Serum

Fresh BALB/c mouse serum was obtained from a research project conducted by Dr. Christina Boros, Department of Paediatrics, University of Adelaide, South Australia.

2.2.6 Biotinylated tetanus toxoid

Biotinylated tetanus toxoid was obtained from a stock preparation made by Ms Claire Jessup for a research project conducted at the Child Health Research Institute, Women's and Children's Hospital, Adelaide, South Australia. Tetanus toxoid was conjugated to biotin succinimide ester based on methods described previously (Muzykantov *et al.*, 1986; Kenny *et al.*, 1990).

2.3 Vaccines

2.3.1 PRP conjugate vaccines

Liquid PedvaxHIB® vaccine (PRP-OMP; *Haemophilus influenzae* type b PRP conjugated to meningococcal outer membrane protein) (Merck Sharp & Dome, New South Wales, Australia). The vaccine (0.5 ml) contains 7.5 μg of purified PRP capsular polysaccharide covalently linked to 125 μg of *N. meningitidis* outer membrane protein component.

Hiberix™ vaccine (PRP-T; *Haemophilus influenzae* type b PRP conjugated to TT) (SmithKline Beecham Biologicals, Rixensart, Belgium). The vaccine (0.5 ml) contains 10 μg of purified PRP capsular polysaccharide covalently bound to 30 μg of TT.

HibTITER® vaccine (PRP-CRM; *Haemophilus influenzae* type b PRP conjugated to diptheria CRM_{197} protein) (Wyeth-Lederle, New York, USA). The vaccine (0.5ml) contains purified capsular polysaccharide of the Eagan *Haemophilus influenzae* type b strain 10 μg conjugated to 25 μg of diphtheria CRM_{197} protein (non toxic variant of diptheria toxin).

2.3.2 Tetanus toxoid vaccines

ADT® vaccine (diphtheria-tetanus toxoid) (CSL, Victoria, Australia). The vaccine (0.5 ml) contains TT 6 Lf and DT 2 Lf absorbed on to aluminium phosphate; thiomersal 0.01% w/v.

Infanrix® vaccine (DTPa; diphtheria-tetanus-acellular pertussis) (SmithKline Beecham Biologicals, Rixensart, Belgium). The vaccine (0.5 ml) contains DT 25 Lf, TT 10 Lf, pertussis toxoid 25 μg, pertussis filamentous haemagglutinin 25 μg, and pertactin 8 μg adsorbed on to aluminium hydroxide; phenoxyethanol as preservative.

2.4 Methods

2.4.1 Biotinylation of PRP

Purified PRP polysaccharide antigen was derivatised with biotin via adipic acid hydrazide groups by cyanogen bromide activation based on the method described by Vann and colleagues (Vann *et al.*, 1990).

Two milligrams of purified PRP polysaccharide (Aventis-Pasteur, Lyon, France) reconstituted in 2 ml of milli-Q water was adjusted to pH 10.5 with 0.1M NaOH. One hundred and sixty micrograms of cyanogen bromide (Sigma Chemical Co., Missouri, USA), freshly dissolved in acetonitrile (Sigma Chemical Co., Missouri, USA) at 10 mg/ml, was added to the PRP, and the reaction mixture was maintained at 4°C and pH 10.5. After 6 minutes, 400 µl of 0.5 M adipic acid dihydrazide (Sigma Chemical Co., Missouri, USA) in 0.5M Na_2CO_3 (pH 8.5) was added and the solution was stirred overnight at 4°C. The solution was desalted on a PD-10 Sephadex® G-25 M Column (Pharmacia Biotech, Uppsala, Sweden) using Milli-Q water and lyophilized using a Dynavac freeze drying unit model FD1.

Lyophilized PRP hydrazide was dissolved in 0.2ml of PBS, and added to 2.5mg of biotin-N-hydroxysuccinimide ester (Sigma Chemical Co., Missouri, USA) dissolved at 10mg/ml in anhydrous amine free dimethylformamide (Sigma Chemical Co., Missouri, USA) at 4°C. The mixture was stirred overnight at 4°C and desalted on a PD-10 Sephadex® G-25 M Column. The biotinylated PRP polysaccharide was eluted in 3.5 ml of Milli-Q water. The concentration was calculated as 2 mg of biotinylated PRP in 3.5 ml. For short-term storage the biotinylated PRP was stored at 4°C. Long term storage was at -70°C.

2.4.2 PRP biotinylation and antigenicity testing

The biotinylation process and the antigenicity of the PRP was tested by a streptavidin coated ELISA, based on the methods published by previous research groups (Barra *et al.*, 1988; Vann *et al.*, 1990) and techniques developed by Ms Leonie Dinan in the Department of Paediatrics, University of Adelaide (MPH thesis).

One hundred microlitres of biotinylated PRP or unbiotinylated PRP (control) at 1 μg/ml diluted in 3% PBS-BSA were added to the wells of a Nunc™ streptavidin coated ELISA plate (Nalge Nunc International, New York, USA) and incubated for 1hour at room temperature. The plate was washed with PBS-Tween and unbound sites were blocked with 3% PBS-BSA for 2 hours at 37°C in a humid chamber. The wells were again washed as before and 100 μl of differing dilutions of USFDA standard anti-Hib antibody serum and quality control negative serum diluted in 1% casein were added. The plates were incubated at room temperature for 3 hours and washed as before. Affinity isolated sheep anti-human IgG peroxidase conjugate diluted in 1:1000 in 1% casein was added (100 μl per well). After incubating for 1 hour at 37°C in a humid chamber the plates were washed with PBS-Tween. The colour reaction was developed by adding 100 μl of ABTS and 33% w/v hydrogen peroxidase (40 μl of H_2O_2 per 100 ml ABTS) to each well. Streptavidin bound biotinylated PRP-antibody complex was detected by the development of colour measured with a Dynatech MR7000 plate reader set at a wavelength of 410 nm. The readings were recorded when the optical densities of dilutions of USFDA standard serum 1:100 reached over 2.0 units or 1:250 reached over 1.5 units in 20-30 minutes. Optical densities four-fold greater than the background were considered as positive.

The specificity and reproducibility of the ELISA is as per the assay developed by Ms L. Dinan in the Department of Paediatrics, University of Adelaide (MPH thesis, Chapter 3, page 45). Here the coefficient of variation of the anti-PRP IgG assay is recorded as 4.8% to 15.9% and 5.2% to 16.7% for high and low quality control sera respectively.

2.4.3 PRP tyramination

Purified PRP antigen (Aventis-Pasteur, Lyon, France) was conjugated to tyramine (Sigma Chemical Co., Missouri, USA) using cyanogen bromide (Sigma Chemical Co., Missouri, USA) based on the method of Insel and Anderson (Insel and Anderson, 1976), to enhance the binding on to polystyrene ELISA plates in the antigen coating step of the anti-PRP antibody ELISA assay.

Fifty milligrams of cyanogen bromide freshly dissolved in milliQ water at 150 mg/ml were added to 1 mg of PRP in 1 ml of 0.1 M $NaHCO_3$, pH 10.2. The pH of the solution was maintained between 10 and 10.2 with 0.1 M NaOH for 6 minutes and 0.4 mg of tyramine in 1ml 0.1 M $NaHCO_3$ was added and the mixture was maintained at 20° C for 10 minutes. The pH of the mixture was then adjusted to 8.6 with 0.1 M NaOH and was dialysed using PBS and 0.01% methiolate in three 24 hour cycles at 4° C. The concentration of tyraminated PRP was calculated as 1 mg in the final volume at the end of dialysis. The tyraminated PRP antigen was stored at 4° C for short term usage. Long term storage was at -20° C.

A checkerboard titration analysis was performed to determine the optimum concentration of PRP-tyramine to be used in the anti-PRP ELISA as described in chapter 3. Differing concentrations of PRP-tyramine were assayed with dilutions of USFDA standard anti-Hib antibody serum, quality control high antibody serum and quality control negative serum samples. The PRP-tyramine concentration was determined to be optimal when the highest dilution (1/100) of USFDA standard anti-Hib antibody serum reading reached an optical density of 2.0 units at a wavelength of 410 nm in 20-30 minutes.

2.4.4 Separation of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from blood collected into lithium heparin tubes. Blood was diluted in equal volumes of PBS, overlaid on Lymphoprep™ (Nycomed, Oslo, Norway) at two volumes of diluted blood to one volume of Lymphoprep™ and centrifuged at 800g for 20 minutes between 10-20°C. The interface layer was carefully collected, diluted in at least in five parts of PBS and centrifuged at 2000g for 10 minutes. After another similar wash, the PBMC cells were resuspended in PBS.

2.4.5 Immunofluorescence staining

Two colour immunofluorescence staining was performed on human peripheral blood mononuclear cells/murine spleen leucocytes using biotinylated antigen, CD19-PE-Cy5 (human B cells marker) or CD45R/B220-FITC (murine B cell marker) and streptavidin-PE to identify antigen specific B cells. All samples were processed in parallel on each day. Staining was performed at 5×10^5 cells per FACS tube on melting ice, according to the layout given below (Table 2.3). Fifty microlitres of biotinylated antigen at 5 and 10 μg/ml concentrations were used to label antigen specific cells by binding on to the antigen receptor. To determine the specificity of the assay, 50 μl of unbiotinylated antigen at 100 μg/ml concentration was added to control tubes to competitively inhibit biotinylated antigen binding on to the antigen receptors. B cell lineage was stained with 5 μl of CD19-PE-Cy5 or CD45R/B220-FITC accordingly. Finally 50 μl of 1/50 dilution streptavidin-PE were added to identify antigen receptor bound biotinylated antigen. Each staining stage was

incubated for 30 minutes and washed using ice cold PBS-Azide containing 1% fetal calf serum.

Table 2.3

Immunofluorescence staining layout

Tube 1 (Control)	**Tube 2 (Test)**	**Tube 3 (Specificity)**	**Tube 4 (Test)**	**Tube 5 (Specificity)**
Ag (100 μg/ml)	Ag-biotin (5 μg/ml)	Ag-biotin (5 μg/ml)	Ag-biotin (10 μg/ml)	Ag-biotin (10 μg/ml)
B cell Marker	B cell Marker	Ag (100 μg/ml)	B cell Marker	Ag (100 μg/ml)
SAPE	SAPE	B cell Marker	SAPE	B cell Marker
		SAPE		SAPE

Ag: Antigen (PRP or TT), Ag-biotin: Biotinylated antigen (PRP-biotin or TT-biotin), SAPE: Streptavidin-PE

2.4.6 Flow cytometric analyses for detection of antigen specific B cells

Flow cytometric analyses were performed immediately after staining was completed. All samples were maintained at 4°C during analysis. Data were recorded using an COULTER EPICS® Elite ESP Flow cytometer / cell sorter (Coulter, Florida, USA). The machine was calibrated with Flow-Check™ Fluorosphere beads (Beckman Coulter, USA) prior to analysis. All cells in each tube were counted. The live cell population was gated on the scatter plot for each sample. Two colour (either FITC or

PE-Cy5 and PE) analysis was performed using either FL 1 (520-530 nm) or FL 4 (665-685 nm) and FL 2 (570-580 nm) channels respectively using an argon laser (488 nm). Compensation was adjusted using control tubes.

2.5 Statistical analyses

Data were analysed using the Microsoft Excel program. Data are presented as means where appropriate, with the standard deviation or standard error of the mean being shown. Statistical significance was determined by Student's paired t-test. $P < 0.05$ was considered significant.

2.6 Ethics

All experimental procedures were approved by the human and animal ethics committees at the Women's and Children's Hospital, and the University of Adelaide, Adelaide, South Australia.

The experiments were performed in the University Department of Paediatrics, University of Adelaide and the Child Health Research Institute at the Women's and Children's Hospital, South Australia.

CHAPTER 3

ATTEMPTED ISOLATION OF PRP SPECIFIC B CELLS FROM PERIPHERAL BLOOD FROM IMMUNISED ADULTS BY FLOW CYTOMETRY

3.1 Introduction

According to the literature reviewed in Chapter 1, the most promising antigen specific cell isolating technique would be magnetic cell separation combined with flow cytometric cell sorting. At the start of the project a magnetic cell separation apparatus was not available. Flow cytometric cell sorting by itself should be able to produce high purity antigen specific B cells, and was used initially with a view to combining with magnetic separation later if the results were sufficiently promising. Furthermore, flow cytometry should also be useful in analysing the detected antigen specific B cell population in terms of phenotyping and characterisation.

Flow sorting has been used widely for isolating antigen specific B cells for a range of antigens using immunofluorescence staining as reviewed in Chapter 1. TNP specific B cells and HPH specific B cells have been isolated from primed mouse spleen cells with purities of over 80% using this method (Greenstein *et al.*, 1980; Hoven *et al.*, 1989). Splenic cells specific for the TI type 2 antigen dextran have been separated from immunised mice with a purity of 70% by flow sorting (Specht *et al.*, 1999). However, the isolation of polysaccharide antigen specific B cells from humans by flow cytometry does not appear to have been reported in the literature.

In the experiments reported in this Chapter, immunofluorescence staining followed by flow cytometric cell analysis was performed on PBMC from Hib conjugate vaccine immunised adult human donors, in an attempt to establish a method for identifying PRP specific B cells with a high yield and purity. Immunised adult PBMC were used in this initial isolation as the immune system of the adults is believed to be more competent in producing polysaccharide antigen specific B cells compared to that of young children (see Chapter 1).

Immunised adult PBMC were stained with the B cell marker CD19 to identify cells of B lineage, and biotinylated PRP antigen followed by SA-PE was used to label B cells through the antigen specific B cell receptor of PRP specific B cells. A flow cytometric analysis was performed on stained PBMC to identify PRP specific B cells.

This chapter also describes a similar attempt to isolate TT specific B cells from immunised adult PMBC by immunofluorescence staining and flow cytometric analysis as a comparison to the isolation of PRP specific B cells. Immunised PBMC were stained with CD19 and biotinylated TT antigen followed by streptavidin-PE to identify TT specific B cells.

3.2 Methods

3.2.1 Recruitment of adult volunteers

Six healthy adult volunteers employed at the Women's and Children's Hospital, Adelaide, South Australia, were recruited for the study after obtaining written informed consent. None of the volunteers had experienced invasive Hib infection

previously. They were not on any immune suppressive drugs nor did they have any immune deficiency illness.

3.2.2 Immunisation with PRP conjugate vaccine

Three of the adult volunteers (V1, V2 and V3) received an intramuscular immunisation with a full dose of liquid PedvaxHIB®. The remaining three adults (V4, V5 and V6) were not immunised for the study, had not received Hib immunisation previously and functioned as the non vaccinated negative control group. One adult (V1) in the immunised group had had prior Hib immunisation with the same vaccine (Table 3.1).

Table 3.1

Characteristics of participating volunteers

Volunteer	Age (yrs)	Sex	Past Hib vaccination	Study Hib vaccination	Past invasive Hib infection
V1	33	Male	Yes (1½ yrs ago)	Yes	No
V2	36	Female	No	Yes	No
V3	25	Female	No	Yes	No
V4	33	Male	No	No	No
V5	24	Female	No	No	No
V6	35	Male	No	No	No

Median age, 33 years; range, 24-36 years

3.2.3 Blood sampling, serum and PBMC collection

Blood samples were collected from the immunised adults (V1, V2 and V3) prior to immunisation (day 0) and on day 5, 10, 14, 20 and 25 post immunisation (day 14 was used, as day 15 of the experiment fell on a public holiday). Blood samples were also collected in parallel from the unimmunised adults (V4, V5 and V6) on day 5, 10, 14, 20 and 25. Ten millilitres of blood were collected from each adult and 2 ml were transferred into serum collecting tubes and 8 ml into lithium heparin blood collecting tubes.

The blood samples in the serum collecting tubes were left at room temperature for 1 hour before being centrifuged at 2000 g for 10 minutes. Serum was then collected and stored at -20°C until anti-PRP antibody levels were measured.

Mononuclear cells were obtained from blood as described in Chapter 2. The cells were resuspended in PBS at a final concentration of 1×10^7 viable cells/ml.

3.2.4 Anti-PRP antibody levels in serum

Anti-PRP IgG antibody titres were measured by an ELISA technique previously developed in the University of Adelaide, Department of Paediatrics laboratory by Ms Leonie Dinan.

Costar® high binding ELISA plates (Corning Inc., New York, USA) were coated with 100 µl per well of optimal concentration of PRP-tyramine (1 µg/ml) diluted in 0.05 M carbonate buffer and incubated at 37°C for 90 minutes in a humid chamber and then at 4°C overnight. The wells were washed three times with PBS-Tween. Unbound sites were blocked with 1% casein solution for 2 hours at 37°C in a humid chamber.

The wells were again washed as before and 100 µl of differing dilutions of USFDA standard anti-Hib antibody serum, quality control serum and test serum diluted in 1% casein buffer were added. The plates were incubated at room temperature for 4 hours and washed as before. Affinity isolated sheep anti-human IgG peroxidase conjugate diluted 1/1000 in 1% casein solution was added (100 µl per well). After incubating for 1 hour at 37°C in a humid chamber the plates were washed with PBS-Tween. The colour reaction was developed by adding 100 µl of ABTS and 33% w/v hydrogen peroxidase (40 µl of H_2O_2 per 100 ml ABTS) to each well. Bound anti-PRP antibodies were detected by the development of colour measured with a Dynatech MR7000 plate reader set at a wavelength of 410 nm. The readings were recorded when the optical density of the highest concentration of the USFDA standard serum (1/100) reached more than 2.0 units with in 20-30 minutes.

An Immunoassay Data Management Program, Multicalc, (Pharmacia, Sweden) was used to quantitate the anti-PRP IgG antibody concentrations in the serum samples of the study volunteers. The results were expressed as µg of antibody/ml of serum in relation to the concentration of anti-PRP IgG antibody in the USFDA standard serum.

3.2.5 Immunofluorescence staining and flow cytometric analyses

Two colour immunofluorescence staining was performed on peripheral blood mononuclear cells as described in Chapter 2 using biotinylated PRP antigen (5 µg/ml and 10 µg/ml), CD19-PE-Cy5 and streptavidin-PE to identify PRP antigen specific B cells.

Flow cytometric analyses were performed immediately after staining was completed. PRP antigen specific B cells were expected to be double stained with CD19-PE-Cy5 and streptavidin-PE. All samples were processed in parallel on each day.

3.2.6 Blood sampling and PBMC collection for TT specific B cell isolation

Blood samples were collected from healthy adult volunteers employed at the Women's and Children's Hospital, immunised with a full dose of intramuscular ADT® vaccine (diphtheria-tetanus toxoid) as part of the risk management policy of the hospital. In Australia, children for many years have been routinely immunised with diphtheria-tetanus toxoid-pertussis vaccine at 2, 4, 6 and 18 months of age and booster immunisation with diphtheria-tetanus toxoid at 4 years. Booster vaccines are given during later teenage years. During adult life, further booster immunisations are given if injuries occur which potentially could lead to tetanus. All adult donors had been immunised in childhood against tetanus. Adult volunteers who had not been immunised recently with TT were recruited as negative controls. Ten millilitres of blood was collected from each adult volunteer into a lithium heparin tube after obtaining consent.

Mononuclear cells were separated from blood samples as described in Chapter 2. The cells were resuspended in PBS at 1×10^7 viable cells/ml.

3.2.7 Immunofluorescence staining and flow cytometry for TT specific B cell isolation

Two colour immunofluorescence staining was performed on PMBC using biotinylated TT (10 μg/ml), CD19-PE-Cy5 and streptavidin-PE as described in Chapter 2 to identify TT antigen specific B cells. In some immunofluorescence assays, cells were stained with additional markers as mentioned below.

Flow cytometric analyses were performed immediately after staining was completed. TT antigen specific B cells were expected to be double stained by CD19-PE-Cy5 and streptavidin-PE.

3.3 Results

3.3.1 Anti-PRP antibody response in blood following immunisation

An anti-PRP ELISA was performed to measure anti-PRP IgG antibody concentrations in blood at the time points of the sample collection for the immunofluorescence assays. The immunised adults (V1, V2 and V3) had anti-PRP IgG antibody titres that rose following immunsation (Fig. 3.1A). V1 had the highest starting anti-PRP IgG titre probably due to previous PRP immunisation, and reached an IgG antibody concentration of 13.56 μg/ml by day 14 post immunisation. The antibody titre decreased to 11.75 μg/ml on day 20 and rose again to 14.33 μg/ml on day 25. Although the starting anti-PRP IgG titre of V2 was lower than V1, there was a rapid response with a peak IgG antibody concentration of 12.48 μg/ml on day 20 post immunisation. V3 had the lowest anti-PRP IgG titre prior to immunisation and

demonstrated the weakest anti-PRP IgG antibody response after immunisation, with a maximum concentration of 3.38 μg/ml on day 20. The anti-PRP IgG antibody titres of the unimmunised adults V5 and V6 were below 2.00 μg/ml (Fig. 3.1B). The anti-PRP IgG antibody concentration of unimmunised adult V4 was slightly higher than the peak level for V3.

3.3.2 Flow cytometric analyses for PRP specfic B cell isolation

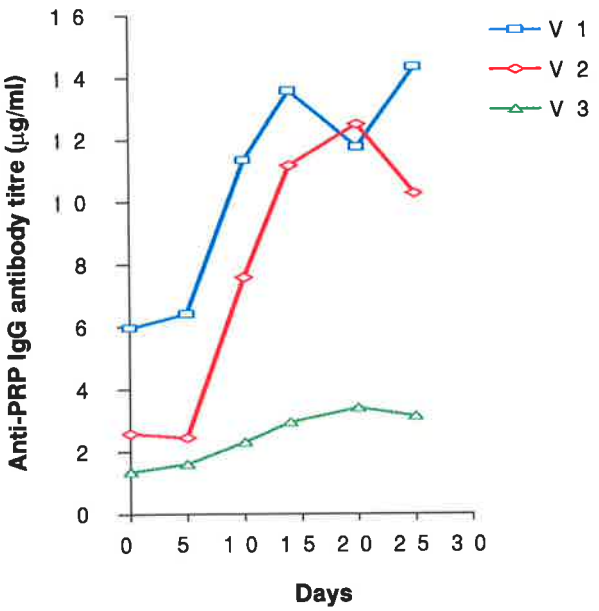
PRP specific B cells were not detected definitively by flow cytometry. Very low and variable percentages of B cells (less than 0.2%) bound streptavidin-PE in all sample analyses throughout the study. However, neither the streptavidin-PE reactive cell percentages nor the FACS patterns demonstrated any marked differences between the samples from immunised volunteers and the samples from unimmunised volunteers, or between test samples and control samples (stained with unbiotinylated PRP and streptavidin-PE) (Table 3.2 A-D).

The flow patterns of PBMC stained with PRP-biotin 5 μg/ml, streptavidin-PE and CD19-PE-Cy5 of V1 and V6 are shown and are representative of all results for all samples (Fig. 3.2 and Fig. 3.3-3.13). Since V1 demonstrated the greatest antibody response to PRP immunisation, it was expected that PRP specific B cells would be most likely to be detected in the flow cytometric analyses from day 14 onwards. However, PRP specific B cells could not be identified in these samples.

Inhibition of streptavidin-PE positive cells by use of excess amounts of unbiotinylated PRP to prevent binding of biotinylated PRP did not result in any significant or consistent inhibition throughout the study (Fig. 3.3-3.13).

Interestingly, the mean fluorescence intensity (MFI) of B cells binding with streptavidin-PE of immunised and unimmunised adults on few occasions on a some days demonstrated very high values compared to the values for the other days (Table 3.2A-D). When the flow cytometric dot plots of these samples were examined closely one or two very brightly streptavidin-PE positive events were seen on the right outer edge of the plot, resulting in the high MFI values. The MFI values of B cells binding with streptavidin-PE in all other samples were not significantly different between immunised and unimmunised samples or between test, inhibition and control samples.

A.



B.

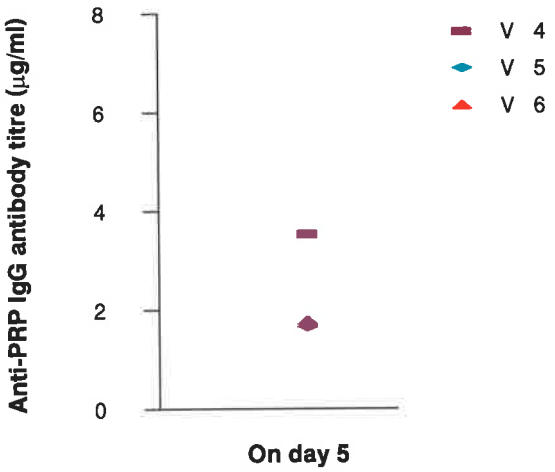
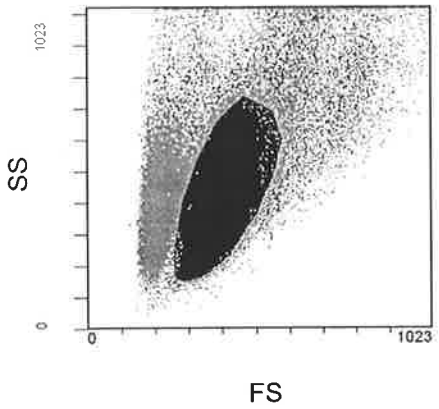


Fig. 3.1 Anti-PRP IgG antibody concentrations in serum of study participants. **A.** Anti-PRP titres of immunised volunteers (V1-V3) demonstrated rising titres with time following immunisation. VI and V2 had the greatest responses. **B.** Anti-PRP antibody titres of unimmunised adults (negative controls) tested on day 5 of the experiment. The titres were similar to the pre immunisation titres of immunised adults.

A.



B.

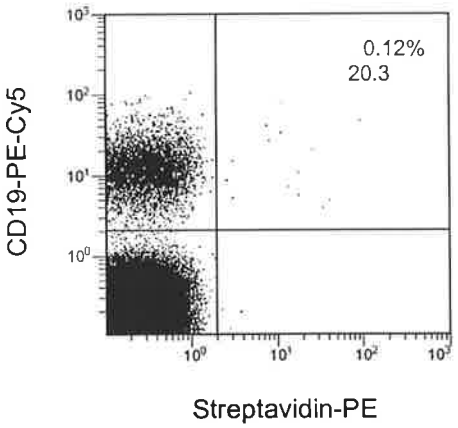


Fig. 3.2 Flow cytometric analysis of stained unimmunised PBMC of V6 (negative control) on day 5. **A.** Live lymphocytes gated according to light scatter properties (forward scatter vs side scatter) for two colour analysis. Similar gate patterns were applied in all unimmunised and immunised PBMC sample analysis. **B.** The cells stained with unbiotinylated PRP (100 μg/ml), CD19-PE-Cy5 and streptavidin-PE as a control to determine background staining. The percentage of B cells staining with streptavidin-PE was 0.12%. The MFI of the staining was 20.3. Similar background staining effects were seen in control samples at all time points.

Table 3.2 A

Percentage and MFI of B cells staining with streptavidin-PE in PBMC of immunised V1 donor

Day	Sample				Staining			
	Test sample PRP-Bi (5 µg/ml) CD19-PE-Cy5 SAPE		*Inhibition sample PRP-Bi (5 µg/ml) PRP (100 µg/ml) CD19-PE-Cy5 SAPE*		Test sample PRP-Bi (10 µg/ml) CD19-PE-Cy5 SAPE		*Inhibition sample PRP-Bi (10 µg/ml) PRP (100 µg/ml) CD19-PE-Cy5 SAPE*	
	B cell %	MFI	*B cell %*	*MFI*	B cell %	MFI	*B cell %*	*MFI*
Pre boost	0.11	19.5	*0.15*	*11.5*	0.10	15.4	*0.10*	*20.8*
Day 5	0.09	**108.1*** (8.4)	*0.05*	*12.7*	0.10	23.6	*0.05*	*21.1*
Day 10	0.07	**172.9*** (7.7)	*0.05*	*4.4*	0.04	**169.5*** (6.1)	*0.06*	*23.3*
Day 14	0.04	41.4	*0.06*	*9.0*	0.05	13.5	*0.15*	*14.4*
Day 20	0.12	9.1	*0.07*	*11.4*	0.03	4.4	*0.10*	*13.1*
Day 25	0.05	5.8	*0.06*	*8.4*	0.08	27.9	*0.17*	*12.6*

B cell %: Percentage of B cells stained with streptavidin-PE (SAPE).

MFI: Mean fluorescence intensity of PE channel of the double positive population.

* Very bright PE positive events at the outer margin of the dot plot. The MFI excluding the very bright signal is given within brackets.

In control PBMC samples stained with unbiotinylated PRP, CD19-PE-Cy5 and streptavidin-PE, the percentage of B cells staining with streptavidin-PE ranged from 0.05 - 0.12. The MFI ranged between 4.6 and 14.8.

Table 3.2 B

Percentage and MFI of B cells staining with streptavidin-PE in PBMC of immunised V2 donor

Day	Sample				Staining			
	Test sample		*Inhibition sample*		Test sample		*Inhibition sample*	
	PRP-Bi (5'µg/ml) CD19-PE-Cy5 SAPE		*PRP-Bi (5 µg/ml) PRP (100 µg/ml) CD19-PE-Cy5 SAPE*		PRP-Bi (10 µg/ml) CD19-PE-Cy5 SAPE		*PRP-Bi (10 µg/ml) PRP (100 µg/ml) CD19-PE-Cy5 SAPE*	
	B cell %	MFI	*B cell %*	*MFI*	B cell %	MFI	*B cell %*	*MFI*
Pre boost	0.16	6.2	*0.00*	*0.0*	0.06	7.2	*0.19*	*31.4*
Day 5	0.07	9.3	*0.08*	***140.0**** *(13.7)*	0.10	21.7	*0.08*	*6.7*
Day 10	0.05	29.3	*0.04*	*7.8*	0.11	11.7	*0.11*	*13.3*
Day 14	0.06	16.4	*0.18*	*8.1*	0.04	3.6	*0.06*	*6.4*
Day 20	0.05	5.3	*0.04*	*27.2*	0.10	5.1	*0.07*	*22.5*
Day 25	0.11	7.9	*0.08*	*8.1*	0.09	**101.1*** (9.1)	*0.19*	*36.8*

B cell %: Percentage of B cells stained with streptavidin-PE (SAPE).

MFI: Mean fluorescence intensity of PE channel of the double positive population.

* Very bright PE positive events at the outer margin of the dot plot. The MFI excluding the very bright signal is given within brackets.

In control PBMC samples stained with unbiotinylated PRP, CD19-PE-Cy5 and streptavidin-PE, the percentage of B cells staining with streptavidin-PE ranged from 0.06 - 0.16. The MFI ranged between 4.2 and 17.0.

Table 3.2 C

Percentage and MFI of B cells staining with streptavidin-PE in PBMC of immunised V3 donor

Day	Sample				Staining			
	Test sample		*Inhibition sample*		Test sample		*Inhibition sample*	
	PRP-Bi (5 μg/ml) CD19-PE-Cy5 SAPE		*PRP-Bi (5 μg/ml) PRP (100 μg/ml) CD19-PE-Cy5 SAPE*		PRP-Bi (10 μg/ml) CD19-PE-Cy5 SAPE		*PRP-Bi (10 μg/ml) PRP (100 μg/ml) CD19-PE-Cy5 SAPE*	
	B cell %	MFI	*B cell %*	*MFI*	B cell %	MFI	*B cell %*	*MFI*
Pre boost	0.05	18.2	*0.02*	*25.4*	0.14	24.8	*0.09*	*36.4*
Day 5	0.03	19.8	*0.08*	*5.4*	0.10	12.3	*0.09*	*27.0*
Day 10	0.04	4.0	*0.07*	*13.7*	0.05	7.6	*0.03*	*11.2*
Day 14	0.03	10.8	*0.05*	*23.2*	0.06	4.9	*0.08*	*6.5*
Day 20	0.08	9.7	*0.08*	*13.7*	0.10	6.0	*0.02*	*4.6*
Day 25	0.11	4.2	*0.13*	*7.0*	0.09	11.3	*0.16*	*4.1*

B cell %: Percentage of B cells stained with streptavidin-PE (SAPE).

MFI: Mean fluorescence intensity of PE channel of the double positive population.

In control PBMC samples stained with unbiotinylated PRP, CD19-PE-Cy5 and streptavidin-PE, the percentage of B cells staining with streptavidin-PE ranged from 0.04 - 0.12. The MFI ranged between 2.5 and 19.7.

Table 3.2 D

Percentage and MFI of B cells staining with streptavidin-PE in PBMC of unimmunised V6 donor (negative control)

Day	Sample				Staining			
	Test sample		*Inhibition sample*		Test sample		*Inhibition sample*	
	PRP-Bi (5 μg/ml) CD1-PE-Cy5 SAPE		*PRP-Bi (5 μg/ml) PRP (100 μg/ml) CD19-PE-Cy5 SAPE*		PRP-Bi (10 μg/ml) CD19-PE-Cy5 SAPE		*PRP-Bi (10 μg/ml) PRP (100 μg/ml) CD19-PE-Cy5 SAPE*	
	B cell %	MFI	*B cell %*	*MFI*	B cell %	MFI	*B cell %*	*MFI*
Day 5	0.14	7.8	*0.13* ***115.9****	*(15.4)*	0.17	8.3	*0.12*	*18.6*
Day 10	0.08	28.0	*0.09*	*15.9*	0.19	23.4	*0.11*	*26.2*
Day 14	0.13	12.5	*0.13*	*12.1*	0.16	21.3	*0.08*	*15.9*
Day 20	0.09	5.4	*0.15*	*14.9*	0.08	6.7	*0.06*	*9.2*
Day 25	0.13	16.3	*0.15*	*21.6*	0.18	7.5	*0.10*	*7.5*

B cell %: Percentage of B cells stained with streptavidin-PE (SAPE).

MFI: Mean fluorescence intensity of PE channel of the double positive population.

* Very bright PE positive events at the outer margin of the dot plot. The MFI excluding the very bright signal is given within brackets.

In control PBMC samples stained with unbiotinylated PRP, CD19-PE-Cy5 and streptavidin-PE, the percentage of B cells staining with streptavidin-PE ranged from 0.03- 0.13. The MFI ranged between 5.8 and 20.3.

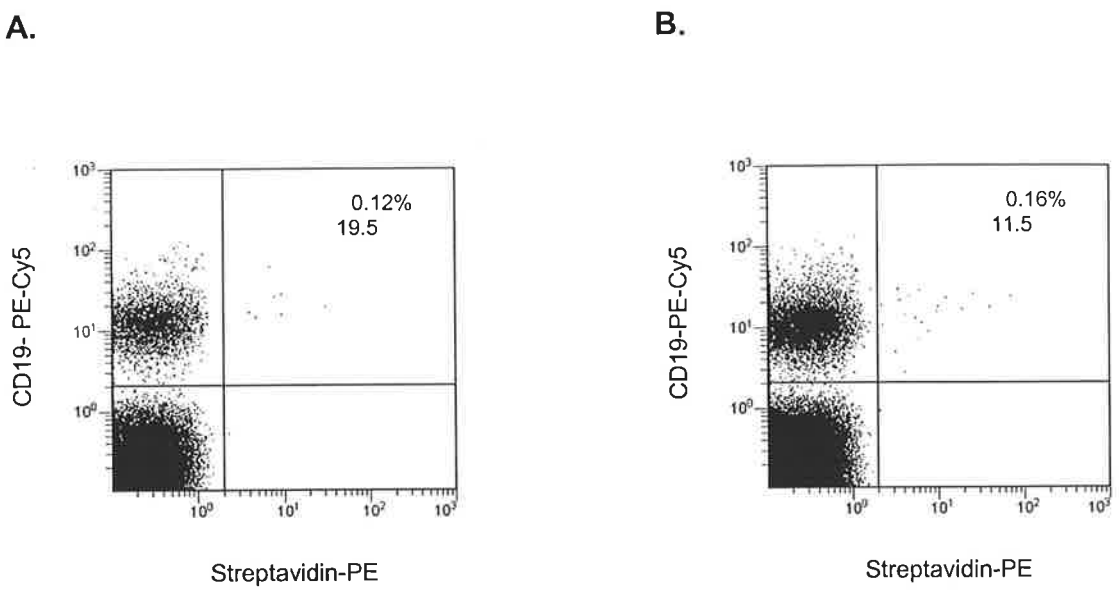


Fig. 3.3 Flow cytometric analysis of PBMC from V1 donor before immunisation . **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.12%. The MFI was 19.5. **B.** The cells stained with PRP-biotin (5 μg/ml), unbiotinylated PRP (100 μg/ml), CD19-PE-Cy5 and streptavidin–PE as an inhibition sample for testing specificity of the PRP specific cells. The percentage of B cells staining with streptavidin-PE was 0.16%. The MFI was 11.5. Total B cell populations in **A** and **B** samples were 7.69% and 10.07% respectively.

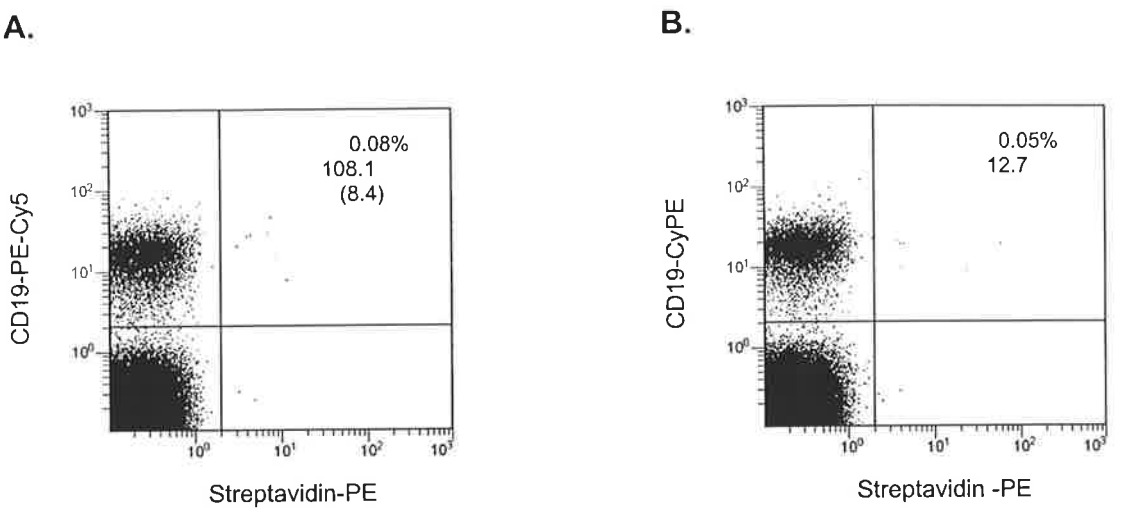


Fig. 3.4 Flow cytometric analysis of PBMC from V1 donor on day 5 post immunisation. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.09%. The MFI was 108.1. **B.** The Percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.06%. The MFI was 12.7. Total B cell populations in **A** and **B** samples were 7.69% and 10.07% respectively.

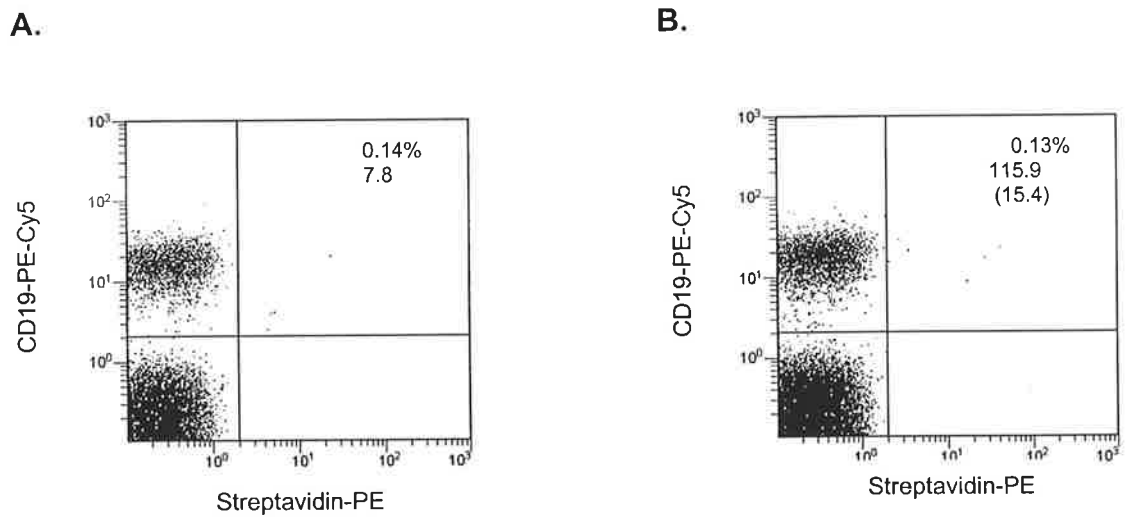


Fig. 3.5 Flow cytometric analysis of PBMC from unimmunised V6 donor (negative control) on day 5 of the experiment. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.10%. The MFI was 8.7. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.14%. The MFI was 15.9. Total B cell populations in **A** and **B** samples were 11.71% and 11.68% respectively.

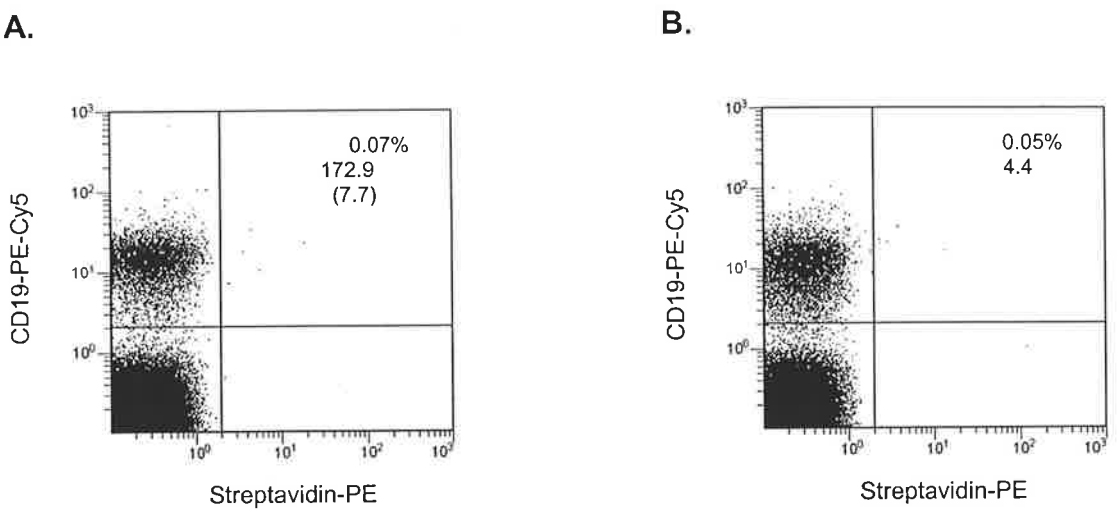


Fig. 3.6 Flow cytometric analysis of PBMC from V1 donor on day 10 post immunisation. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.07%. The MFI was 172.9. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.05%. The MFI was 4.4. Total B cell populations in **A** and **B** samples were 9.52% and 10.91% respectively.

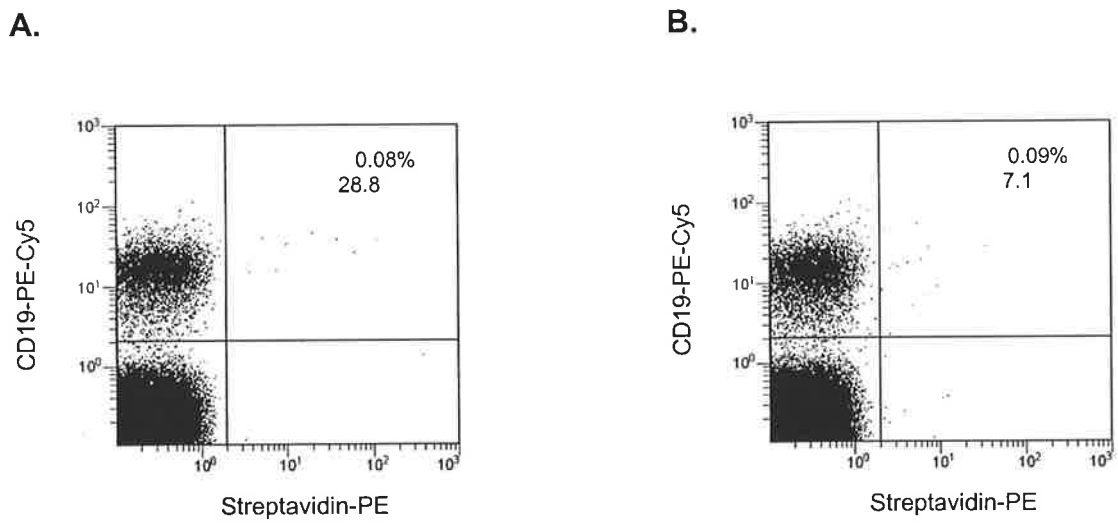


Fig. 3.7 Flow cytometric analysis of PBMC from unimmunised V6 donor (negative control) on day 10 of the experiment. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.08%. The MFI was 28.0. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.09%. The MFI was 7.1. Total B cell populations in **A** and **B** samples were 6.65% and 7.13% respectively.

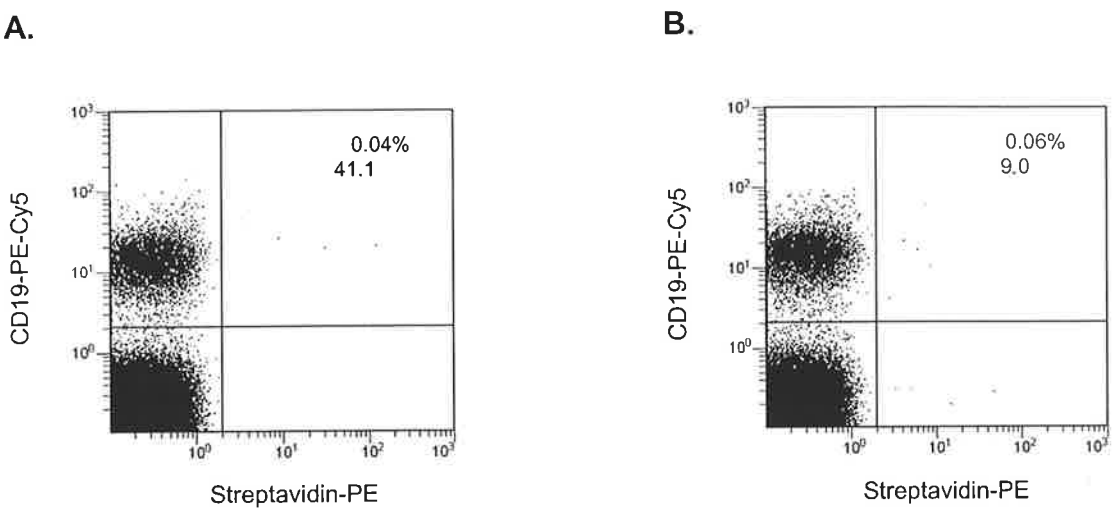


Fig. 3.8 Flow cytometric analysis of PBMC from V1 donor on day 14 post immunisation. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin was 0.04%. The MFI was 41.1. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.06%. The MFI was 9.0. Total B cell populations in **A** and **B** samples were 7.07% and 9.96% respectively.

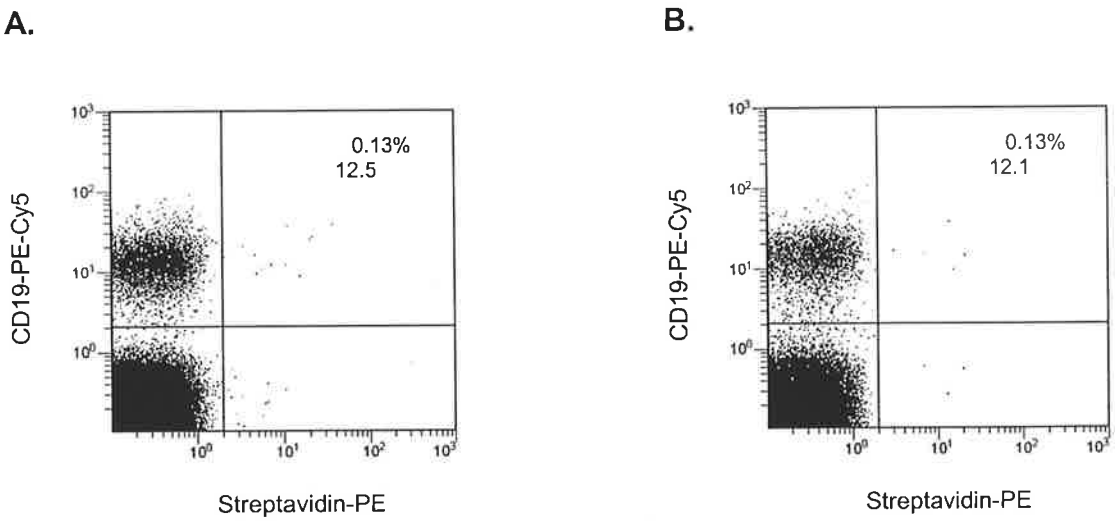


Fig. 3.9 Flow cytometric analysis of PBMC from unimmunised V6 donor (negative control) on day 14 of the experiment. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.13%. The MFI was 12.5. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.13%. The MFI was 12.1. Total B cell populations in **A** and **B** samples were 5.55% and 4.59% respectively.

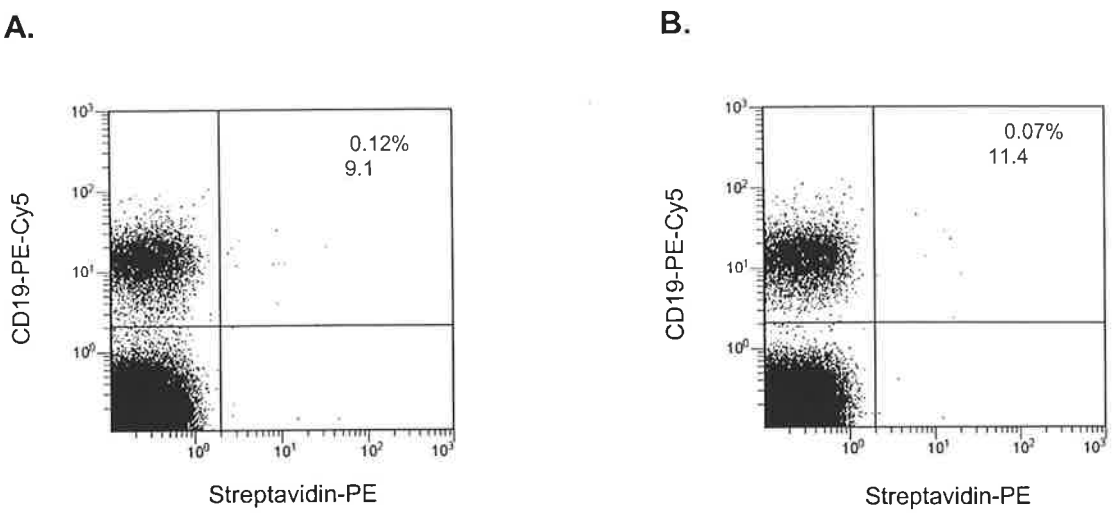


Fig. 3.10 Flow cytometric analysis of PBMC from V1 donor on day 20 post immunisation. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.12%. The MFI was 9.1. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.07%. The MFI was 11.4. Total B cell populations in **A** and **B** samples were 7.08% and 10.23% respectively.

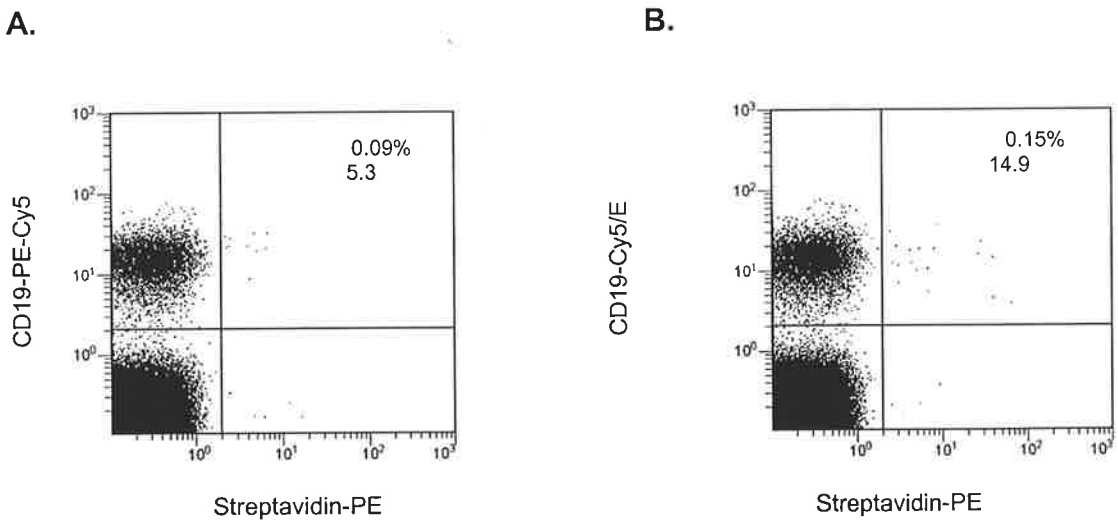


Fig. 3.11 Flow cytometric analysis of PBMC from unimmunised V6 donor (negative control) on day 20 of the experiment. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.09%. The MFI was 5.3. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.15%. The MFI was 14.9. Total B cell populations in **A** and **B** samples were 7.00% and 6.56% respectively.

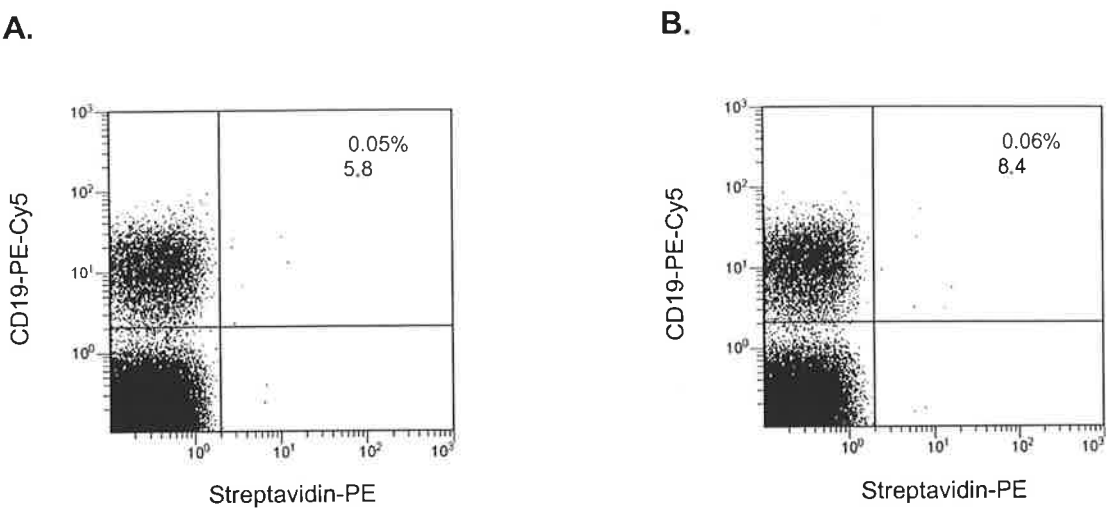


Fig. 3.12 Flow cytometric analysis of PBMC from V1 donor on day 25 post immunisation. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.05%. The MFI was 5.8. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.06%. The MFI was 8.4. Total B cell populations in **A** and **B** samples were 10.21% and 11.60% respectively.

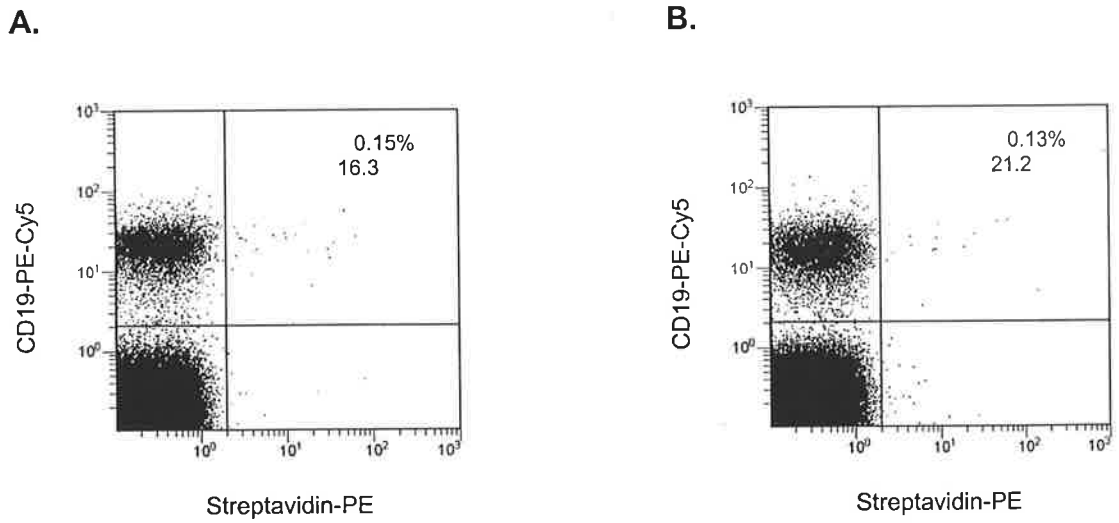


Fig. 3.13 Flow cytometric analysis of PBMC from unimmunised V6 donor (negative control) on day 25 of the experiment. **A.** The cells were stained with PRP-biotin (5 μg/ml), CD19-PE-Cy5 and streptavidin-PE. The percentage of B cells staining with streptavidin-PE was 0.15%. The MFI was 16.3. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.13%. The MFI was 21.2. Total B cell populations in A and B samples were 7.64% and 7.41% respectively.

3.3.3 Flow cytometric analyses for TT specific B cell isolation

PBMC obtained on day 21 from a 45 year old adult volunteer immunised with ADT® vaccine and PBMC obtained from a 57 year old adult volunteer who had not been immunised for tetanus during the past 5 years (negative control) were stained and flow cytometric analyses were performed as described in Chapter 2. The percentages of B cells binding with streptavidin-PE in immunised and non-recently immunised PBMC stained with biotinylated TT were 2.28% and 0.63% respectively (Fig. 3.14-3.16). These percentages were significantly higher than background (0.15%) of the control sample stained with unconjugated TT, streptavidin-PE and CD19-PE-Cy5. Therefore, the streptavidin-PE binding B cells were likely to be TT binding cells. The TT binding was competitively inhibited by approximately 30% and 20% when unbiotinylated TT was added to immunised and non-recently immunised samples respectively. A large population of TT binding non-B cells (10.30%) was detected only in PBMC from the recently immunised adult. These cells were completely inhibited with unbiotinylated TT.

Several flow cytometric experiments jointly conducted with Ms Claire Jessup, a collaborator in the research team, also demonstrated similar populations of TT binding non-B cells in peripheral blood from 5 of 7 recently immunised adults tested between day 10 and day 20 post immunisation. TT binding B cells were detected in PBMC from all 7 recently immunised adults. The TT binding non-B cells were almost completely inhibited by unbiotinylated TT in contrast to the partial inhibition of TT binding B cells. Some preliminary analyses of the TT binding non-B cells were performed. None of the non-recently immunised adults (4 negative controls) demonstrated TT binding non-B cells.

3.3.3.1 Phenotyping of TT binding non-B cells.

Flow cytometric analyses of PBMC from an adult on day 19 post immunisation revealed that approximately 57% of TT binding non-B cells were positive for the NK cell marker, CD56 (Fig. 3.17). The percentage positive for the monocyte marker CD14 was nearly 8%. PBMC from another adult on day 20 post TT immunisation demonstrated that 7.2% of non B cells were binding to TT. The percentages of CD3 and CD5 positive cells in this population were 5% and 2.6% respectively. Furthermore, approximately 92% of these TT binding non B cells were positive for CD16 (Fig. 3.18A). CD16 is FcγRIII, a low affinity IgG binding receptor found mainly on NK cells and on some monocytes and T cells. Less than 2% of the TT binding non B cells were positive for CD64, the FcγRI IgG receptor found on monocytes, and macrophages.

3.3.3.2 Binding of TT to non B cells through Ig

In PBMC obtained on day 20 from the adult described in Fig. 3.18 A, nearly 100% of TT binding non B cells stained with anti human Ig, indicating the presence of human immunoglobulin on the surface of all cells (Fig. 3.18 B). PBMC from an adult, collected on day 16 post TT immunisation, were preincubated in sheep serum (3×10^6/ml) at 37°C for 1hour and then stained for TT binding cells (Fig. 3.19). The TT binding non B cell population (7.2%) detected in the non pre incubated sample was absent in the pre incubated sample. Furthermore, when pre incubated PBMC were stained for surface Ig light chains lamda (λ) and kappa (κ), cells positive for both λ and κ were reduced from 7.3% to 0.5%. The percentage of TT binding B cells

in the pre incubated sample was 3.1% compared to 3.3% of B cells binding with TT in the non pre incubated sample.

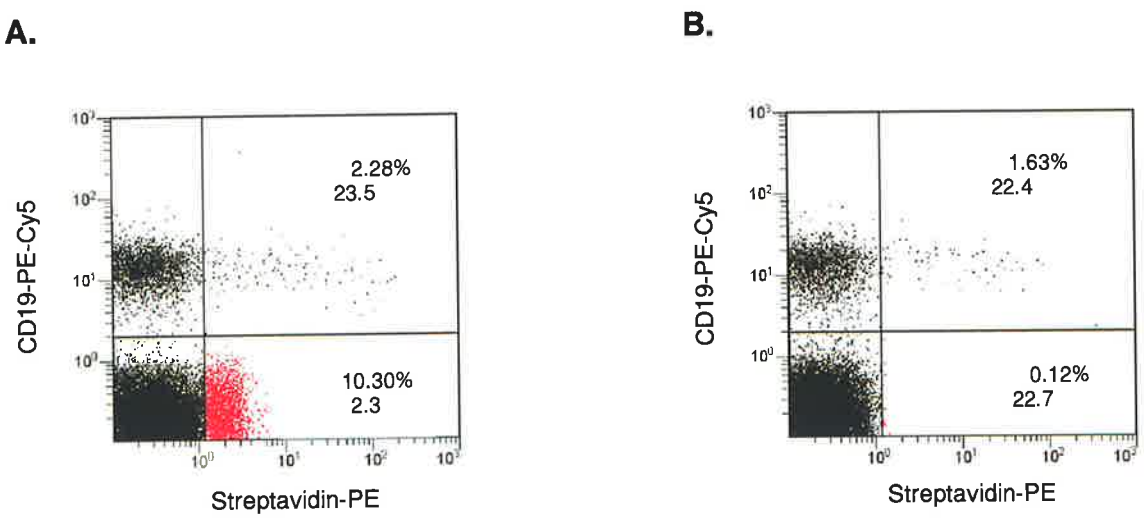


Fig. 3.14 Flow cytometric analysis of PBMC from an adult on day 21 post TT immunisation. The cells were stained with TT-biotin, CD19-PE-Cy5 and streptavidin-PE. **A.** The percentage of B cells staining with streptavidin-PE was 2.28%. The MFI was 23.5. The percentage of non B cells (in red) staining with streptavidin-PE was 10.30%. The MFI was 2.3. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 1.63%. The MFI was 22.4. The non B cell population staining with streptavidin-PE was reduced by 99% to background level. The total B cell populations in **A** and **B** samples were 9.95% and 7.53% respectively

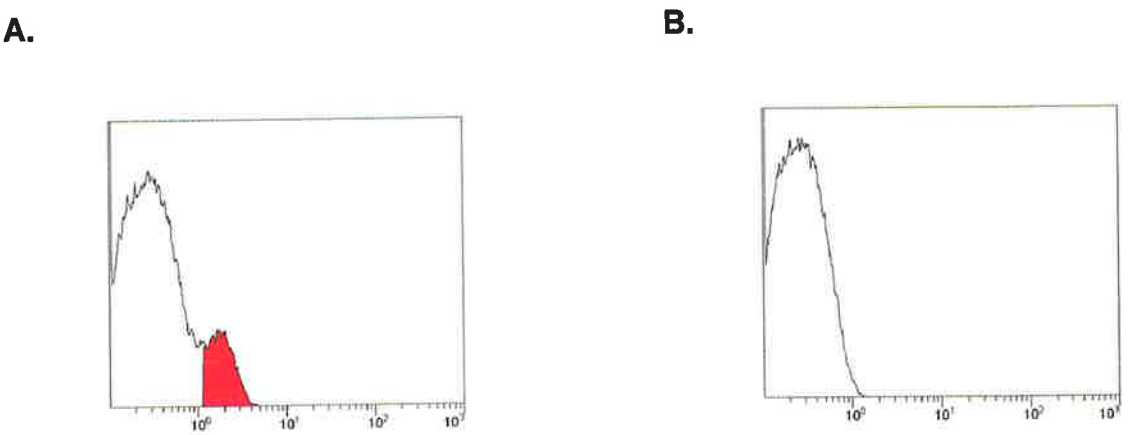


Fig. 3.15 Streptavidin-PE histogram of the PBMC in the above CD19 PE-Cy5 negative populations. **A.** A distinct low fluorescent streptavidin-PE positive cell population was seen (in red). **B.** The streptavidin-PE positive cell population was absent in the inhibition sample.

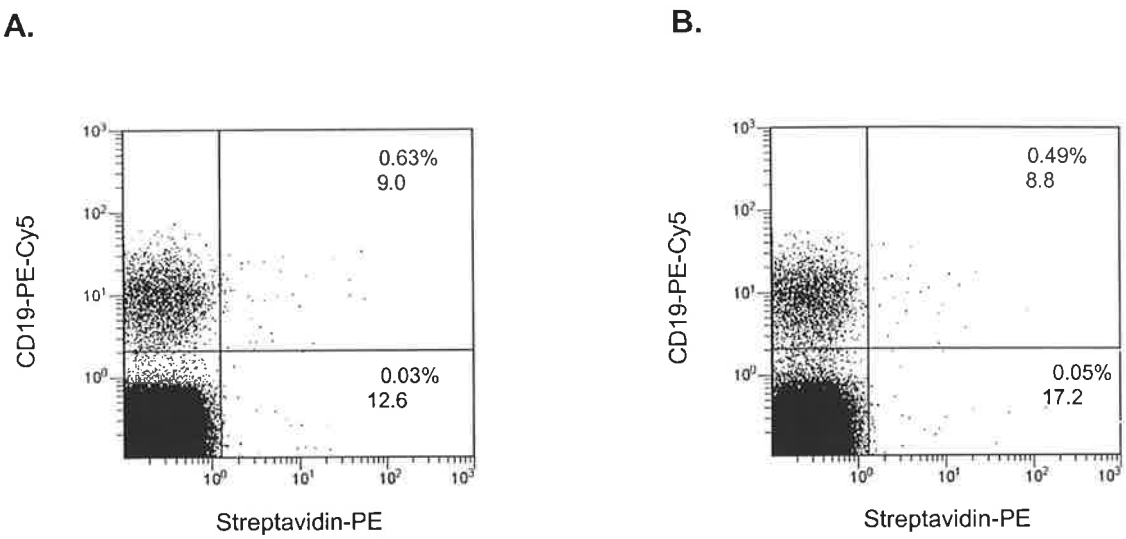


Fig. 3.16 Flow cytometric analysis of PBMC from a non recently immunised adult. The cells were stained with TT-biotin, CD19-PE-Cy5 and streptavidin-PE. **A.** The percentage of B cells staining with streptavidin-PE was 0.63%. The MFI was 9.0. The streptavidin-PE staining non B cell population was 0.03%, similar to background staining with streptavidin-PE in the control sample. **B.** The percentage of B cells staining with streptavidin-PE in the inhibition sample was 0.49%. The MFI was 8.8. The total B cell populations in **A** and **B** samples were 5.78% and 6.92% respectively

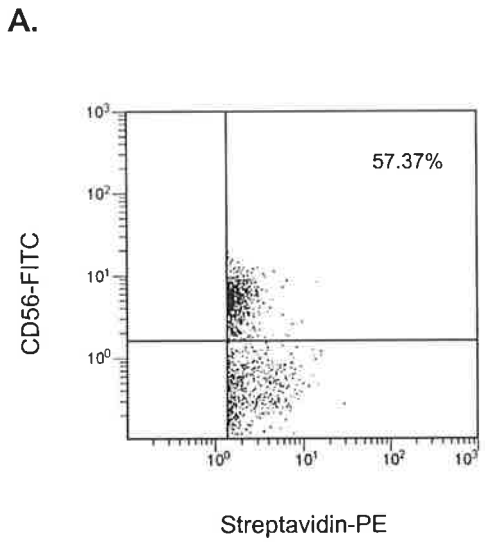


Fig.3.17 Flow cytometric analysis of PBMC from an immunised adult on day 19 post TT immunisation. The cells were stained with TT-biotin, CD19-PE-Cy5, CD56-FITC and streptavidin-PE. Streptavidin-PE staining (TT binding) non B cell population (3.4%) was gated and analysed. Approximately 57% of cells were positive for CD56, a marker for the NK cells.

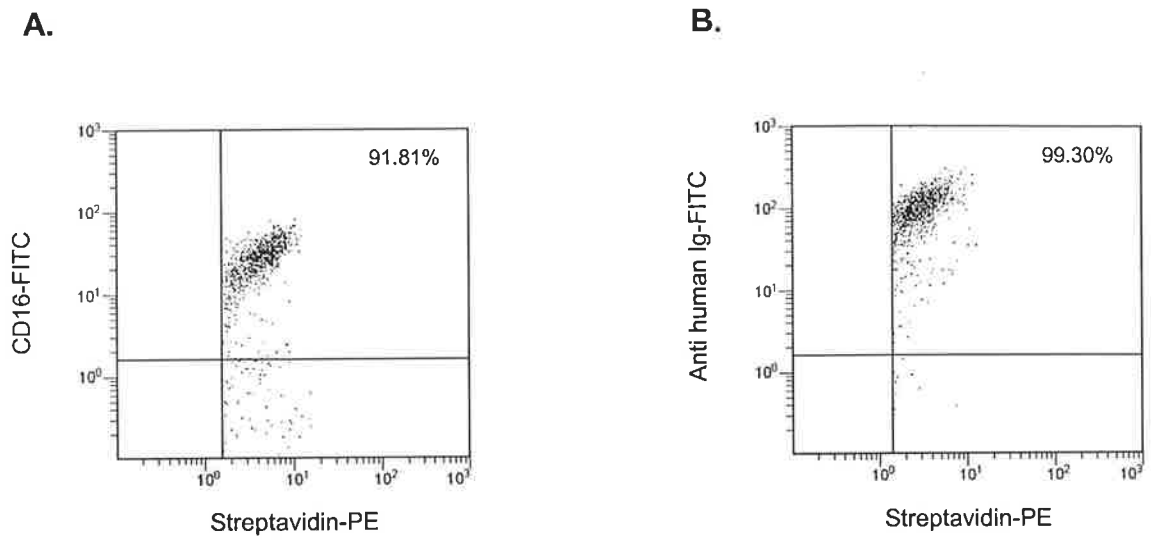


Fig.3.18 Flow cytometric analysis of PBMC from an adult on day 20 post TT immunisation. The cells were stained with TT-biotin, CD19-PE-Cy5, streptavidin-PE and CD16-FITC or anti human Ig-FITC. Streptavidin-PE staining (TT binding) non B cell population (7.2%) was gated and analysed **A.** Approximately 92% of cell were positive for CD16, the maker for FcγRIII. **B.** Nearly 100% of cells were stained with anti human Ig-FITC indicating the presence of surface Ig on TT binding non B cells.

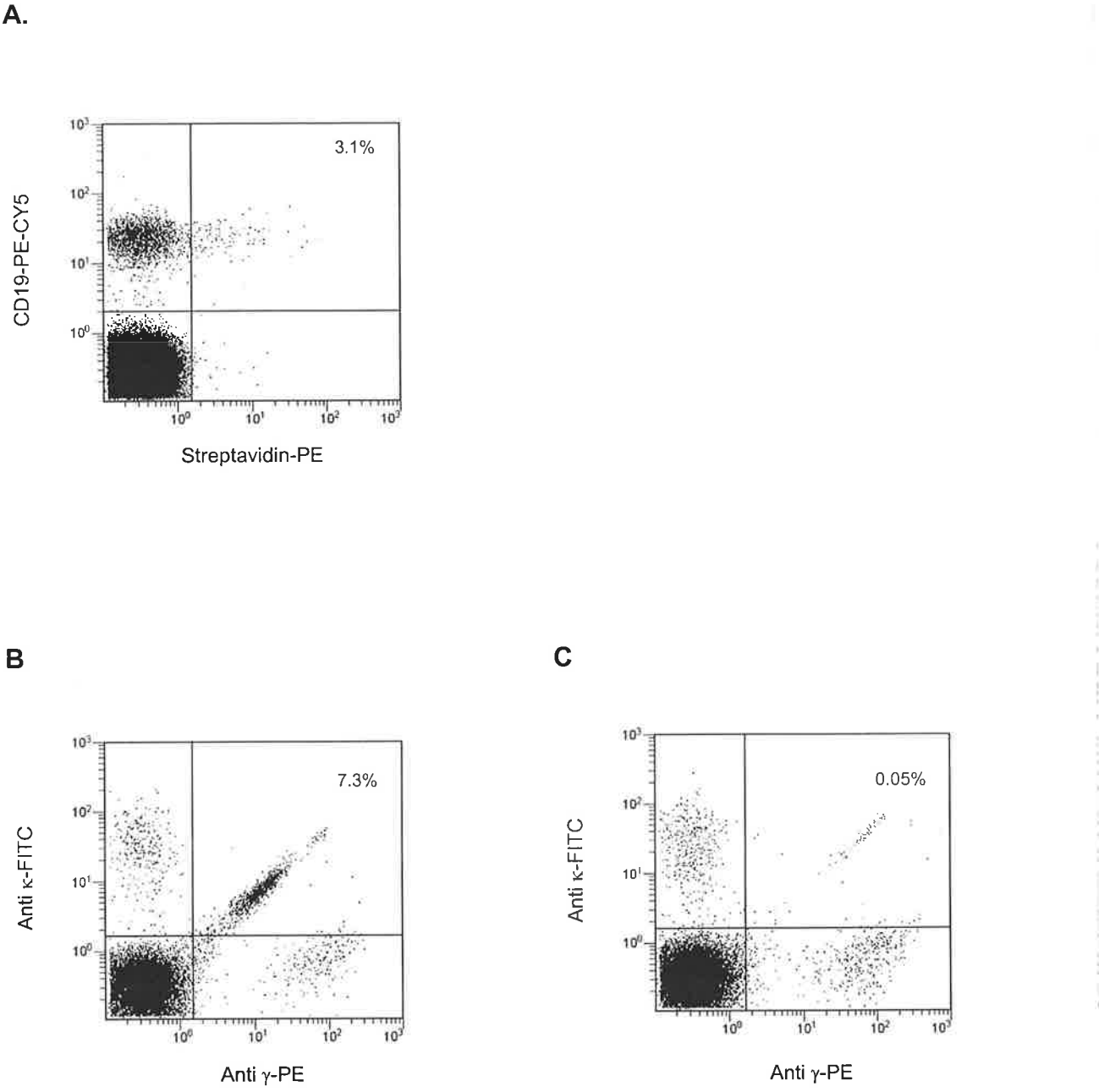


Fig.3.19 Flow cytometric analysis of PBMC from an adult on day 16 post TT immunisation. The cells were stained with TT-biotin, CD19-PE-Cy5 and streptavidin-PE. The percentages of streptavidin-PE staining (TT binding) B cells and non B cells were 3.3% and 7.2% respectively. **A.** When PBMC were preincubated with sheep serum (3×10^6/ml) at 37°C the percentage of TT binding B cells was reduced to 3.1% and the TT binding non B cell population was not seen. **B.** PBMC stained with anti human λ-PE and κ-FITC conjugates. The double positive cell percentage was 7.3%. **C.** When PBMC were pre incubated with sheep serum at 37°C and stained with anti human λ-PE and κ-FITC conjugates, the double positive cell percentage was reduced to 0.5%.

3.4 Discussion

All adult volunteers demonstrated anti-PRP IgG antibody titres in blood at the time of commencement of the study, presumably due to natural exposure to the PRP antigen of the Hib bacterium during adult life. The adult volunteers immunised with Hib conjugate vaccine PRP-OMP, mounted an anti-PRP IgG antibody response with time (Fig 3.1). V1 demonstrated the greatest response probably due to being primed by previous Hib immunisation. The moderate response for V2 and a weak response for V3 may represent the normal physiological variation in mounting immune responses among individuals.

3.4.1 PRP antigen specific B cells

PRP specific B cells were not detected above background levels in the flow cytometric analyses. Only very small percentages of B cells were stained with streptavidin-PE in all samples from both the immunised and unimmunised groups throughout the study. The streptavidin-PE staining B cell percentages and the flow cytometric patterns did not show any significant differences between the test samples, the inhibition samples and the negative control samples. Therefore, if there were any B cells expressing antibody against PRP on their surface, they were not distinguished from non specific background staining. Although the controls indicated that it is mainly non specific binding of streptavidin-PE directly to cells that contributes to non-specific staining, non specific binding of PRP-biotin to cells and subsequent binding of streptavidin-PE cannot be ruled out.

The MFI of B cells staining with streptavidin-PE of immunised and unimmunised adults on some days showed high values in comparison to the values of all other

samples throughout the study. When the dot plots of these analyses were examined closely a few very bright streptavidin-PE positive events were seen on the outer margin of the plots. These events are usually considered as non specific noise signals in flow cytometic analyses. However, as a very rare occurrence it is possible that these events may represent occasional PRP specific B cells. The MFI is not a reliable index in interpreting results of small populations as it depends on the number of events as well as the fluorescent intensity of each event.

It is possible that in the process of biotinylating PRP antigen, the antigenic epitope configuration had been altered, preventing binding with B cell antigen receptors. The antigenicity was tested after biotinylation with the use of anti-PRP serum in a streptavidin coated ELISA. However, it is possible that serum contains a variety of anti-PRP antibodies including some against epitopes that were not modified by biotinylation, while the corresponding B cells might be extremely rare.

Furthermore, it is suggested that PRP specific memory B cells may be confined mainly to secondary lymphoid tissues, while only a very small number of cells recirculate in peripheral blood, which cannot be distinguished above background.

Alternatively, it is possible that since PRP antigen is a polysaccharide with repeating epitopes, it binds to multiple surface receptors and becomes internalised in B cells during the immunofluorescence assay, in spite of performing the assays at cold temperatures and in the presence of azide to prevent such internalisation. This hypothesis could be tested by staining cells with radio labeled PRP and measuring radioactivity in the cytoplasm at the end of the assay.

Since polysaccharide antigens usually result in the production of low affinity antibodies, it is possible that the affinity of the antigen receptors on the B cells is low and binds only weakly with PRP antigen. This could result in dissociation of the PRP antigen from the receptor during the immunofluorescence staining. The argument against this suggestion is that although the affinity of the binding site is low the avidity may be high due to the antigen binding to multiple receptors due to it's repeating epitope characteristics. Modifying the assay procedure by reducing the number of washing steps could help to prevent any dissociation of antigen from the receptor, although non specific background might be greater.

Furthermore, it is possible that the PRP-biotin preparation contained significant amounts of unbiotinylated PRP that may have competitively inhibited the binding of PRP-biotin to antigen receptors of specific B cells. This possibility cannot be ruled out, as no method was used to purify the PRP-biotin preparation by removing unbiotinylated PRP, technically, a relatively difficult procedure. Although a streptavidin coated ELISA was used to check the biotinylation and the antigenicity of PRP-biotin, this would not indicate the presence of any unbiotinylated PRP in the preparation.

There seem to be no previous publications describing the isolation of PRP specific B cells. However, Hougs *et al* have separated anti-PRP antibody secreting cells (ASC) from PBMC from adults immunised with Hib conjugate vaccine (PRP-T) using antigen coated magnetic beads (Hougs *et al.*, 1993). Separated cells were immediately tested in plaque forming assays (ELISPOT) for anti-PRP antibody secretion. The percentages of anti-PRP ASC from the PBMC population subjected to purification varied between 0.1% to 0.75% for the four adults from day 7 to day 9-

post immunisation. The recovery of anti-PRP ASC from peripheral blood by magnetic bead separation was between 35-85%. The specificity of the separated cells was estimated to be nearly 98% by an indirect method considering the enrichment factor. However, this may not be the true purity of the separated PRP binding cell populations because of the possibility of cells binding to magnetic beads through non specific mechanisms. Furthermore, the specificity of the ELISPOT assay system was also not tested.

Hougs *et al* separated cell populations may have contained PRP antigen specific B cells. However, the cells detected in the ELISPOT assay are most likely to be anti-PRP antibody secreting plasmablasts or plasma cells rather than PRP antigen specific B cells. PRP specific B cells could have been isolated from the separated cell fractions by further purification using pure B cell markers.

3.4.2 Detection of TT specific B cells

In contrast, in this study, TT specific B cells were readily identified by using immunofluorescence staining followed by flow cytometric analysis, similar to the technique used in the attempt at isolating PRP specific B cells. This was performed in order to assess the ability to identify antigen specific B cells by flow cytometry and to see if the difficulties experienced with PRP were restricted to polysaccharide antigens.

TT binding B cells were identified among PBMC from a recently immunised adult donor. This population was only partially inhibited even with a 10 fold excess of free unbiotinylated TT. Does this mean that the majority of TT binding to B cells is non specific binding? This question cannot be answered at present.

The detection of a small TT binding B cell population in unimmunised PMBC could be due to the presence of TT specific memory B cells in the circulation as the adult donor had received several previous TT immunisations with the latest being about 5 years ago.

3.4.3 Detection of TT binding non B cells

Another interesting feature of this experiment was the detection of a TT binding non B cell population in the recently immunised donor. This population was completely inhibited by excess free antigen in contrast to the TT binding B cell population. The presence of a marked population of TT binding non B cells in recently immunised human peripheral blood has not been reported in the literature to date.

The presence of TT binding non B cells in the PBMC from recently immunised adult donors was confirmed by the experiments jointly conducted with Ms Claire Jessup. Subsequent flow cytometric analyses of PBMC from two immunised donors demonstrated that TT binding non B cells of one adult donor decreased to less than 1% after one month post immunisation, whereas in the other adult donor they continued to be present in peripheral blood at a higher percentage (8.5%) even on day 50 post immunisation.

Flow cytometric analyses of PBMC from adult donors immunised with TT have revealed that all TT binding non B cells were positive for human Ig on the cell surfaces. Furthermore, about 90% of TT binding non B cells were positive for CD16, the FCγRIII receptor for IgG. When PBMC were pre incubated at 37°C the TT binding non B cell population almost disappeared. These findings suggest that TT was indirectly bound to non B cells through anti-TT antibodies attached to cells

mainly via FCγRIII. When PBMC were incubated at 37°C, in the absence of serum containing antibody, it was possible that Fc receptor bound anti-TT antibodies were eluted resulting in a complete reduction of TT binding non B cell population. This explanation is supported by the finding that pre-incubation of PBMC markedly reduced the κ and λ light chain double positive cell population in the flow cytometric analysis. The cells coexpressing κ and λ light chains are cells that have absorbed Ig from serum.

FCγRIII receptor is mainly expressed on NK cells and also on monocytes, macrophages and at low levels on T cell subsets. The results of phenotyping assays revealed that the majority of TT binding non B cells were NK cells while a small proportion was monocytes and T cells. However, cells that possess other types of Fc receptors such as FCγRI and FCγRII could also bind to TT through anti-TT antibodies. Further studies are required to confirm this.

A recent publication from Bell and Gray bears some relationship to this interesting finding (Bell and Gray, 2003). After immunising mice with PE, they detected PE binding B220 positive and B220 negative populations in mice. The PE binding B220 negative cells (non B cells) were detected in spleen cells on day 14 post immunisation, peaked around 3 weeks and then the cell numbers reduced slightly and were maintained for at least 6 months post immunisation. These cells were also detected in peripheral blood and in bone marrow. After performing extensive flow cytometric analyses they concluded that these B220 negative cells belong to a myeloid lineage and bind antigen through monomeric IgG via FcγRI surface receptors.

3.4.4 Previous studies of isolation of TT specific B cells

Previous studies describing isolation of TT specific B cells have produced compatible results (Oshiba *et al.*, 1994; Leyendeckers *et al.*, 1999). Some of these isolations have resulted in detecting TT binding non B cell, but they have not been characterised or described as a distinct population (Lenzner *et al.*, 1998; Zafiropoulos *et al.*, 2000).

In an attempt to isolate TT specific B cells from B cells of previously immunised adults, Oshiba *et al* showed that 0.34% of total B cells bound to TT coated immunomagnetic beads (Oshiba *et al.*, 1994). The mean binding percentage reached a maximum of 2%, 4 days after booster immunisation. These results are similar to the results of the TT immnufluoresence assays described in this chapter. However, the binding of B cells to TT coupled magnetic beads was competitively inhibited up to 90% when pre-incubated with excess amounts of free TT. When PBMC from previously immunised adults were incubated with TT coated magnetic beads, approximately 12% of the rosetting population consisted of CD3 positive T cells. When T cell fractions from previously immunised adults were incubated with antigen coated beads, the percentages of T cells binding to TT were less than 0.02%. Similar percentages of non B cells binding to TT antigen were also seen in the experiments described in this chapter on PBMC from previously TT immunised adults. The percentage of T cells binding to antigen coated magnetic beads following TT booster immunisation was not reported by Oshiba *et al.*

Flow cytometric experiments performed by Leyenderkers *et al* indicated that 0.14% of B cells from an adult immunised with TT during childhood stained with TT conjugated PE (Leyendeckers *et al.*, 1999). Some of the flow cytometric experiments

reported in this chapter have also shown similar small numbers of B cells from previously immunised adult donors binding to biotinylated TT antigen.

Lenzner *et al* separated TT binding cells from PBMC of previously immunised adults by immunoselecting with TT pulsed monocytes (Lenzner *et al.*, 1998). Flow cytometric analysis of the immunoselected cells in culture showed the presence of both B and T cells. Although details of the B and T cell populations were not described, this indicates binding of T cells to TT antigen during immunoselection.

In another set of experiments in which TT binding cells were isolated from *in vitro* immunised adult PBMC using biotinylated TT and streptavidin coated paramagnetic beads, only 85% of the isolated cells were CD19 positive B cells when assessed with flow cytometry (Zafiropoulos *et al.*, 2000). This demonstrates selection of non B cells during the isolation process. However, further details of the TT binding B and non B cell populations were not reported.

3.4.5 Comparison of isolating PRP and TT specific B cells

TT is a protein antigen and the immune response to TT is a "true" TD response unlike the "converted" TD response to PRP conjugate antigen. Therefore, it is possible that TT immunisation leads to a greater and a more long lasting antibody response than the antibody response to PRP conjugate antigen, generating a greater number of antigen specific memory B cells. This could be a reason for being able to detect TT specific B cells in peripheral blood, but not PRP specific B cells.

All adult volunteers who participated in this TT study had been routinely immunised with several doses of TT during childhood and had received TT boosters during adult

life. Therefore, subsequent TT immunisation would result in enhanced antibody responses, generating greater numbers of TT specific B cells. The adult volunteers for this PRP study had not been previously immunised with PRP conjugate vaccine (except for V1 receiving a PRP vaccine one and a half years ago), but may have been repeatedly exposed to natural PRP antigen of the Hib bacterium during life. However, since PRP antigen is a TI antigen, it is possible that the immune systems of these adults were not well primed to mount stronger antibody responses similar to the responses of the TT immunised adults.

Although the adult volunteer (V1) who had received a previous immunisation with the same type of PRP conjugate vaccine demonstrated high level of anti-PRP antibodies, PRP specific B cells were not detected in peripheral blood by flow cytometry. A definitive explanation cannot be given, as there was only one subject in this category.

The isolation of TT specific B cells proved that when antigen specific B cells are present in the peripheral circulation in significant numbers it is possible to distinctly identify these cells by the techniques of immunofluorescence staining and flow cytometry analysis used in this study. Furthermore, it led to the discovery of a large percentage of antigen binding non B cells in the circulation of recently immunised adults. Antigens binding non B cell populations in peripheral blood of humans have not been well described before. This population needs thorough analysis to derive firm conclusions.

The next approaches were to attempt to detect PRP specific B cells, by (1) using unbiotinylated PRP antigen and (2) testing the hypothesis that PRP specific B cells

may be confined mainly to secondary lymphoid tissues. Chapters 4 and 5 deal with the production of anti-PRP monoclonal antibodies and the development of an indirect immunofluorescence assay using unbiotinylated PRP for detection of PRP specific B cells by flow cytometry. Chapter 6 describes the use of a murine model to detect PRP specific cells in peripheral blood and the splenic tissues.

CHAPTER 4

PRODUCTION AND CHARACTERISATION OF MURINE ANTI-PRP MONOCLONAL ANTIBODIES

4.1 Introduction

Monoclonal antibodies are an important tool in the field of immunology. In general they are widely used for characterisation of cell surface antigens, cell phenotyping and separation, antigen purification and diagnostic assays. They have been used in a limited capacity in treating malignancies, graft rejection and some specific inflammatory conditions.

Monoclonal antibodies to PRP, the capsular polysaccharide of Hib, were initially developed by Gigliotti and Insel in the early 1980's (Gigliotti and Insel, 1982). Since then, anti-PRP monoclonal antibodies have been produced by several investigators for use in the study of immune response to Hib infection (Hunter *et al.*, 1982; Gigliotti *et al.*, 1984; van Alphen *et al.*, 1996). In most of these studies, the antibody repertoire to PRP antigen has been analysed using human anti-PRP monoclonal antibodies (Carrol *et al.*, 1992; Adderson *et al.*, 1993a; Lucas *et al.*, 1994; Pinchuk *et al.*, 1995; Adderson *et al.*, 1998b).

Only two publications have described the production of murine anti-PRP monoclonal antibodies to date (Gigliotti and Insel, 1982; Bunse and Heinz, 1994). These PRP antibodies were shown to be bactericidal in vitro (Gigliotti and Insel, 1982; Bunse and

Heinz, 1994) and effective in eradicating bacteraemia in Hib infected rats (Gigliotti and Insel, 1982).

This chapter describes the production and characterisation of murine anti-PRP monoclonal antibodies for use in indirect immunofluorescence assays in detecting PRP antigen specific B cells.

4.2 Methods

4.2.1 Immunisation of mice and spleen cell collection

Mouse hybridomas were developed to produce monoclonal antibodies against PRP capsular polysaccharide antigen. Two six week old BALB/c mice (A and B) were immunised with Hiberix™ vaccine (PRP-T). The immunisation was performed with ¼ strength of human vaccine dose using a two dose schedule as reported by the vaccine manufacturer (unpublished data).

A vial of PRP-T vaccine antigen was reconstituted in 0.5 ml of sterile 0.9% saline diluent according to the product information. The vaccine antigen was then mixed with a vial of GERBU adjuvant (10μg/0.1ml) (GERBU Biotechnik, Gaiberg, Germany) to enhance the immune response (Grubhofer, 1995). From this preparation, 150 μl (2.5 μg of PRP conjugated to 7.5 μg of TT) was injected subcutaneously at the nape of neck. A booster dose of vaccine antigen prepared without GERBU adjuvant was injected in similar fashion after four weeks. The mice were sacrificed five days after the booster dose using CO_2 inhalation, and were pinned to a corkboard, cleaned with 70% alcohol and placed inside a laminar flow cabinet. A mid line incision was

made and the spleen was dissected out under aseptic conditions. The spleens were placed on petri dishes and rinsed in sterile PBS. The spleen capsules were incised and the spleen tissues were crushed with the blunt end of 10 ml plastic syringe barrels, allowing the cells to drain into sterile PBS. The suspensions were then filtered through sterile Falcon® 70 μm cell strainers (Becton Dickinson Labware, New Jersey, USA). The cell suspensions were diluted in five parts of sterile PBS and centrifuged at 1000 g for 10 minutes. The supernatants were discarded and the cells were resuspended in 5 ml of NH_4Cl red cell lysis solution for 5 minutes. The recovered cells were diluted in five parts of sterile PBS, centrifuged at 1500 g for 10 minutes and the supernatants were discarded. Two further similar washes were performed and the cells were finally re suspended in 1 ml of sterile PBS. Viability counts were performed by trypan blue exclusion. The viable cell counts were adjusted to a concentration of 4×10^7/ml.

4.2.2 Hybridoma production

Murine hybridomas were produced based on methods described previously (Kohler and Milstein, 1975; Zola, 1987; Carrol *et al.*, 1992).

Cells from a mouse myeloma cell line P3-X63-Ag8.653 (Kearney *et al.*, 1979), were harvested and resuspended in 1ml of sterile PBS and a viability count was performed. Mouse spleen cells were then added to myeloma cells at a two to one viable cell ratio and then centrifuged at 200 g for 5 minutes. The supernatant was removed and 1ml of warm (37°C) polyethylene glycol 1500 (BDH Laboratory Chemicals, Poole, England) was added. The cell suspension was mixed well with a pipette for 1 minute. Three millilitres of RF10 medium were added drop wise over 10 minutes followed by 10 ml

of warm (37°C) RF10 medium drop wise over 10 minutes. The cell mixture was then centrifuged at 200 g for 5 minutes and resuspended in 20 ml of RF10 and left in a pre gassed petri dish for 2-3 hour in an incubator at 37°C with 5% CO_2. All cells were harvested from the petri dish and centrifuged at 200g for 5 minutes and then resuspended in RF10 medium containing HAT media supplement (50×) (Sigma Chemical Co., Missouri, USA) 1/50 and HFCS (50×) (Boehringer Mannheim GmbH, Germany) 1/50. The suspension was plated in 24 well plates in 2 ml aliquots. The cells were fed weekly with new RF10 medium containing HT media supplement (50×) (Sigma Chemical Co., Missouri, USA) 1/50 and HFCS (50×) 1/100. After approximately 3-4 weeks, when the medium turned yellow the supernatants were tested for anti-PRP antibody production by enzyme immunoassay.

4.2.3 Selecting anti-PRP antibody secreting hybridomas

Anti-PRP antibody secreting hybridomas were identified by a PRP ELISA similar to the technique used for measuring anti-PRP antibody concentrations in human adult volunteers.

Costar® high binding ELISA plates were coated with 100 µl per well of optimal concentration of PRP-tyramine (1 µg/ml) diluted in 0.05 M carbonate buffer. The plates were incubated at 37°C for 90 minutes in a humid chamber and then at 4°C overnight. The wells were washed three times with PBS-Tween and unbound sites were blocked with 1% casein solution for 2 hours at 37°C in a humid chamber. The wells were again washed as before and 100 µl of differing dilutions of USFDA standard anti-Hib antibody serum and quality control serum diluted in 1% casein

solution were added to control wells. One hundred microlitres of PRP hybidoma supernatant were added to each test well. The plates were incubated at room temperature for 4 hours and then washed as before. Affinity isolated sheep anti-human IgG peroxidase conjugate and affinity isolated sheep anti-mouse immunoglobulin peroxidase conjugate diluted in 1% casein solution at 1/1000 were added at 100 µl per well to the control and test wells respectively. After incubating for 1 hour at 37°C in a humid chamber, the plates were washed with PBS-T. The colour reaction was developed by adding 100 µl of ABTS and 33% w/v hydrogen peroxidase (40 µl of H_2O_2 per 100ml ABTS) to each well. Bound anti-PRP antibodies were detected by the development of colour measured with a Dynatech MR7000 plate reader set at a wavelength of 410 nm. The readings were recorded when the optical density of the highest dilutions of the USFDA standard serum (1/100) reached more than 2.0 units. Optical densities of four fold greater than background were considered as positive

Positive hybridoma cultures were cloned by a limiting dilution technique (Goding, 1980), in RF10 medium containing HT (50×) 1/50 and HFCS (50×) 1/100 in 0.2 ml aliquots in 96 well plates. The cells were fed weekly with the same medium. When the culture medium turned yellow the clones were tested for anti-PRP monoclonal antibody production as described above.

The binding specificity of the monoclonal antibodies to PRP antigen was confirmed by an inhibition ELISA. PRP antigen (10 µg/ml) was added (100 µl per well) along with the supernatants to competitively inhibit the binding of anti-PRP antibodies in the supernatants to PRP-tyramine in the PRP ELISA. The supernatants of hybridoma clones (A1, B2, B10 and B14) that demonstrated inhibition of more than 85% were

selected. The hybridoma clone B10 demonstrated the strongest reaction in repeated PRP ELISA testing. Therefore, this clone was expanded and supernatant was collected for further analysis.

4.2.4 Isotyping

Isotype testing was performed on supernatants of all selected hybridoma clones (A1, B2, B10, and B14) to determine the isotypes of anti-PRP monoclonal antibodies secreted by the respective clones.

The IsoStrip™ Mouse Monoclonal Antibody Kit (Roche Diagnostics Corporation, Indianapolis, USA) was used for isotyping. Testing was performed according to the manufacturer's recommendations.

4.2.5 Specificity of anti-PRP monoclonal antibody

The specificity of anti-PRP antibodies in the B10 supernatant was tested by an inhibition immunoassay and an agglutination assay with Hib bacteria.

4.2.5.1 Inhibition assay

An inhibition assay was performed by adding *H. influenzae* type b bacterial culture suspension to the PRP enzyme immunoassay to demonstrate the competitive inhibition of the binding of PRP monoclonal antibodies to the PRP antigen coated on the plate.

H. influenzae type b bacteria (ATCC 33533 strain) (Microbiology Department, Women's and Children's Hospital, Adelaide, South Australia) were grown on a 5%

haemolysed and heated horse blood agar (chocolate agar) (Oxoid, Basingstoke, UK) plate at 37°C in 5% CO_2.

A pneumococcal bacterial suspension was used as a negative control. *Streptococcus pneumoniae* (pneumococcal) bacteria (ATCC 49619) (Microbiology Department, Women's and Children's Hospital, Adelaide, South Australia) were grown on a 5% horse blood agar (Oxoid, Basingstoke, UK) plate at 37°C in 5% CO_2.

The bacterial colonies were harvested and washed in PBS and then resuspended in 1% formalin saline at 37°C for 1hour. The suspension was washed twice in PBS and the formalin inactivated bacteria were resuspended in PBS at a final concentration of 1×10^7/ml.

A PRP ELISA was set up as described before. Formalin inactivated Hib bacterial suspension was added (100 µl per well) to the inhibition testing wells and formalin inactivated pneumococcal suspension was added (100 µl per well) to the negative control wells along with the hybridoma supernatants. The plate was incubated and the colour reaction was developed as described before.

4.2.5.2 *Agglutination assay*

An agglutination assay was performed to determine whether anti-PRP monoclonal antibodies had any agglutination activity for Hib bacteria. Pneumococcal and *Streptococcal agalactiae* group B bacteria were used as a negative control to demonstrate the specificity of the agglutination.

Streptococcus agalactiae group B (GBS) bacteria (Isolate 51145) (Microbiology Department, Women's and Children's Hospital, Adelaide, South Australia) were grown on a 5% horse blood agar (Oxoid, Basingstoke, UK) plate at 37°C in 5% CO_2.

Formalin inactivated Hib, pneumococcal and *Streptococcus agalactiae* group B bacterial suspensions were prepared as described above. After incubating 100 µl of each bacterial suspension with 100 µl of B10 supernatant at 37°C for 3 hours, microscope slides were prepared with small aliquots of the mixtures. Cover slips were placed and the slides were examined under high power (×100) using a light microscope (LEITZ DMRB, Leica, Germany).

USFDA standard anti-Hib antibody serum 1/10 dilution in PBS and FMC 41 supernatant (murine IgM κ monoclonal antibody against human blood group A antigen on red cells from Flinders Medical Centre, South Australia) were used as controls.

4.2.6 Biological activity of anti-PRP monoclonal antibodies

Functional characteristics of anti-PRP monoclonal antibodies in B10 supernatant were determined by performing bactericidal and chemiluminescence assays using Hib bacteria and human PMN.

4.2.6.1 Bactericidal Assay

An antibody mediated bactericidal assay was performed based on the method reported by Ferrante *et al* with modifications (Ferrante *et al.*, 1993).

Neutrophils were obtained from the blood of a healthy adult volunteer, using the rapid single step method (Ferrante and Thong, 1982). Blood was collected into a lithium heparin tube and layered onto Hypaque Ficoll medium of density 1.114 (prepared by Department of Immunopathology, Women's and Children's Hospital, Adelaide, South Australia) at a 3:2 ratio and then centrifuged at 400 g for 30 minutes. The neutrophils were carefully recovered from the second leukocyte band and were washed three times in PBS. A viability count was performed by trypan blue exclusion and the cells were resuspended at 1×10^7/ml viable cells in PBS.

Supernatant from the B10 clone subcultured several times in RF 10 culture medium prepared without penicillin and streptomycin antibiotics was used for the bactericidal assay.

Live Hib and pneumococcal bacterial suspensions were prepared from bacterial cultures at 1×10^7/ml concentrations. The assay was set up in screw top tubes as in Table 4.1.

Table 4.1

Bactericidal assay layout

Tube	Hib	Pn	SN B10	MS	PMN
1	100 µl	-	-	-	-
2	100 µl	-	100 µl	-	-
3	100 µl	-	-	-	50 µl
4	100 µl	-	100 µl	-	50 µl
5	100 µl	-	100 µl	40 µl	50 µl
6	-	100 µl	100 µl	-	-
7	-	100 µl	100 µl	-	50 µl
8	-	100 µl	100 µl	40 µl	50 µl

Pn: Pneumococcal suspension, SN: Supernatant, MS: Fresh mouse serum, PMN: Neutrophils

Tube three containing Hib suspensions with PMN and tube one containing only Hib suspension functioned as controls. The final volume of each tube was brought to 500 µl by adding sterile PBS. The tubes prepared with pneumococcal suspensions were negative controls.

All tubes were gassed with a CO_2 air mixture and incubated at 37°C for 2 hours with end to end mixing. A sample of 50 µl from each tube was taken at 0, 1 hour and 2 hours during incubation and diluted in 10ml of sterile milliQ water. Fifty microliters of each dilution were inoculated onto an agar plate and incubated at 37°C in 5% CO_2. The inoculations were performed in duplicate. Chocolate agar plates were used for Hib cultures and blood agar plates were used for pneumococcal cultures. Bacterial colonies were counted after overnight incubation and mean bacterial counts were calculated.

4.2.6.2 Chemiluminescence assay

A chemiluminescence assay was performed to further evaluate the biological function of anti-PRP monoclonal antibodies in the B10 supernatant by activating neutrophils to phagocytose Hib bacteria. The superoxide released from activated neutrophils was measured by monitoring chemiluminescence resulting from the oxidation of lucigenin substrate (Gyllenhammar, 1987; Hardy *et al.*, 1994).

Live Hib and pneumococcal bacterial suspensions were prepared from cultures and resuspended in Hank's balanced salt solution (HBSS) (Invitrogen Corporation, NY, USA) at 2×10^7/ml concentrations. Fresh mouse serum was used as a source of complement.

Neutrophils were obtained from the blood of a healthy adult volunteer as described in the previous experiment. A viability count was performed and the viable neutrophil count was adjusted to 1×10^7/ml in HBSS. The neutrophils were allowed to stand at room temperature for 2 hours to reduce background activation.

The assay was set up in five polystyrene measuring tubes as in Table 4.2 and incubated at 37°C for one hour.

Table 4.2

Chemiluminescence assay layout

Tube	Hib	Pn	SN B10	MS
1	100µl	-	150µl	50µl
2	-	100µl	150µl	50µl
3	100µl	-	-	50µl
4	-	100µl	-	50µl
5	-	-	-	-

Pn: Pneumococcal suspension, SN: Supernatant, MS: Fresh mouse serum

Bacterial suspensions in tubes one and two were pre incubated with the B10 supernatant and mouse serum in order to opsonise the organisms for neutrophil phagocytosis. Sample tubes three, four and five functioned as controls for the assay.

After pre incubation, 100 µl of neutrophils were added to all five tubes and sufficient HBSS was added to bring the volume of each tube to 500 µl. Lucigenin (Sigma Chemical Co., Missouri, USA) dissolved in 500 µl of HBSS (250 µM) was added to each tube. The tubes were immediately placed in the 37°C water jacket chamber of the Luminescence Analyser 1251 (Bio-Orbit, Turku, Finland) and superoxide production from neutrophil activation was recorded by chemiluminescence over a period of 10 minutes. Results were expressed as the maximum rate of chemiluminescence response (in mV) achieved during the 10 minute period.

4.3 Results

4.3.1 Isotyping

All four PRP hybridoma clones selected were secreting IgM antibodies with *k* as light chains (Table 4.3).

Table 4.3

Anti-PRP antibody secreting hybridoma clones

Hybridoma clone	Mean PRP ELISA reading (n = 3)	PRP antibody isotype
A1	1.100	IgM κ
B2	0.626	IgM κ
B10	2.324	IgM κ
B14	0.663	IgM κ

4.3.2 Inhibition assay

The optical density values of the B10 clone supernatant in the PRP ELISA were competitively inhibited when Hib bacterial suspension was added. No inhibition was seen by adding pneumococal bacterial suspension (Fig. 4.1).

4.3.3 Agglutination assay

Agglutination was seen with Hib bacteria incubated with B10 supernatant (Fig. 4.2). *Streptococcus pneumoniae* and *Streptococcus agalactiae* group B bacteria did not agglutinate when incubated with B10 supernatant. Hib bacteria formed aggregates with USFDA serum, which functioned as a positive control. None of the bacterial species agglutinated with negative control FMC 41 supernatant. The anti-PRP antibodies in the supernatant of the B10 clone appeared to be binding specifically with the Hib bacteria to form aggregates.

4.3.4 Bactericidal assay

All bacterial cultures performed at the beginning of the incubations produced similar number of colonies (Fig. 4.3). Cultures from Hib bacterial samples incubated with B10 supernatant, supernatant with neutrophils and supernatant with neutrophils and mouse serum produced significantly fewer colonies compared to the pneumococcal controls. The number of colonies produced by these Hib samples demonstrated a downward trend with time except for the sample incubated with supernatant alone. The Hib sample incubated with neutrophils only produced slightly less number of colonies in subsequent cultures but remained within the range of colonies produced by the controls.

4.3.5 Chemiluminescence assay

Neutrophils added to Hib bacteria preincubated with supernatant and mouse serum produced the highest rate of chemiluminescence response (Fig. 4.4). The neutrophil

response to pneumococcal bacteria preincubated with supernatant and mouse serum was significantly less and was similar to the responses of the control samples.

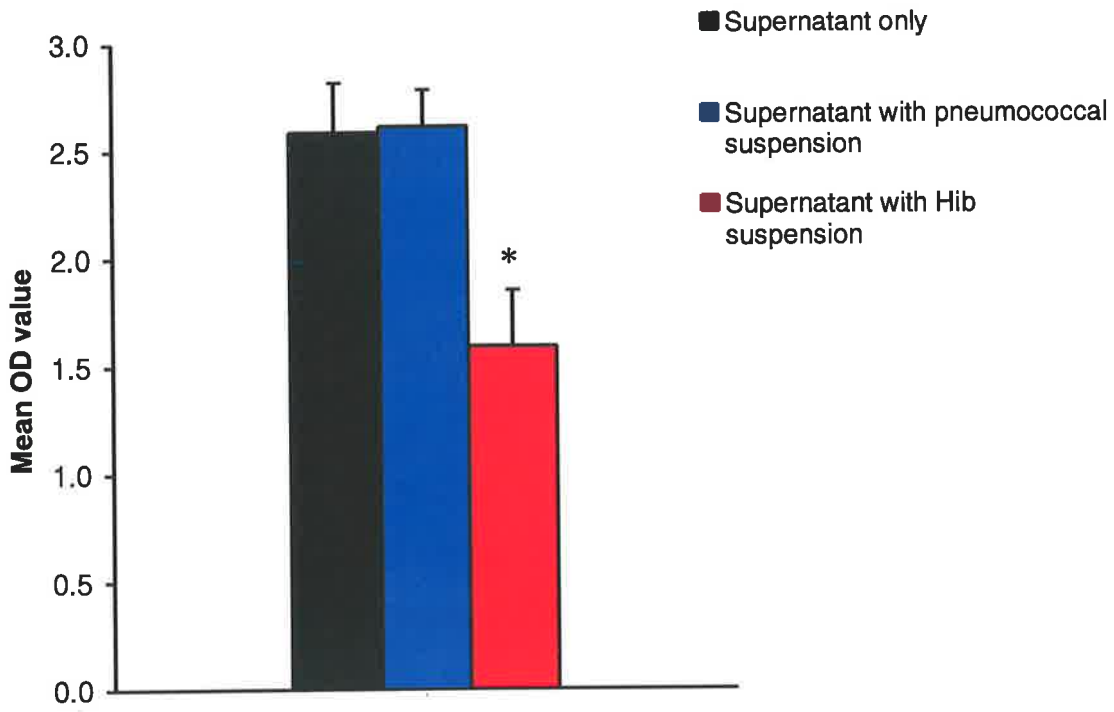
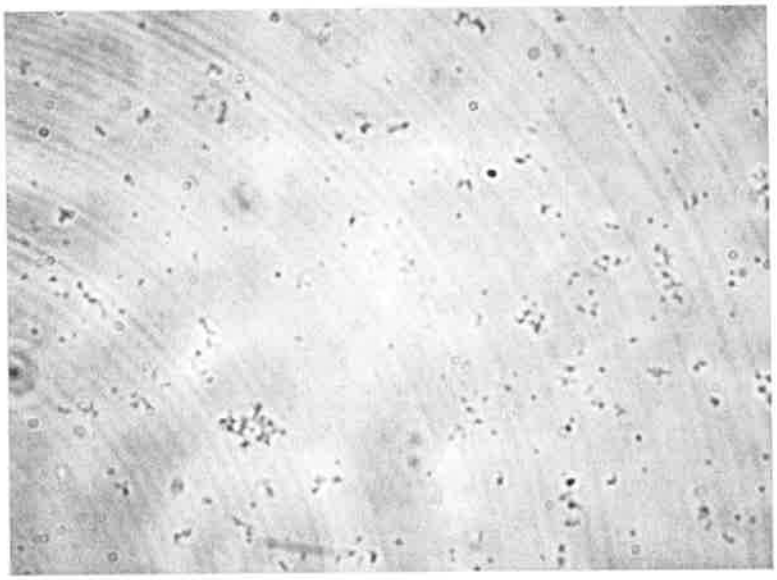


Fig. 4.1 The mean optical density (OD) value of the B10 supernatant in the PRP ELISA. The OD value was inhibited by adding Hib suspension. No inhibition was seen when pneumococcal suspension was added to the supernatant in control wells. Error bars represent the standard deviation of three values. *The inhibition is statistically significant ($P < 0.05$).

A



B

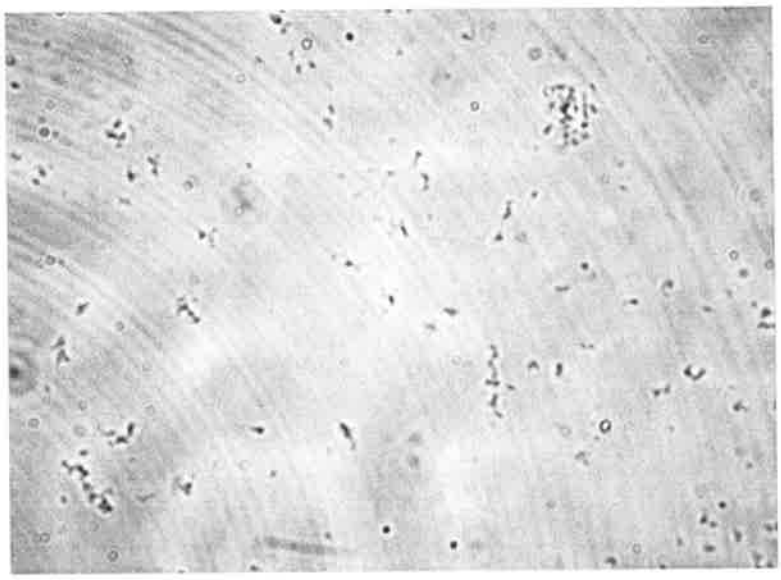
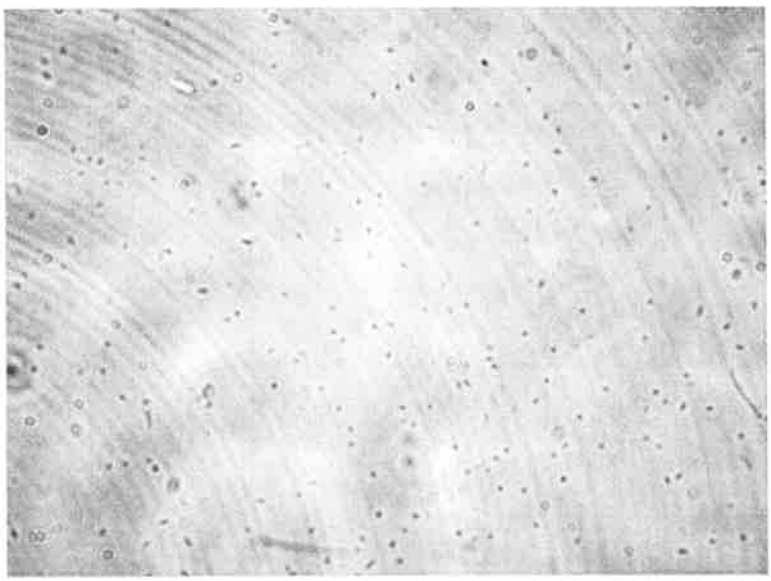


Fig 4.2 Agglutination assay to demonstrate agglutination activity of anti-PRP monoclonal antibodies in B10 supernatant. **A.** Hib incubated with B10 supernatant. **B.** Hib incubated with USFDA serum (positive control). Agglutination seen in both samples.

C



D

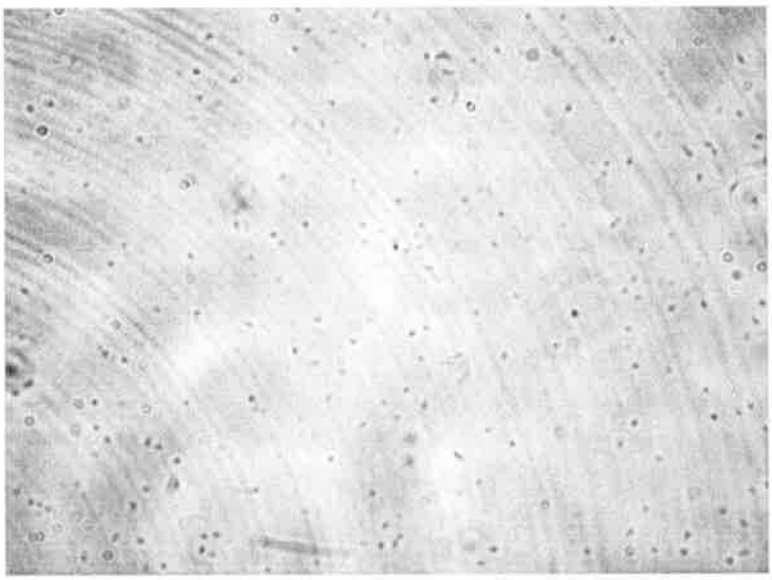
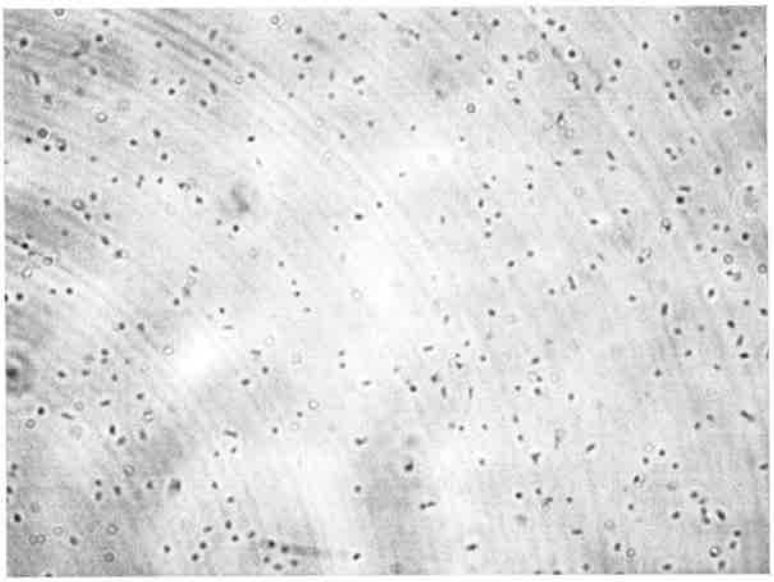


Fig. 4.2 C. Hib incubated with RF10 medium. **D.** Hib incubated with unrelated IgM monoclonal antibody FMC 41. Agglutination was not detected in both samples (negative controls).

E



F

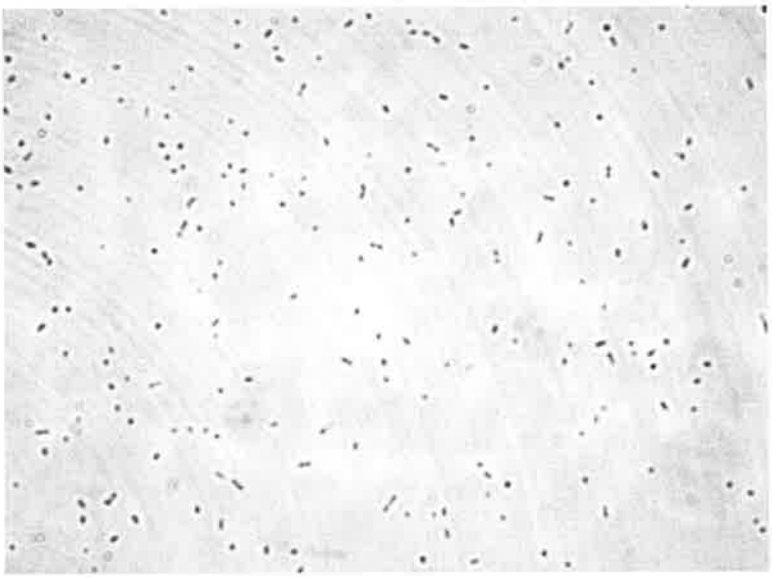


Fig 4.2 E. Pneumococcal bacteria incubated with B10 supernatant. **F.** Group B streptococcal bacteria incubated with supernatant. Agglutination was not detected in both samples (controls).

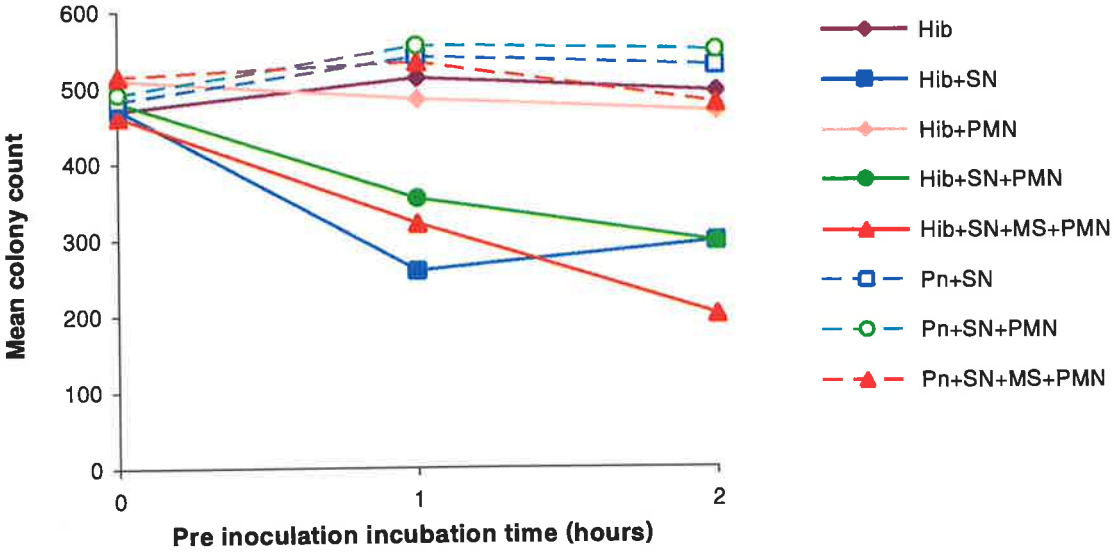


Fig. 4.3 Bactericidal assay used for evaluation of the functional activity of anti-PRP antibodies in the B10 supernatant. Results are expressed as the mean colony count of duplicate cultures. The numbers of bacterial colonies produced in the bactericidal assay by Hib samples incubated with B10 supernatant, B10 supernatant with neutrophils and B10 supernatant with neutrophils and mouse serum were less compared to the control samples. The B10 supernatant inhibited the growth of Hib bacteria with time, but not the growth of pneumococcal bacteria, suggesting the anti-PRP antibody in the B10 supernatant is bactericidal for Hib.

SN: Supernatant PMN: Neutrophils MS: Fresh mouse serum

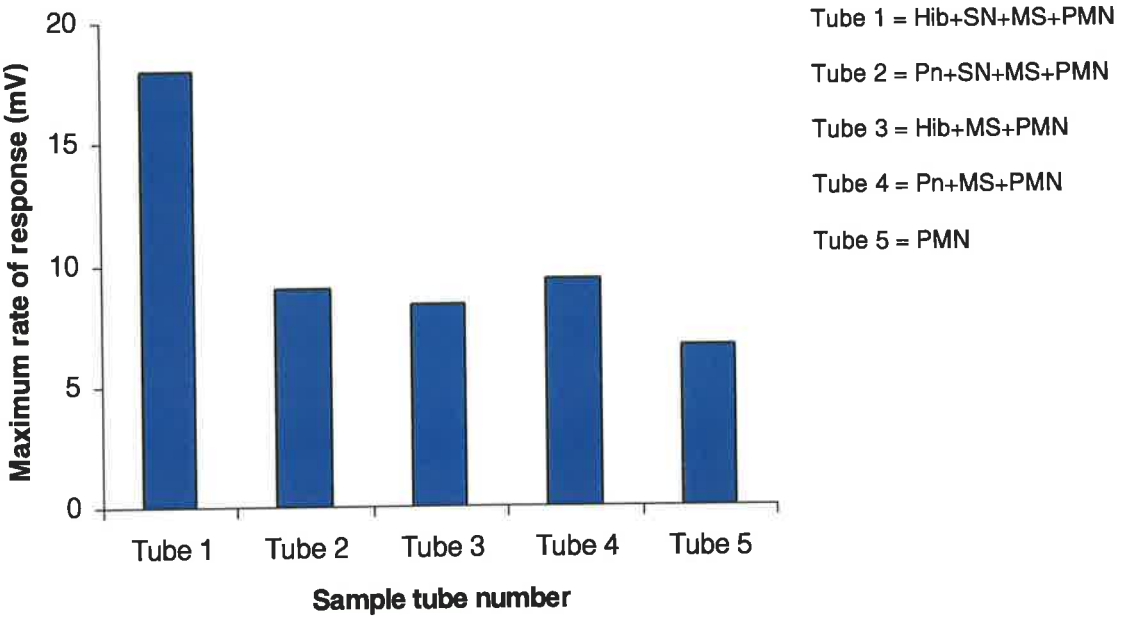


Fig. 4.4 Chemiluminescence assay used for evaluating the functional activity of anti-PRP antibodies in the B10 supernatant. Results are expressed as the maximum rate of neutrophil oxidative response measured in single testing. Neutrophils were activated by anti-PRP antibodies in the supernatant in the presence of Hib.

SN: Supernatant PMN: Neutrophils MS: Fresh mouse serum

4.4 DISCUSSION

The production and characterisation of anti-PRP murine monoclonal antibodies are described in this chapter. Monoclonal antibodies to PRP polysaccharide have been produced by hybridoma techniques during the past two decades. According to the literature, anti-PRP monoclonal antibodies have been used to determine the antibody response to Hib disease by studying the antibody generation, the diversity of the antibody repertoire, and the functional and protective efficacy of the antibodies. These studies have been mainly performed with human monoclonal antibodies. The production of anti-PRP mouse monoclonal antibodies has been reported in only two publications.

A hybridoma secreting anti-PRP monoclonal antibody was initially developed by fusion of splenic lymphocytes from a mouse immunised with several intra peritoneal injections of formalin treated Hib organisms with a myeloma cell line (Gigliotti and Insel, 1982). The antibodies were of IgM class with κ light chains and demonstrated in vitro bactericidal activity. In protective efficacy studies, these antibodies were effective in reducing both the level and incidence of bacteremia in Hib infected rats. As several different *in vivo* immunisation attempts did not produced detectable anti-PRP antibody levels in mice, Bunse and Heinz considered PRP antigen as a weak immunogen in mice (Bunse and Heinz, 1994). To overcome this suggested "limitation", they used a combined *in vivo* and *in vitro* immunisation technique for producing anti-PRP monoclonal antibodies (Bunse and Heinz, 1994). Spleen cells from a mouse immunised with intraperitoneal and intrasplenic injections of PRP-D conjugate vaccine were stimulated in vitro with pure PRP antigen for five days and then fused with a mouse myeloma cell line. One hybridoma secreting anti-PRP

monoclonal antibodies was developed. The antibodies were of the IgM class with κ light chains and were shown to agglutinate several Hib strains using immunoflorescence microscopy. Specificity was further demonstrated by dot blot analysis. The biological activity of the antibodies was shown by neutrophil phagocytosis of Hib bacteria.

Although *in vivo* immunisation per se was not considered to be effective by Bunse and Heinze in producing anti-PRP mouse hybridomas, in this present study one hybridoma clone from mouse A and three hybridoma clones from mouse B were successfully developed using splenic lymphocytes immunised with 2 doses of PRP-T conjugate vaccine *in vivo*.

The monoclonal antibodies of all four hybridoma clones were of the IgM class with κ as light chains. This was consistent with the isotyping results of the two previous reports of murine anti-PRP monoclonal antibody production (Gigliotti and Insel, 1982; Bunse and Heinz, 1994). It appears that the two dose *in vivo* immunisation schedule with PRP-T conjugate vaccine has not resulted in class switching of the anti-PRP antibody response in mice.

In contrast, anti-PRP human monoclonal antibodies produced in previous studies from adult lymphocytes were of IgG and IgA classes, using an immunisation protocol of one dose of either pure PRP vaccine or PRP conjugate vaccine (PRP-D or PRP-CRM) (Gigliotti *et al.*, 1984; Adderson *et al.*, 1992; Carrol *et al.*, 1992; Adderson *et al.*, 1993b; Lucas *et al.*, 1994). This is likely to be a result of class switching by stimulating already existing PRP memory B cells with immunisation, as indicated by pre and post immunisation PRP antibody titre measurements (Adderson *et al.*, 1992; Carrol *et al.*, 1992; Adderson *et al.*, 1993b; Pinchuk *et al.*, 1995).

The results of previous mouse studies and the present study suggest that the antibody response to PRP antigen may be restricted to the IgM class in mice irrespective of the form of PRP antigen used for immunisation. This warrants further investigation.

The binding of monoclonal antibody to purified PRP antigen was inhibited with Hib organisms by about 40% in the inhibition assay. Possibly a stronger inhibition may have been achieved if higher concentrations of Hib organisms were used. Inhibition and agglutination assays clearly demonstrated that the B10 monoclonal antibody was binding to Hib bacteria, presumably to the PRP capsule of the bacteria. This indicates that the configuration of the binding sites of PRP monoclonal antibodies was compatible with the PRP antigenic epitopes of the intact Hib organism. Since agglutination was not demonstrated with pneumococcal and *Streptococcus agalactiae* group B bacteria and it appears that the PRP monoclonal antibody binding to Hib bacteria was specific. However, ideally further specificity testing should be performed to rule out any degree of cross reactivity with other bacteria such as *Streptococcus pyogenes* group A, *Neisseria meningitidis* and *Escherichia coli* K1 carrying surface polysaccharide antigens.

The bactericidal assay indicated that the PRP monoclonal antibody was biologically active against Hib bacteria in comparison to the activity against pneumococcal bacteria. Hib bacteria incubated with monoclonal antibodies, fresh mouse serum and neutrophils produced the greatest decline in the number of colonies in relation to incubation time. The monoclonal antibody together with mouse serum complement facilitated the neutrophil phagocytosis of the Hib bacteria through opsonisation.

Previous studies have also demonstrated the bactericidal effect of IgM anti-PRP monoclonal antibodies in the presence of complement (Gigliotti and Insel, 1982). A

considerable decrease of colonies was also seen in the sample incubated with neutrophils and monoclonal antibodies without mouse serum. This was unexpected as IgM is thought not to be recognised by Fc receptors on neutrophils and usually does not mediate phagocytosis by itself without complement fixation. However, chemiluminescence assays performed by Bunse and Heinz have shown evidence of neutrophil phagocytosis when Hib was incubated with anti-PRP IgM antibodies (Bunse and Heinz, 1994). Since the PRP capsule of Hib is a repetitive polymer found in high density on the bacterial surface, it is possible that IgM anti-PRP antibodies may have agglutinated Hib organisms and produced large antigen antibody complexes triggering direct neutrophil phagocytosis.

When Hib organisms were incubated with only monoclonal antibodies without neutrophils, the organisms were partially inactivated in the initial phase and then multiplied during the second hour of incubation. Although monoclonal antibodies are thought not to usually possess direct bactericidal capabilities (Oroszlan and Nowinski, 1980), it is possible that binding of monoclonal antibodies to Hib may have completely or partially inhibited the growth of some bacteria. Multiplication of the non inhibited or partially inhibited bacteria could have resulted in producing a higher colony count at the end of two hour incubation than the count at the end of one hour incubation. This argument is supported by the study of van Alphen *et al* (van Alphen *et al.*, 1996). They have shown that anti-PRP antibodies inhibit the multiplication of Hib bacteria. The inhibition depended on the antibody concentration; high concentrations completely inhibited the growth, whereas the inhibition with low concentrations was temporary. It was suggested that the binding of anti-PRP antibodies to the capsule forms a negatively charged layer around the bacterium and may act as a diffusion barrier for nutrients into bacteria.

The functional activity and specificity of the monoclonal antibodies were further confirmed by chemiluminescence assay. An enhanced neutrophil oxidative burst was achieved by opsonisation of Hib bacteria with monoclonal antibodies in the presence of fresh mouse serum as a source of complement.

Anti-PRP monoclonal antibodies may be useful in the diagnosis of Hib infection. At present a confirmatory diagnosis of invasive Hib infections is made by blood or cerebrospinal fluid (CSF) culture. This process may take up to 24-36 hours. During Hib infection the PRP capsular antigen is present in body fluids (serum, CSF and urine). Anti-PRP polyclonal antibody coated latex particles are used to perform a rapid provisional diagnosis of Hib infection from body fluids (Kumar *et al.*, 1980). The latex agglutination test (Wellcogen* Bacteria Antigen Kit, Murex Biotech, UK) has a specificity and a sensitivity of over 95%. If monoclonal antibodies are used in coating latex beads, it should further increase the specificity of the test, as the binding sites of the antibodies will posses only a single configuration leading to minimal cross reactivity. However, this possibly could compromise the sensitivity of the diagnostic test.

Anti-PRP monoclonal antibodies could be used in the development of a rapid immunochromatography test as an alternative to the latex agglutination test for diagnosis invasive Hib disease. This could be achieved by adopting a similar technique used in the development of the immunochromatography test for the diagnosis of *Haemophilus ducreyi* bacterial infection, a sexually transmitted disease causing genital ulcers (Patterson *et al.*, 2002). In this diagnostic test highly specific mouse monoclonal antibodies to a receptor protein of *Haemophilus ducreyi* were used

in the preparation of immunochromatography test strips. The results of this test were determined in 15 minutes with a specificity of 100%.

Serum antibody titres to Hib organisms are measured by in house laboratory immunoassays similar to the PRP ELISA used in this study. The immunoassays are performed using PRP antigen coated plates. It is possible that the specificity and precision of the immunoassays could be improved if the ELISA plates were coated with PRP monoclonal antibodies and a sandwich immunoassay system is used.

When anti-PRP monoclonal antibodies were initially developed, the bactericidal effects of the antibodies were studied in attempts to use them as a therapeutic agent during Hib infection (Gigliotti and Insel, 1982; Hunter *et al.*, 1982). Since very effective antibacterial medications have been developed against Hib, use of monoclonals as a therapeutic agent is of more theoretical than practical interest. However, administration of anti-PRP antibodies could be useful as passive immunisation for the prevention of Hib infection in high risk patients who cannot be immunised adequately with Hib vaccines (Santosham *et al.*, 1987; Siber *et al.*, 1992). In this situation, polyclonal anti-PRP antibodies may be more useful than the monoclonals as the protective efficacy of the former would be greater, although monoclonal antibody is more amenable to manipulation to prevent the formation of recipient antibody in this form of immunotherapy.

In summary, this chapter has described the development of anti-PRP monoclonal antibodies by simple *in vivo* immunisation with PRP-protein conjugate antigen for the first time. The anti-PRP monoclonal antibodies were specific and functionally active against Hib bacteria. The following chapter reports the attempted use of anti-PRP

monoclonal antibodies in immunofluorescence assays in order to detect PRP specific B cells by flow cytometry.

CHAPTER 5

USE OF ANTI-PRP MONOCLONAL ANTIBODIES IN INDIRECT IMMUNOFLUORESCENCE STAINING FOR ISOLATION OF PRP SPECIFIC B CELLS

5.1 Introduction

Direct labeling of PBMC with biotinylated PRP did not allow clear identification of PRP specific B cells by flow cytometry (Chapter 3). Since one possible explanation was that the biotinylated PRP was not binding with antigen specific B cells as expected because of the chemical modification required for biotinylation, it was felt that the use of unmodified pure PRP, detected by anti-PRP monoclonal antibodies may produce definitive results.

As described in Chapter 4, anti-PRP monoclonal antibodies were produced for use with pure PRP antigen in indirect immunofluorescence staining to identify PRP specific B cells by flow cytometry as mentioned above. The experiments were designed to be performed by incubating PBMC from immunised donors with pure PRP antigen and then identifying PRP bound B cells with PRP monoclonal antibodies detected by sheep anti-mouse fluorescent conjugate. PBMC were also to be stained with labelled CD19 antibodies to identify the B cell lineage, and stained with other relevant labelled antibodies for the characterisation of the PRP antigen specific B cells.

Furthermore, since PRP polysaccharide is a long chain with multiple repeating antigenic epitopes, several anti-PRP monoclonal antibodies may bind to the PRP and subsequently be detected by secondary fluorescent antibodies resulting in an amplified and a brighter signal. Increasing the amount of fluorescence on each cell may facilitate the identification of positive cells, provided background staining is not increased at the same time. Therefore, this technique could also be a better choice for detecting the PRP specific B cells, if the signal strength of the positive cells was not adequate.

However, it was important to be certain that the anti-PRP monoclonal antibodies produced would not bind PBMC, especially B cells, through non PRP molecules during the immunofluorescence staining. Therefore, some initial immunoflorescence assays were performed by incubating PBMC from unimmunised donors with hybridoma supernatants containing anti-PRP monoclonal antibodies, sheep anti mouse fluorescent conjugate and CD19 fluorescent conjugate to determine any "non specific binding" of anti-PRP monoclonal antibodies to B cells, which could interfere with PRP specific B cell detection.

5.2. Methods

Prior to performing the immunofluorescence assays, supernatants from all four selected PRP hybridoma clones (A1, B2, B10 and B14) were retested for anti-PRP activity by PRP ELISA. Supernatants from B10 and A1 clones demonstrated anti-PRP activities, as described previously in Chapter 4. Clones B2 and B14 had completely ceased anti-PRP antibody secretion. Therefore, supernatants B10 and A1

were used in indirect immunofluorescence assays with flow cytometry to determine the extent of the "non specific binding".

5.2.1 Lymphocyte preparation

Peripheral blood lymphocytes were separated from blood of adult healthy volunteers as described in Chapter 2. Viability counts were performed by trypan blue exclusion and the cell numbers were adjusted to 1×10^7 viable cells/ml of PBS.

5.2.2 Immunofluorescence staining

The indirect immunofluorescence staining was performed as illustrated in table 5.1. PBMC (50 µl aliquots) were added to a set of three immunofluorescence tubes. Fifty microlitres of PRP antibody supernatant, either neat or in 1/4 dilution in PBS or in 1/8 dilution in PBS, were added to the appropriate tube and incubated at 4°C for 30 minutes. Similar sets of tubes were prepared with a blocking step in an attempt to block the anticipated non-specific binding of PRP antibodies. Prior to the addition of the PRP antibody supernatant to these tubes, either 15 µl or 30 µl of neat sheep serum (Cytostain, Adelaide, Australia) were added to each tube and incubated at 4°C for 15 minutes. In some immunofluorescence assays 15 µl of neat fetal calf serum were used as the blocking agent. After the incubation, the cells were washed with PBS-Azide as described in Chapter 2 and then anti-mouse immunoglobulin-FITC 50µl of 1/50 dilution in PBS were added to each tube. After incubating at 4°C for 30 minutes the cells were washed as above. Unbound sites on the anti-mouse immunoglobulin-FITC were blocked by adding 5 µl of mouse serum to each tube and incubating at 4°C

for 15 minutes. CD19-PE-Cy5 5 µl was added to each tube and incubated at 4°C for 30 minutes and then washed as above.

Table: 5.1

Indirect immunofluorescence assay layout

TEST			BLOCK		
Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
SN neat	SN 1/4	SN 1/8	Block	Block	Block
Am Ig-FITC	Am Ig-FITC	Am Ig-FITC	SN neat	SN 1/4	SN 1/8
CD19-PE-Cy5	CD19-PE-Cy5	CD19-PE-Cy5	Am Ig-FITC	Am Ig-FITC	Am Ig-FITC
			CD19-PE-Cy5	CD19-PE-Cy5	CD19-PE-Cy5

SN: Supernatant, Am Ig-FITC: Anti-mouse immunoglobulin-FITC

Control tubes were prepared by incubating PBMC either with 50 µl of RF 10 culture medium (culture medium for PRP hybridoma) or with 50 µl of B14 supernatant (anti-PRP activity lost clone) or with 50 µl of FMC 41 supernatant (murine IgM κ monoclonal antibody against human blood group A antigen on red cells from Flinders Medical Centre, South Australia) at 4°C for 30 minutes and subsequently staining with anti-mouse immunoglobulin-FITC and CD19-PE-Cy5 as described above.

In some experiments, the supernatants were spun at 13,000 rpm for 15 minutes in a micro centrifuge and upper portions of the supernatants were incubated with the

PBMC. This was performed to remove possible aggregates of PRP antibodies in the supernatants in an attempt to reduce "non specific binding".

5.2.3 Flow cytometric analyses

Flow cytometric analyses were performed immediately after staining was completed. All samples were maintained at 4°C during analysis. Data were recorded using an EPICS® ELITE ESP Flow cytometer / cell sorter (Coulter, USA). The machine was calibrated with Flow-Check™ Fluorosphere beads (Beckman Coulter, USA) prior to analysis. Over 20,000 live lymphocytes were gated according to the light scatter pattern of each analysis. Two colour (FITC and PE-Cy5) analyses were performed using FL2 (515-535 nm) and FL4 (665-685 nm) channels respectively using argon laser (488 nm). Compensation was adjusted with the help of control tubes containing cells stained separately with sheep anti mouse immunoglobulin FITC conjugate and CD19-PE-Cy5

5.3 Results

In the control sample of PBMC incubated with RF10 medium, 0.18% of B cells and 0.06% of non B cells were stained with FITC (Fig. 5.1 A). This was the background staining by anti-mouse immunoglobulin-FITC conjugate. In the negative control samples, when PBMC were incubated with FMC 41 supernatant, 0.14% of B cells and 0.05% non B cells were stained with FITC (Fig. 5.1 B). When B14 supernatant (anti-PRP activity lost clone) was incubated with PBMC, 0.17% of B cells and 0.09% of non B cells were stained with FITC (Fig. 5.1 C).

The mean percentages of B and non B cells binding with PRP monoclonal antibodies in B10 supernatant are given in table 5.2 and 5.3. When the supernatant was added neat, 2.79% of B cells were stained with FITC (Table 5.2). When the supernatant was added at 1/4 and 1/8 dilutions, the FITC stained B cell mean percentages were 1.38% and 1.12% respectively. When PBMC were preincubated with sheep serum the B cells mean percentages staining with FITC were increased to 2.53%, 1.96% and 2.33% respectively. The mean percentages of B cells stained with FITC after PBMC were pre-incubated with fetal calf serum were 9.20%, 3.85% and 1.93% respectively (Table 5.3). In all samples the mean percentages of non B cells stained with FITC were less than 1%. The flow cytometric patterns for a selected donor are shown in figures 5.2 and 5.3. The flow cytometric patterns of all analyses were similar to these dot plots.

The mean percentages of B and non B cells binding with anti-PRP monoclonal antibodies in high speed centrifuged B10 and A1 supernatants are shown in tables 5.4 and 5.5 respectively. The mean percentages at a 1/8 dilution of the B10 supernatant were 1.17%, 1.02% and 0.94% respectively. The mean percentages of B cells binding to PRP antibodies in A1 supernatants were 1.92%, 1.62% and 1.01% respectively. Pre incubation of PBMC with sheep serum either 15 µl or 30 µl increased the mean percentages of B cells staining with FITC. The mean percentages of non B cells stained with FITC in all occasions were less than 1%. The flow cytometric patterns of all analyses were similar to the dot plots shown in figures 5.2 and 5.3.

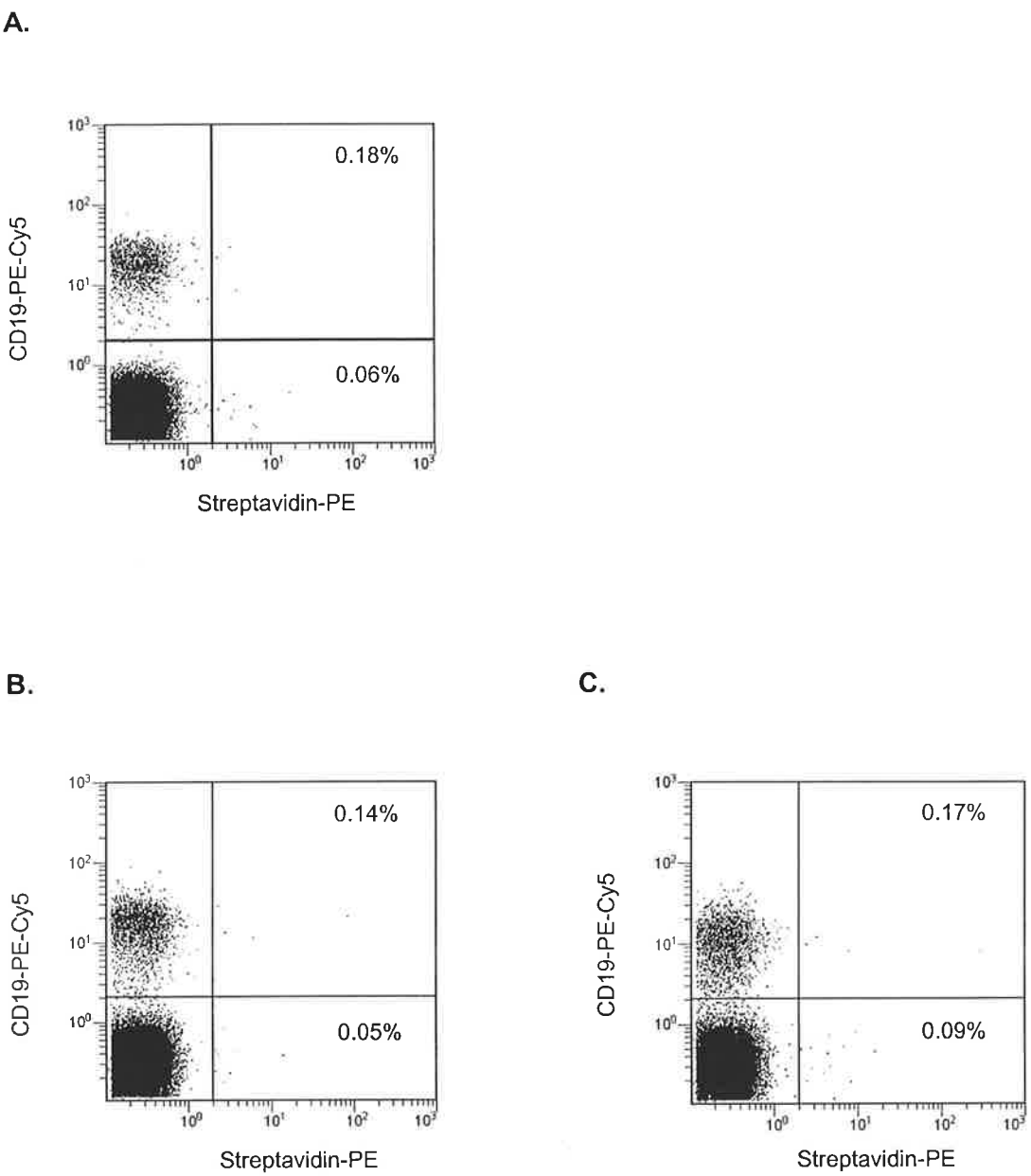


Fig. 5.1 Flow cytometric analysis of PBMC from an adult donor as controls. **A.** The cells were incubated with RF10 culture medium and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5 to determine background staining. Percentages of B cells and non B cells stained with FITC were 0.18 and 0.06 respectively. **B.** The cells were incubated with FMC 41 supernatant and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5 as a negative control. Percentages of B cells and non B cells stained with FITC were 0.14 and 0.05 respectively. **C.** The cells were incubated with B14 supernatant and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5 as a negative control. Percentages of B cells and non B cells stained with FITC were 0.17 and 0.09 respectively.

Table 5.2

Mean percentages of B and non B cells binding with PRP monoclonal antibodies in the B10 supernatant (blocked with sheep serum)

Blocking	SN Neat		SN 1/4 Dilution		SN 1/8 Dilution	
	B cell %	Non B cell %	**B cell %**	Non B cell %	**B cell %**	Non B-cell %
No blocking	**2.79** **(1.14)**	0.47 (0.18)	**1.38** **(0.22)**	0.27 (0.03)	**1.12** **(0.21)**	0.18 (0.04)
SS 15 µl	**2.53** **(0.19)**	0.61 (0.25)	**1.96** **(0.38)**	0.38 (0.09)	**2.33** **(0.84)**	0.24 (0.03)

Cell %: Mean percentage of B or non B cells stained with FITC. SS: Sheep serum
SN: Supernatant
Number of donors four. Standard error of mean (SEM) given within brackets.

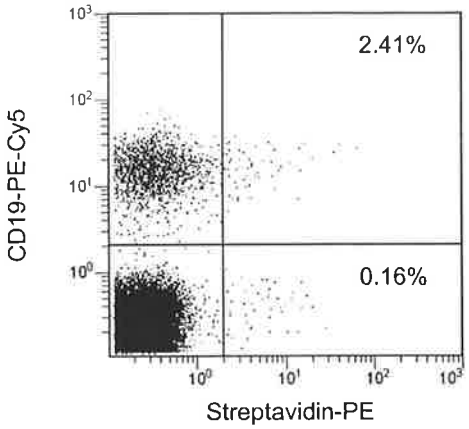
Table 5.3

Mean percentages of B and non B cells binding with PRP monoclonal antibodies in the B10 supernatant (blocked with fetal calf serum)

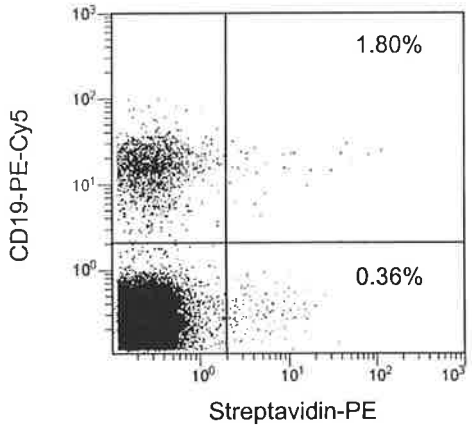
Blocking	SN Neat		SN 1/4 Dilution		SN 1/8 Dilution	
	B cell %	Non B cell %	**B cell %**	Non B cell %	**B cell %**	Non B cell %
No blocking	**3.62** **(2.49)**	0.78 (0.01)	**1.23** **(0.40)**	0.25 (0.04)	**0.88** **(0.30)**	0.22 (0.05)
FCS 15 µl	**9.20** **(6.84)**	1.57 (0.12)	**3.85** **(2.80)**	0.47 (0.07)	**1.93** **(1.41)**	0.18 (0.04)

Cell %: Mean percentage of B or non-B cells stained with FITC. FCS: Fetal calf serum
SN: Supernatant
Number of donors two. SEM given within brackets.

A.



B.



C.

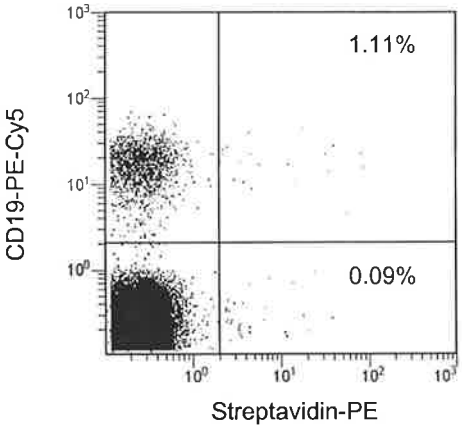
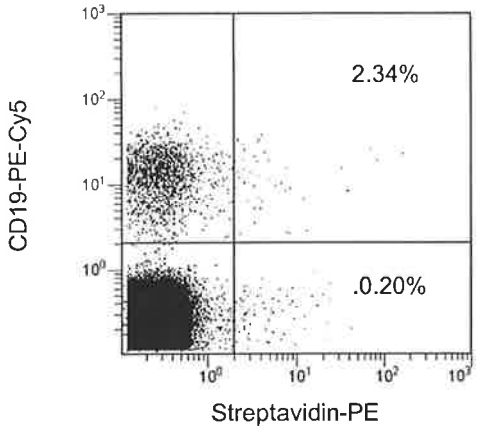
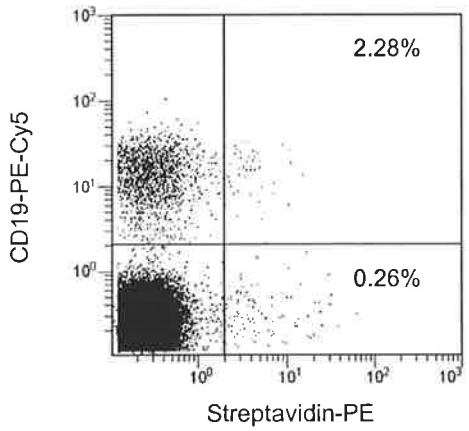


Fig. 5.2 Flow cytometric analysis of PBMC from an adult donor to determine "non specific binding" of anti-PRP monoclonal antibodies in the B10 supernatant to lymphocytes. **A.** The cells were incubated with neat supernatant and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 2.41 and 0.16 respectively. **B.** The cells were incubated with B10 supernatant at 1/4 concentration and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 1.80 and 0.36 respectively. **C.** The cells were incubated with B10 supernatant at 1/8 concentration and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 1.11 and 0.09 respectively.

A.



B.



C.

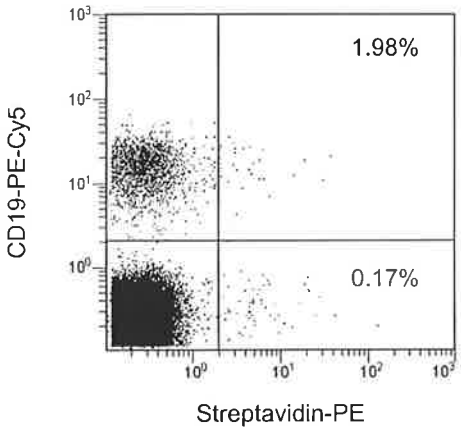


Fig. 5.3 Flow cytometric analysis of PBMC from the donor in Fig. 5.2 to determine "non specific binding" of PRP monoclonal antibodies in the B10 supernatant to lymphocytes following pre incubating with neat sheep serum in an attempt to block the non specific binding **A.** The cells were then incubated with neat B10 supernatant and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 2.34 and 0.20 respectively. **B.** The cells were incubated with B10 supernatant at 1/4 concentration and were stained with anti-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 2.28 and 0.26 respectively. **C.** The cells were incubated with the B10 supernatant at 1/8 concentration and were stained with ant-mouse Ig-FITC and CD19-PE-Cy5. Percentages of B cells and non B cells stained with FITC were 1.98 and 0.17 respectively.

Table 5.4

Mean percentages of B and non B cells binding with PRP monoclonal antibodies of B10 supernatant following high speed centrifugation to remove aggregates

Blocking	SN Neat		SN 1/4 Dilution		SN 1/8 Dilution	
	B cell %	Non-B cell %	**B cell %**	Non-B cell %	**B cell %**	Non-B cell %
No blocking	**1.17** **(0.28)**	0.29 (0.06)	**1.02** **(0.21)**	0.24 (0.11)	**0.94** **(0.10)**	0.20 (0.08)
SS 15 µl	**1.61** **(0.34)**	0.25 (0.09)	**1.11** **(0.19)**	0.17 (0.04)	**2.05** **(0.54)**	0.30 (0.09)
SS 30 µl	**1.69** **(0.29)**	0.25 (0.09)	**1.20** **(0.12)**	0.19 (0.07)	**1.78** **(0.47)**	0.20 (0.08)

Cell %: Mean percentage of B or non B cells stained with FITC. SS: Sheep serum
SN: supernatant
Number of donors four. SEM given within brackets.

Table 5.5

Mean percentages of B and non B cells binding with PRP monoclonal antibodies of A1 supernatant following high speed centrifugation to remove aggregates

Blocking	SN Neat		SN 1/4 Dilution		SN 1/8 Dilution	
	B cell %	Non-B cell %	**B cell %**	Non-B cell %	**B cell %**	Non-B cell %
No blocking	**1.92** **(0.55)**	0.46 (0.17)	**1.62** **(0.43)**	0.32 (0.21)	**1.01** **(0.19)**	0.30 (0.32)
SS 15 µl	**2.59** **(1.19)**	0.53 (0.32)	**2.97** **(0.46)**	0.54 (0.40)	**1.53** **(0.45)**	0.24 (0.15)
SS 30 µl	**3.97** **(2.91)**	0.85 (0.73)	**1.73** **(0.25)**	0.36 (0.17)	**2.27** **(0.74)**	0.29 (0.14)

Cell %: Mean percentage of B or non B cells stained with FITC. SS: Sheep serum
SN: Supernatant
Number of donors three. SEM given within brackets.

5.4 Discussion

Binding of anti-PRP monoclonal antibodies to B and non B cells through non PRP molecules was considered as "non specific binding" in these experiments as it was not expected to happen in ideal situations. The non specific staining of B cells and non B cells by anti-mouse immunoglobulin-FITC in the control samples (background staining control and negative controls) was less than 0.2%. Therefore, when PBMC were incubated with supernatant B10 or A1 the staining of B cells and non B cells by anti-mouse immunoglobulin-FITC was mainly due to "non specific binding" of anti-PRP monoclonal antibodies to lymphocytes. The mean percentages of B cells binding PRP monoclonal antibodies were invariably more than 1.0%, whereas the mean non B cell percentages were less than 1.0%. The "non specific binding" of PRP monoclonal antibodies to B cell and non B cells was highly variable between donors as indicated by the SEM values.

Due to the lack of anti-PRP antibodies in the supernatant, the FITC staining in PBMC incubated with B14 supernatant was low (negative control).

IgM monoclonal antibody activity in the FMC 41 supernatant was confirmed by performing an agglutination slide test with blood group A red blood cells prior to use in the immunofluorescence staining in the control sample (results not shown). It is not clear why the IgM antibodies in the FMC 41 did not produce high levels of non specific staining of the PBMC similar to the non specific staining produced by anti-PRP IgM antibodies in the B10 and A1 supernatant.

Serial dilution of the B10 and A1 supernatants reduced the "non specific binding" in some assays. However, even at a 1/8 dilution the "non specific binding" of B cells

was more than 0.50% (individual sample data not shown). This is a significant percentage when attempting to isolate antigen specific B cells present in low or very low frequency. Further dilution of supernatant may have reduced the "non specific binding" but the concentrations may not be adequate to detect PRP specific B cells.

When the supernatant was centrifuged at high speed to remove aggregates, the binding of anti-PRP monoclonal antibodies to B cells and non B cells was reduced, but remained much higher than the background values of the control samples.

Attempted inhibition of the "non specific binding" of PRP monoclonal antibodies by pre incubating PBMC with sheep serum at two different concentrations (15 µl and 30 µl) as well as with fetal calf serum (15 µl) did not result in any reduction of the non specific binding. In fact in most instances, the "non specific binding" was increased.

The PRP monoclonal antibodies in B10 and A1 supernatants were of IgM class. Although IgM Fc receptors have been reported to be present on some B cells (Shibuya *et al.*, 2000; Shimizu *et al.*, 2001), the "non specific binding" of the PRP monoclonal antibodies to B cells appears unlikely to be through their Fc receptors as the binding was not reduced by pre incubating the PBMC with sheep serum or fetal calf serum. Therefore, use of F(ab) fragments or single chain PRP monoclonal antibodies (Ig without Fc portions) in the indirect immunofluorescence assays may not produce any significant improvement in the non specific binding.

FITC labeled secondary antibodies were the detecting reagent in the indirect immunofluorescence assays described in this Chapter. However, switching from PE (streptavidin-PE was used in the direct immunofluorescence assays in Chapter 3) to FITC reduces the signal strength, as the quantum yield of FITC is approximately 2.5

fold less than the yield of PE (Zola, 1998). Therefore, FITC is less sensitive than PE in immunofluorescence staining. Since high background staining of PBMC was seen due to "non specific binding" of PRP monoclonal antibodies, the use of a secondary antibody conjugated to PE would be unlikely to have been helpful.

It can be concluded that the use of anti-PRP monoclonal antibodies in the immunofluorescence staining to detect antigen specific B cells by flow cytometry may give rise to false positive results due to non specific binding of the monoclonal antibodies to lymphocytes, especially when the expected antigen specific B cells numbers are very low. Therefore, detection of PRP specific B cells using unbiotinylated pure PRP antigen was not attempted.

The next step of the project was to test the hypothesis that PRP specific B cells are likely to be confined to secondary lymphoid tissues. This is discussed in Chapter 6.

CHAPTER 6

ISOLATION OF ANTI-PRP AND ANTI-TT ANTIBODY SECRETING CELLS FROM IMMUNISED MICE

6.1 Introduction

In Chapter 3, PRP specific B cells could not be definitively identified by flow cytometry in blood from human adults immunised with PRP-OMP conjugate vaccine. As a reason for this finding, it was suggested that PRP specific B cells may be mainly confined to secondary lymphoid tissues and largely absent in blood. Therefore, a murine model was developed to test this hypothesis by attempting to isolate PRP specific B cells from spleen tissues from immunised mice.

The spleen is a secondary lymphoid organ that mounts efficient immune responses to foreign antigens (see Chapter 1). Its structure is highly organised to generate antibody responses to TD and TI antigens. Therefore, spleen cells have been widely used in animal models to study immune responses in the secondary lymphoid tissues.

Two techniques were used in parallel to study the presence of antigen specific B cells in splenic tissues. (1) Immunofluorescence staining and flow cytometric analysis as described in Chapter 3 was used to detect antigen specific B cells. (2) An enzyme linked immunospot (ELISPOT) assay was developed to enumerate antigen specific antibody secreting cells (ASC) as an indirect representation of antigen specific B cells.

The ELISPOT technique was initially developed by Czerkinsky *et al* in the early 1980's as an alternative to conventional haemolytic plaque forming assays for enumeration of cells secreting specific antibody (Czerkinsky *et al.*, 1983). A cell suspension containing antibody secreting cells was incubated in antigen coated polystyrene plates, and bound antibodies were demonstrated by binding of an anti immunoglobulin antibody linked to enzyme, followed by an enzyme substrate reaction performed in agarose, producing dark coloured circular spots. This initial technique was improved by introducing nitrocellulose membrane as a solid support for the coating antigen and performing the enzyme substrate reaction to produce dark coloured spots on the membrane (Moller and Borrebaeck, 1985).

In order to identify PRP specific B cells from lymphoid tissues in this present study, mice were immunised with PRP-T conjugate vaccine and then flow cytometry and ELISPOT assays were performed on spleen cells. TT specific B cells were studied in parallel to compare and contrast the generation of PRP specific B cells in response to the PRP-T conjugate vaccine with that of protein antigen. ELISPOT assays were also performed on peripheral blood from immunised mice to detect the presence of anti-PRP and anti-TT cells.

6.2 Methods

6.2.1 Immunisation of mice

Eight to ten week old BALB/c mice were obtained from the University of Adelaide Laboratory Animal Services, and were housed in cages at the animal care facility at the Women's and Children's Hospital, South Australia.

Hiberix™ vaccine (PRP-T) was obtained as a gift from SmithKline Beecham, Belgium.

A preliminary set of experiments was performed on three mice immunised with one dose of PRP-T vaccine. Antigen specific B cells were not detected definitively from spleen cells by flow cytometric analysis above background staining on day 5 post immunisation (0.22% of B cells). The frequencies of anti-PRP and anti-TT ASC in the splenic tissues detected by ELISPOT assays in immunised mice were also low (230 and 200 per 10^6 spleen cells respectively) and were only marginally higher than in unimmunised mice (155 and 167 per 10^6 spleenocytes respectively). The differences between immunised and unimmunised mice were not statistically significant. On the basis of these results, a two dose immunisation protocol was adopted.

Six mice were immunised subcutaneously at the nape of neck with two injections of PRP-T vaccine, 2 weeks apart. Immunisation was performed with ¼ of the normal human vaccine dose (125 µl containing 2.5 µg of RPR and 7.5 µg of TT) as recommended by the vaccine manufacturer (unpublished data).

6.2.2 Spleens and blood collection

Five days after the second immunisation, the mice were sacrificed using CO_2 inhalation. The mice were placed on a corkboard cleaned with 70% alcohol and mid line incisions were made from the lower abdomen up to the thoracic cage. Blood was collected into lithium heparin tubes by cardiac puncture. The spleens were collected from the abdominal cavity and placed in containers containing sterile PBS. Spleens and blood samples were collected in parallel from unimmunised mice as negative controls. All procedures were performed under aseptic conditions in a laminar flow cabinet.

6.2.3 Obtaining MNC from spleens and leukocytes from blood

All procedures were performed under sterile conditions in a laminar flow cabinet. The spleens were placed in petri dishes containing 3 ml of sterile PBS. The spleen capsules were cut and the splenic tissues were then crushed with the blunt ends of 10 ml plastic syringes allowing the cells to drain into PBS. The suspensions were then filtered through sterile Falcon® 70 μm cell strainers (Becton Dickinson Labware, New Jersey, USA). The cell suspensions were diluted in five parts of PBS and centrifuged at 1000g for 10 minutes. The supernatants were discarded and the cells were then re-suspended in NH_4Cl red cell lysis solution for 5 minutes at room temperature (5 ml per spleen). The cells were then washed three times in PBS and re suspended in RF10 culture medium. Viability counts were performed using the trypan blue exclusion method. Cell counts were adjusted to 1×10^6 viable cells per 1 ml.

The blood samples were mixed with NH_4Cl red cell lysis solution for 5 minutes at room temperature (5 ml per sample) and then washed threes times in sterile PBS as

described above. The remaining blood leucocytes were re suspended in RF10 culture medium at 1×10^6 viable cells per 1 ml.

6.2.4 Immunofluorescence staining and flow cytometric analyses

Two colour immunofluorescence staining was performed on the spleen cells as described in Chapter 2 using biotinylated PRP and TT, CD45R/B220-FITC (murine B cell marker) and streptavidin-PE to identify PRP and TT antigen specific B cells. The biotinylated antigens were used only at 10 μg/ml concentration. The control samples were stained with only CD45R/B220-FITC and streptavidin-PE to determine background staining of streptavidin-PE.

Flow cytometric analysis was performed immediately after staining was completed as described in Chapter 2. PRP and TT antigen specific B cells were expected to be double stained by CD45R/B220-FITC and streptavidin-PE.

6.2.5 ELISPOT assays

Cells secreting anti-PRP and anti-TT antibodies were detected by ELISPOT assays as reported previously, with some modifications (Moller and Borrebaeck, 1985; Munoz and Insel, 1987; Barington *et al.*, 1992). Optimal concentrations of antigens and secondary antibodies and the duration of cell incubation in the assays were determined in preliminary experiments.

Mixed cellulose ester membrane based 96 well MultiScreen™ plates (Millipore, Bedford, USA) were coated with PRP (10 μg/ml) and (TT 10 μg/ml) diluted in 0.05 M carbonate buffer 100 μl per well and incubated overnight at 4°C. The plates were

then sucked dry with a MilliPore vacuum manifold (Millipore, Bedford, USA) and left at room temperature for 1 hour. After being washed twice with PBS using the MilliPore vacuum manifold, the plates were blocked with 1% PBS-BSA (200 µl per well) for 2 hours at 37°C. The plates were then washed once with PBS using the MilliPore vacuum manifold before adding the cells.

Serial dilutions of spleen cell suspensions at 5×10^3, 10×10^3, 50×10^3, and 100×10^3 cells in 100 µl of RF10 culture medium containing affinity isolated sheep anti-mouse immunoglobulin alkaline phosphatase conjugate (AMRAD Biotech, Victoria, Australia) 1/7500 were added into triplicate wells.

The peripheral blood leukocytes were added to triplicate wells at 50×10^3, 100×10^3 and 150×10^3 in 100 µl of RF10 culture medium containing anti-mouse Ig alkaline phosphatase conjugate at a final dilution of 1/7500.

The plates were incubated for 12-15 hours at 37°C in 5% CO_2 in a humid chamber. The plates were rinsed six times with PBS, four times with PBS-Tween using the MilliPore vacuum manifold, and twice with Tris buffer using the MilliPore vacuum manifold. The substrate preparation containing 66 µl of Nitro Blue Tetrazolium (Sigma Chemical Co., Missouri, USA) stock solution (50 mg per 1ml of 70% Dimethyl Formamide) and 33 µl of 5-Bromo-4-Chloro-3-Indolyl Phosphate (Sigma) stock solution (50 mg per 1ml of 100% Dimethyl Formamide) in 10 ml of Tris buffer was added (100 µl per well) and incubated for 15 minutes at 37°C. The reaction was terminated by rinsing the plates with distilled water and the plates were then dried in hot air.

The antigen specificity of the ELISPOT assays was tested by inhibition experiments with excess quantities of free antigen in the culture medium during antibody secretion. PRP and TT antigens were added at 10 μg per well to the incubating cells as competitive inhibitors to inhibit the spot formation.

The plates were read under low magnification using a dissecting microscope to detect black spots representing the presence of antibody secreting cells. A homogeneously stained black single spot with a diffuse periphery was considered to be created by a specific ASC. Two individuals read each plate blind to the experimental conditions, and the mean numbers of black spots were calculated for the triplicate wells. Optimum numbers of specific ASC spots were observed at 1×10^4 spleen cells per well and 1×10^5 blood leucocytes per well. The mean PRP and TT ASC counts for these wells were finally expressed as per 1×10^6 cells.

All experiments were not performed on all mice due to technical difficulties (Table 6.1). The experiments were performed in batches of 2 or 4 mice (equal numbers of immunised and unimmunised mice), except on one occasion when only 1 mouse was used (an unimmunised mouse could not be included).

Table 6.1

Mouse experiment layout

Immunised mice Unimmunised mice	Flow cytometry spleen cells	ELISPOT spleen cells	ELISPOT Blood cells
IM1 UM1	×	×	NI
IM2 UM2	×	×[3]	NI
IM3 UM3	×[1]	×	×
IM4 UM4	ND	×	×
IM5 UM5	×[2]	×	×
IM6 UM6	×	×	×
IM7	ND	×	ND

1: Performed only TT detection due to insufficient number of cells

2: Performed only PRP detection due to insufficient number of cells

3: Performed only TT detection due to spoilt PRP coated ELISPOT plate. Not included in the analysis.

NI: Not included (this component was done only after seeing positive ELISPOT results on spleen cells from IM1 and IM2 mice).

ND: Not done

6.3 Results

6.3.1 Flow cytometric analyses of spleen cells

Flow cytometric analysis was performed on spleen cells from five immunised and five unimmunised mice. Live small spleen cells were gated on forward and side scatter for two colour analysis (Fig 6.1A). PRP or TT binding B cells could not be detected definitively. Small and variable percentages of cells positive for both FITC and PE staining were detected in all samples from immunised and unimmunised mice. There

was no marked difference of these respective streptavidin-PE staining cell percentages or their FACS patterns between immunised and unimmunused mice or between control samples (stained with CD45R/B220-FITC and streptavidin-PE) and test samples. Furthermore, addition of excess amounts of unbiotinylated antigen to compete with the biotinylated antigen did not result in any significant or consistent inhibition throughout the study. The mean percentages of B cells staining with streptavidin-PE for each set of samples of immunised and unimmunised mice are given in Table 6.2 A-B. The MFI of B cells staining with streptavidin-PE of immunised and unimmunised mice also did not demonstrate any marked difference. Flow cytometric dot plots of an immunised and unimmunised mouse are shown as a representation of the results of the study (Figs. 6.2, 6.3 and 6.4).

Large live spleen cells were also gated and analysed as above since B cell blasts would be expected to be in this region (Fig 6.1 B). PRP or TT binding cells were not definitively detected. The percentages of B cells stained with streptavidin-PE were not significantly above the background staining.

Approximately 50% of small spleen cells were stained with CD45R/B220-FITC (Fig 6.5A). The large cells were mainly CD45R/B220 positive (85%) and stained more brightly than small cells (MFI 24.8 vs 13.7) (Fig 6.5 B).

6.3.2 Anti-PRP and anti-TT antibody secreting cells in spleens

ELISPOT assays were performed as described above on spleen cells from all immunised mice and unimmunised mice. Anti-PRP and anti-TT ASC were detected. In immunised mice, the mean number of PRP specific ASC in the spleen cell preparations was 672 per 10^6 cells. The TT specific mean ASC number was nearly

twice that ($1245/10^6$ spleen cells) and was statistically greater (Fig. 6.6 A). PRP and TT spot formations were inhibited by approximately 80% and 90% respectively when cells were incubated with free antigens in the inhibition experiments (Fig. 6.7).

The mean frequencies of anti-PRP and anti-TT ASC in the spleen cell preparations from unimmunised mice were between 180 and 147 per 10^6 cells respectively and the two were not statistically different (Fig. 6.6 B). In the inhibition experiments of the unimmunised mice, there was no difference in the mean number of anti-PRP ASC (data not shown). However, the mean number of anti-TT ASC was reduced by nearly 50%, but the reduction was not statistically significant.

The diameter of the ASC spots in the ELISPOT assays varied, possibly depending on the amount of antibody secreted by individual cells. TT spots were much bigger and darker than PRP spots. The spots produced by spleen cells from unimmunised mice were smaller in size and lightly stained than those of immunised mice. In inhibition experiments of the immunised mice, the characteristics of the spots were similar to the spots of unimmunised mice. There was no change in the appearance of the spots in the inhibition experiments of unimmunised mice.

6.3.3 Anti-PRP and anti-TT antibodies secreting cells in blood

ELISPOT assays were also performed on blood leucocytes from four immunised and four unimmunised mice. Anti-PRP ASC in significant numbers were not detected in blood from immunised mice (Fig 6.8 A). However, anti-TT ASC were present in immunised blood (mean $14/10^6$ leucocytes). Anti-PRP and anti-TT ASC were not detected in blood from unimmunised mice (Fig 6.8 B). Inhibition assays were not performed on blood leucocytes.

Table 6.2 A

Mean percentage of B cells staining with streptavidin-PE in the immunofluoresence assays of spleen cells from immunised mice

Sample				Staining			
Test sample		*Inhibition sample*		Test sample		*Inhibition sample*	
PRP-Bi (10 μg/ml) CD45R/B220-FITC SAPE		*PRP-Bi (10 μg/ml) PRP (100 μg/ml)* CD45R/B220-FITC *SAPE*		TT-Bi (10 μg/ml) CD45R/B220-FITC SAPE		*TT-Bi (10 μg/ml) TT (100 μg/ml)* CD45R/B220-FITC *SAPE*	
B cell %	(SEM)	*B cell %*	(SEM)	B cell %	(SEM)	*B cell %*	(SEM)
0.19	(0.05)	*0.18*	*(0.04)*	0.14	(0.04)	*0.12*	*(0.03)*

In the control spleen cells samples stained with CD45R/B220-FITC and streptavidin-PE, the mean percentage of B cells staining with streptavidin-PE was 0.17 (0.05).

Table 6.2 B

Mean percentage of B cells staining with streptavidin-PE in the immunofluoresence assays of spleen cells from unimmunised mice

Sample				Staining			
Test sample		*Inhibition sample*		Test sample		*Inhibition sample*	
PRP-Bi (10 μg/ml) CD45R/B220-FITC SAPE		*PRP-Bi (10 μg/ml) PRP (100 μg/ml)* CD45R/B220-FITC *SAPE*		TT-Bi (10 μg/ml) CD45R/B220-FITC SAPE		*TT-Bi (10 μg/ml) TT (100 μg/ml)* CD45R/B220-FITC *SAPE*	
B cell %	(SEM)	*B cell %*	(SEM)	B cell %	(SEM)	*B cell %*	(SEM)
0.18	(0.06)	*0.14*	*(0.04)*	0.16	(0.05)	*0.17*	*(0.05)*

In the control spleen cells samples stained with CD45R/B220-FITC and streptavidin-PE, the mean percentage of B cells staining with streptavidin-PE was 0.20 (0.07).

B cell %: Mean percentage of B cells stained with streptavidin-PE (SAPE).

Standard error of the mean is given within parenthesis.

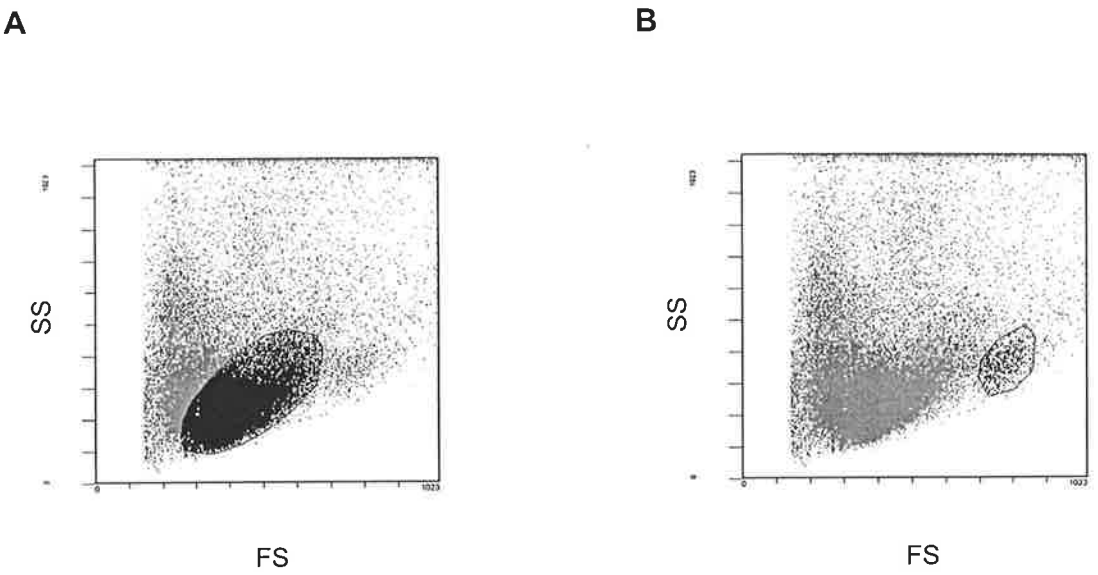


Fig.6.1 Flow cytometric analysis of murine spleen cells after immunofluorescence staining. Live cells gated according to light scatter properties (forward scatter vs side scatter). **A.** Gate for small lymhphocytes. **B.** Gate to select for large cells including lymphoblasts.

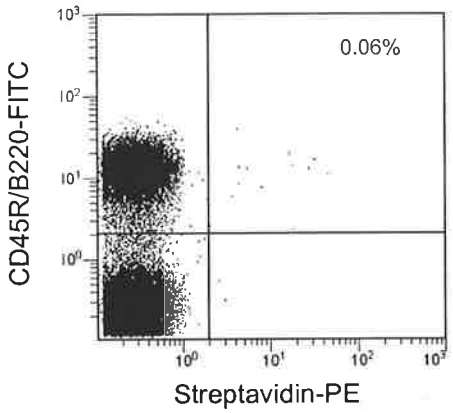


Fig. 6.2 Flow cytometric analysis of spleen cells from a mouse 5 days after immunisation with two doses of PRP-T. Cells stained with CD45R/B220-FITC and streptavidin-PE as a control to determine background staining. The percentage of B cells stained with streptavidin-PE is shown in the upper right quadrant. Similar patterns of background staining were seen in spleen cells from all mice.

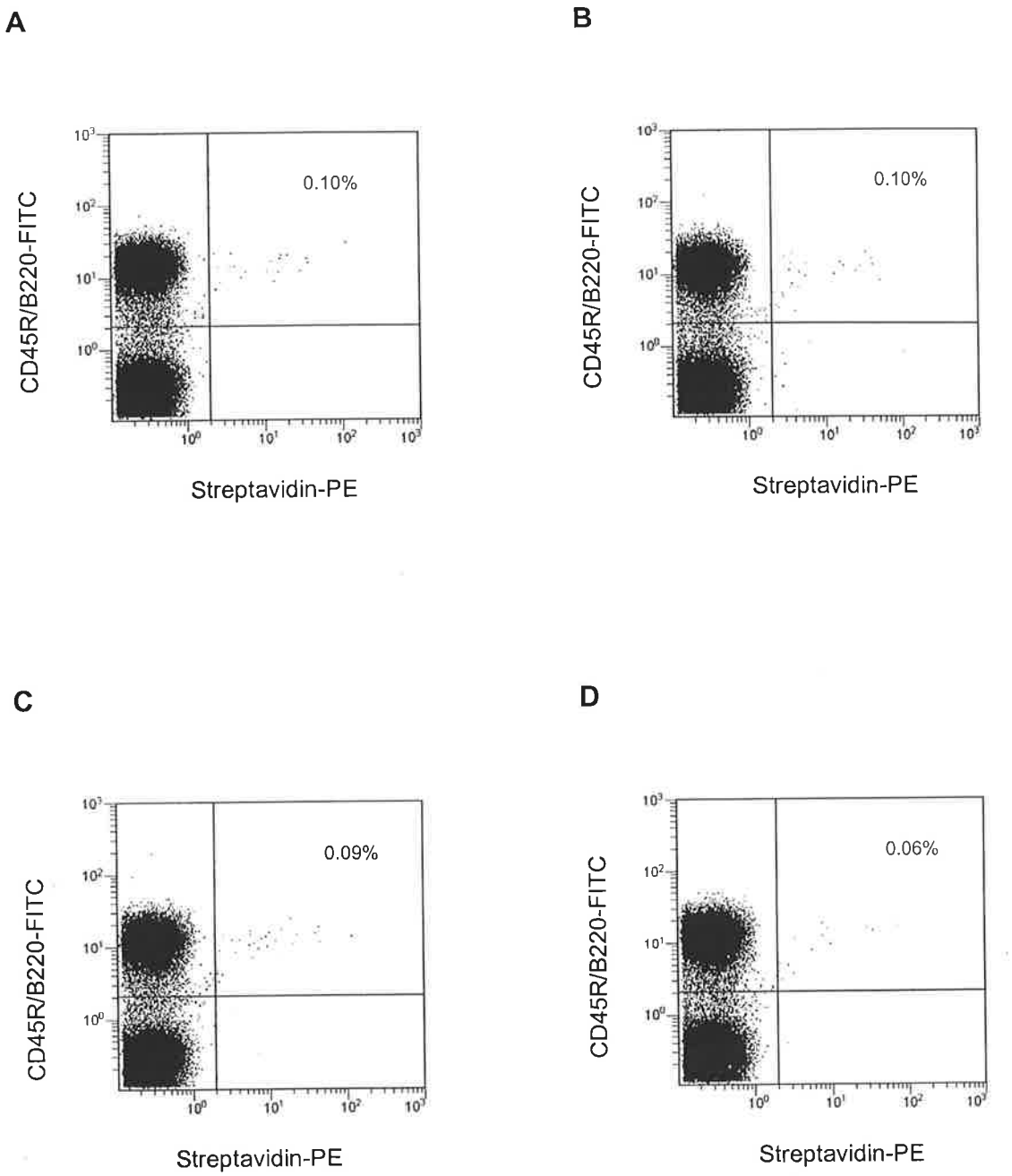


Fig. 6.3 Flow cytometric analysis of spleen cells from the mouse in Fig. 6.2. Cells were stained as follows and gated as in Fig.6.1 A: **A.** CD45R/B220-FITC, PRP-biotin and streptavidin-PE. **B.** CD45R/B220-FITC, PRP-biotin, unbiotinylated PRP and streptavidin-PE in the inhibition sample. **C.** CD45R/B220-FITC, TT-biotin and streptavidin-PE. **D.** CD45R/B220-FITC, unbiotinylated TT, TT-biotin and streptavidin-PE in the inhibition sample. The percentages of B cells staining with streptavidin-PE are shown in the upper right quadrants. CD45R/B220 positive B cell populations in **A, B, C** and **D** were 51.34%, 51.84%, 53.12% and 51.66% of the gated spleen cells respectively.

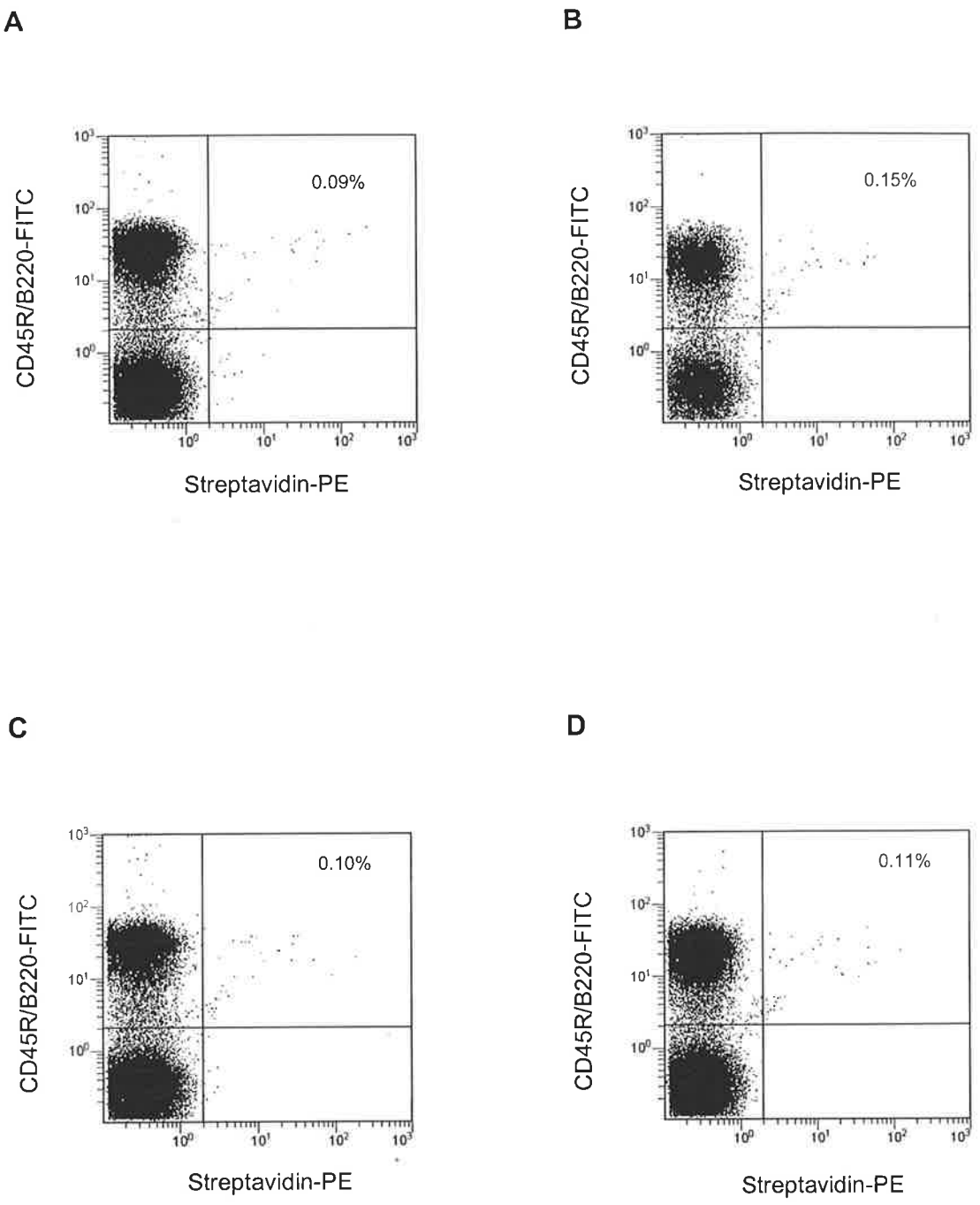


Fig. 6.4 Flow cytometric analysis of spleen cells from an unimmunised mouse. Cells were stained as follows and gated as in Fig.6.1 A: **A.** CD45R/B220-FITC, PRP-biotin and streptavidin-PE. **B.** CD45R/B220-FITC, PRP-biotin, unbiotinylated PRP and streptavidin-PE in the inhibition sample. **C.** CD45R/B220-FITC, TT-biotin and streptavidin-PE. **D.** CD45R/B220-FITC, TT-biotin, unbiotinylated TT, streptavidin-PE in the inhibition sample. The percentages of B cells staining with streptavidin-PE are shown in the upper right quadrant. CD45R/B220 positive B cell populations in **A, B, C** and **D** were 49.90%, 43.75%, 44.81% and 43.43% of the gated spleen cells respectively.

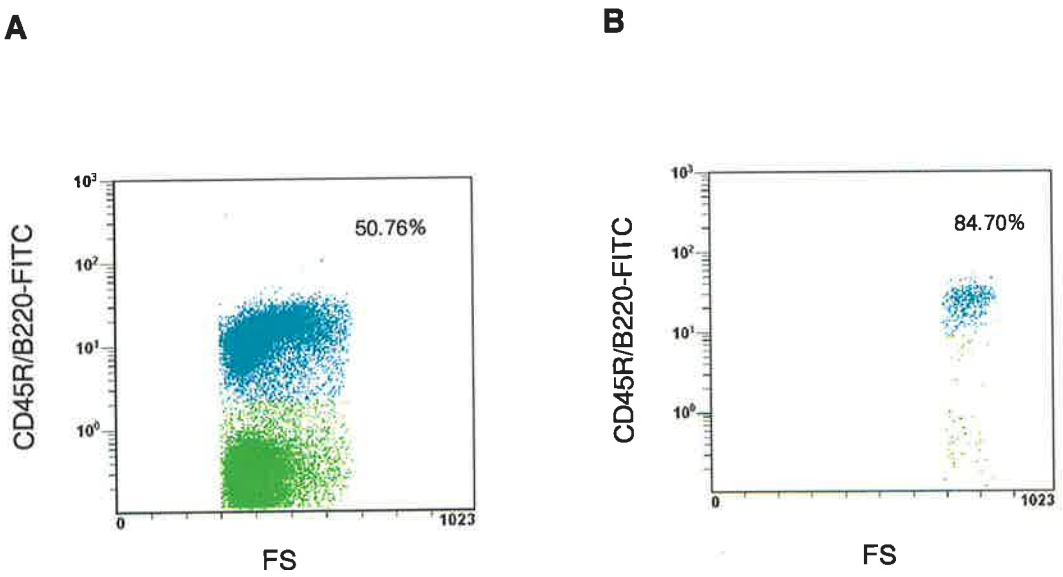
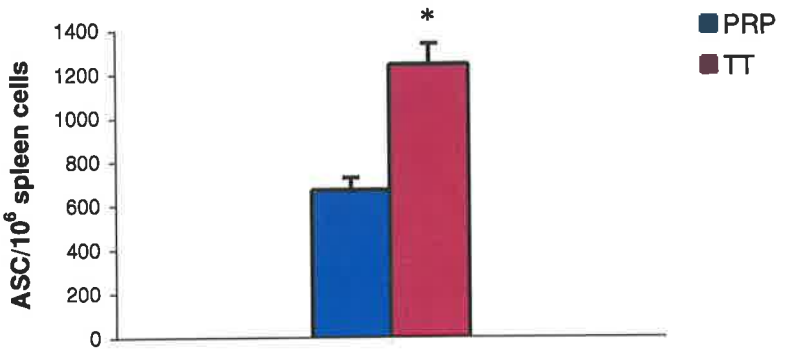


Fig.6.5 Flow cytometric analysis of stained murine spleen cells CD45R/B220 vs forward scatter. **A.** Analysis of live small cells as gated in Fig. 6.1A. **B.** Analysis of live large cells as gated in Fig. 6.1B. The percentages of CD45R/B220 positive B cells in the gated populations are shown in the dot plots.

A



B

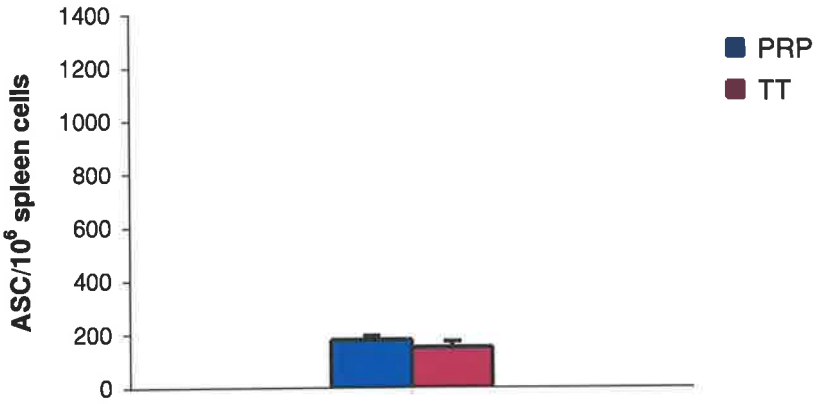
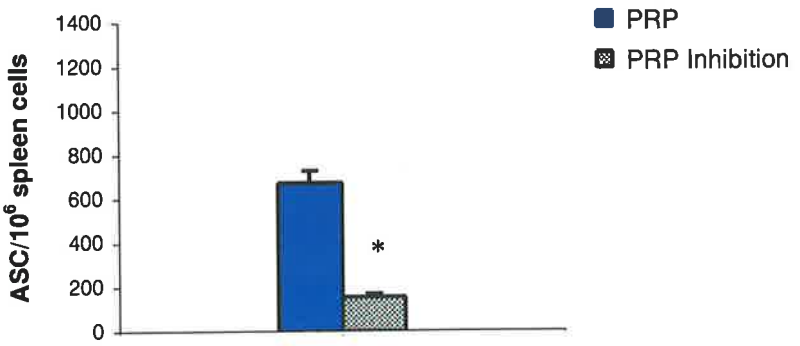


Fig. 6.6 Mean numbers of anti-PRP and anti-TT ASC detected by ELISPOT assays in spleen cell preparations from; **A.** mice 5 days after immunisation with two doses of PRP-T (n = 6). **B.** unimmunised mice as a negative control (n = 5).

Error bars denominate SEM. *The difference between the mean number of anti-PRP and anti-TT ASC was statistically significant ($P < 0.05$).

A



B

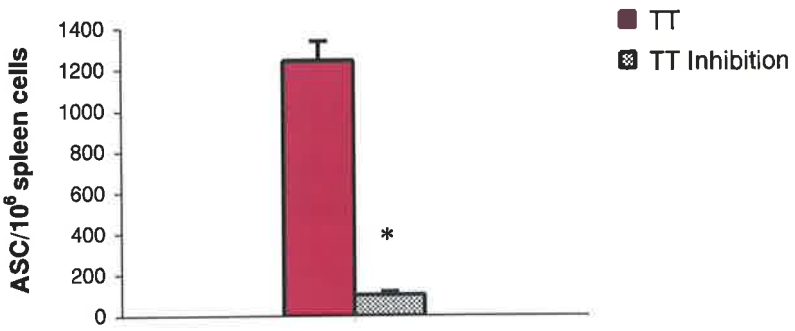
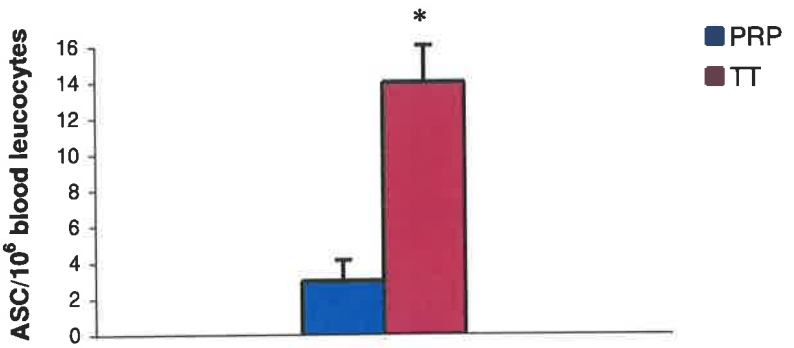


Fig. 6.7 Mean numbers of ASC detected by ELISPOT assays in spleen cells from mice 5 days after immunisation with two doses of PRP-T. Antigen specificity was tested by inhibition experiments with excess quantities of free antigens in the culture medium during cell incubation. **A.** Mean number of anti-PRP ASC with and without inhibition. **B.** Mean number of anti-TT ASC with and without inhibition.

n = 5. Error bars denominate SEM. *The difference between the mean numbers of ASC with and without inhibition was statistically significant ($P < 0.05$).

A



B

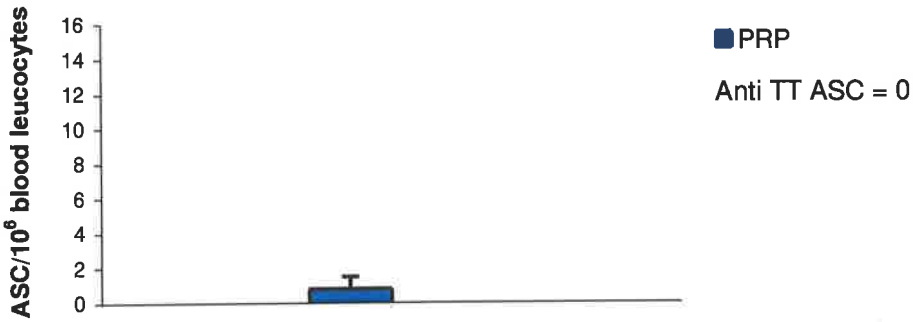


Fig. 6.8 Mean numbers of anti-PRP and anti-TT ASC detected by ELISPOT assays in blood cell preparations from; **A.** mice 5 days after immunisation with two doses of PRP-T (n = 4). **B.** unimmunised mice as a negative control (n = 4).

Error bars denominate SEM. *The difference between the mean number of anti-PRP and anti-TT ASC was statistically significant ($P < 0.05$).

6.4 Discussion

As discussed in Chapter 1, PRP-T conjugate vaccine is known to induce a strong immune response. In this study mice were immunised with PRP-T in order to detect PRP specific cells. Further, by selecting PRP-T as the vaccine it was possible to compare the presence of PRP specific cells with that of TT specific cells.

Based on the results of previous reports that have assessed antibody responses to TI and TD antigens, a day 5 post immunisation time point was selected for the detection of antigen specific B cells and ASC in this study. Immunohistochemical studies have shown that the presence of ASC in spleens from mice primed with the TI-2 antigens NP-Ficoll (Delemarre *et al.*, 1990; van den Eertwegh *et al.*, 1991; Garcia de Vinuesa *et al.*, 1999) and pneumococcal polysaccharide (Soesatyo *et al.*, 1993) reached a maximum around day 5 post immunisation. Furthermore, after mice were immunised with the TI-2 antigens dextran (Kolb *et al.*, 1993), polyvinyl pyrrolidinone (Whitmore *et al.*, 1996) or with TD antigen TNP-*Lactobacillus acidophilus* (Buiting *et al.*, 1996), the numbers of ASC in the splenic tissues were at peak levels at day 5 post immunisation when detected by ELISPOT assays. In another study, spleen cells from human adults immunised with PRP-D vaccine showed optimum antibody production *in vitro* when immunization occurred 5-7 days prior to splenectomy (Ambrosino *et al.*, 1990).

In this present study antigen specific B cells could not be isolated definitively by flow cytometry from spleen cells. As the controls have indicated, the staining of CD45R/B220 positive cells with streptavidin-PE could be due mainly to non specific binding of streptavidin-PE directly to the cells. However, any non specific binding of biotinylated antigen to B cells and subsequent detection by streptavidin-PE cannot be

disregarded. The small lymphocytes consisted of equal proportions of CD45R/B220 positive and negative cells. Large lymphocytes were mainly CD45R/B220 positive and stained slightly brighter than small cells. These cells could predominantly be lymphoblasts in the spleen.

However, antigen specific ASC were detected by ELISPOT assays. All immunised mice generated readily detectable anti-PRP and anti-TT ASC in spleen cell preparations. Although the frequency of ASC spots observed in unimmunised spleens was quite low, it was not expected, as it is very unlikely that mice would have been exposed to PRP and TT antigens. Since the characteristics of these spots were similar to those in inhibition experiments and furthermore as they could not be significantly inhibited by free antigen the possibility of non specific spot formation should be considered. This suggestion is also supported by not being able to inhibit the spot formation completely in immunised mice by excess unbiotinylated antigen. However, higher percentages of inhibition may have been achieved if higher concentrations of excess antigen were used (Barington *et al.*, 1992).

The corresponding anti-PRP and anti-TT ASC numbers detected by ELISPOT assay of spleen cells used in flow cytometric analyses in Fig 6.3 were 767 and 1250 per 10^6 spleen cells respectively (approximately 0.075% and 0.125% respectively).

In contrast to the spleen cell preparations, anti-PRP and anti-TT ASC were not detectable in peripheral blood from immunised mice. This could be due to lack of ASC in the circulation at this given time point of the immune response to PRP-T vaccine.

The small volume of peripheral blood available for collection from mice (less than 1 ml) did not provide adequate cell numbers to perform flow cytometric analyses to detect PRP and TT antigen specific B cells as well as performing ELISPOT assays.

In the ELISPOT assays the number of spots formed did not correlate with the cell numbers incubated in the wells. This could have resulted from cell death during incubation due to competition for nutrients in the culture medium in the wells containing high cell numbers.

According to the literature there seem to be no previous studies that have isolated PRP specific B cells or anti-PRP ASC from immunised mice. However, some studies have reported the detection of TT specific ASC in splenic tissues from immunised mice by ELISPOT assays (Pihlgren *et al.*, 2001; von Hunolstein *et al.*, 2001; Schallert *et al.*, 2002; Pihlgren *et al.*, 2003a).

6.4.1 Previous studies on anti-TT ASC in murine spleens

Approximately 250 anti-TT IgG ASC per 10^6 splenocytes were detected by ELISPOT assay from mice 3 weeks after being immunised with a single dose of intradermal PRP-T injection (von Hunolstein *et al.*, 2001). Anti-PRP ASC were not measured.

In another study, anti-TT specific IgG ASC enumerated by ELISPOT were very few in numbers up to day 7, increased to significant higher numbers (mean of $75/10^6$ cells) on day 10 and then declined to low levels by day 20 in the splenic tissues of mice immunised with an intraperitoneal injection of TT (Pihlgren *et al.*, 2001). Following a booster immunsation after 3 weeks, a transient strong antibody response was induced generating approximately 4000 IgG ASC per 10^6 spleen cells by day 5 post

secondary immunisation and then the ASC decreased to low numbers and disappeared by day 20. Anti-TT ASC spots were not seen in the assays of unimmunised mice. Recent murine studies have also recorded similar patterns in detecting TT specific IgG ASC (Schallert *et al.*, 2002; Pihlgren *et al.*, 2003a).

A recent experiment reported by Pihlgren *et al* recorded higher numbers of anti-TT specific IgG ASC by ELISPOT assays in spleen cell preparations from mice immunised with an intraperitoneal injection of TT and CpG oligonucleotides (CpG-ODN) compared to the numbers from mice immunised with TT and control-ODN as normal controls (Pihlgren *et al.*, 2003b). In contrast to the results of previous studies (Pihlgren *et al.*, 2001; Pihlgren *et al.*, 2003a) anti-TT IgG numbers in both sets of mice peaked on day 27 post immunisation (means of 75 and 45 per 10^6 spleen cells respectively). The ASC numbers were reduced by day 42 but persisted around 25-30/10^6 cells for up to 3 months, much higher than in control mice. This enhancement of the ASC response in the spleen was associated with a four fold increase of anti-TT ASC numbers in the bone marrow at 3 months post immunisation. The authors have discussed the adjuvant effect of CpG-ODN in the immune response to TD antigens.

6.4.2 Previous studies on anti-PRP and anti-TT ASC in blood from humans

No studies were found to have measured anti-PRP or anti-TT ASC in blood from immunised mice. However, there are many studies enumerating anti-PRP and anti-TT cells in peripheral blood from humans. The results of these studies have been highly variable.

Munoz and Insel enumerated ASC in peripheral blood of adults immunised with pure PRP and TT vaccines (Munoz and Insel, 1987). On day 8 post immunisation, the

mean number of anti-TT ASC detected by ELISPOT was approximately four fold higher than the mean of anti-PRP ACS (164 vs 45 per 10^6 PBMC). This demonstrated the stronger antigenicity of TD antigen TT over pure TI antigen PRP, as expected.

A study conducted by Barington *et al* in 1990 detected PRP antibody secreting cells by haemolytic plaque forming assays in peripheral blood from adults immunised with one dose of PRP-D vaccine subcutaneously (Barington *et al.*, 1990). Anti-PRP ASC were detected from day 5 to day 14 post immunisation with a narrow peak around day 8 where antibody secreting cells ranged from about 200 to 11000/10^6 PBMC (median approximately 1500). According to the graph in the paper, anti-PRP ASC numbers on day 5 and day 14 were very small and were close to the baseline values. The specificity of the assay was confirmed by inhibition experiments. Antibody secreting cells to diphtheria toxoid (DT) were not measured.

In contrast to the studies of Barington *et al* in 1990, Tarkowski and his colleagues detected only 900/10^6 MNC (mean) in peripheral blood secreting anti-PRP antibodies by ELISPOT assays from adults imuunised with PRP-D vaccine 1 week after immunisation (Tarkowski *et al.*, 1990). PBMC were pre incubated with the protein synthesis inhibitor cycloheximide to determine that spot formation was due to active secretion of de novo synthesized antibodies. This resulted in a reduction of more than 90% in the number of spots. The specificity of the assay was demonstrated by inhibition of spot formation with excess free antigen. The frequency of anti-DT ASC was not enumerated.

A study conducted by Barington *et al* in 1991 on several adults immunised with subcutaneous single doses of PRP-D vaccine also observed low numbers of anti-PRP

ASC (range 153 to 1179; median 525/10^6 PBMC) in peripheral blood between 6 to 9 days (peak, day 8) after immunisation (Barington *et al.*, 1991). The median frequency of anti-DT cells was 40/10^6 PBMC. The anti-DT antibodies acquired during childhood immunisation correlated positively with the antibody titers and the numbers of PRP and DT ASC in the circulation after immunisation. On the other hand, the antibody levels and the ASC numbers to both PRP and DT in peripheral blood did not correlate with the pre immunisation titres of anti-PRP. After a booster immunisation with the conjugate vaccine administered at 4 weeks, the numbers of PRP ASC detected in peripheral blood were about 10 fold less (median 52/10^6 MNC) but peaked earlier (day 7) than after the first immunisation. The numbers of DT ASC were nearly three fold lower (median 16/10^6 MNC). Pre incubation of MNC with cycloheximide suggested that the spot formation in the ELISPOT assays was not due to the release of passively absorbed antibodies.

Equal numbers of anti-TT ASC (175-220/10^6 PBMC) were detected in peripheral blood from adults 7 days after immunisation with subcutaneous single dose of either TT or PRP-T (Barington *et al.*, 1992). This finding suggests that the immune response to TT is unchanged by conjugating to PRP antigen. The frequencies of anti-PRP ASC ranged between 290-380 per 10^6 PBMC in peripheral blood. The antigen specificity of the ELISPOT assays was confirmed by the demonstration of dose dependent inhibition of spot formation by free antigen.

Hougs *et al* detected anti-PRP ASC ranging from 2000 to 16300/10^6 mononuclear cells (mean, 8170) in peripheral blood from four adults immunised with PRP-T vaccine on day 9 post immunisation. (Hougs *et al.*, 1993). Anti-TT ASC numbers were also measured. When blood from one of the immunised adults was tested on

day 7, the numbers of anti-TT and anti-PRP ASC were almost equal (approximately $8000/10^6$ PBMC) but on day 9, the anti-TT secreting cells were about 30 fold less than those of anti-PRP ASC. PRP and TT spot formation prior to immunisation was almost zero.

Another group of investigators have reported that the number of anti-PRP and anti-TT cells detected by ELISPOT assay were extremely low in peripheral blood from adults (mean values approximately $5/10^6$ MNC) three to four weeks after a single dose of PRP-T by intramuscular injection (Breukels *et al.*, 1999). The specificity of the spot formation was tested by addition of excess free antigen.

A recent study has shown that in adults immunised with a subcutaneous single dose of PRP-CRM_{197}, the numbers of anti-PRP ASC in the peripheral circulation peaked around 1 week (80 per 10^6 MNC) and then gradually declined to about $35/10^6$ MNC by 6 weeks (Kamboj *et al.*, 2001). The frequency of PRP spots detected by ELISPOT in pre immunised blood was less than $20/10^6$ MNC.

In the two studies of Barington *et al* and Hougs *et al* the PRP and TT antibody secreting cell numbers among subjects was highly variable (Barington *et al.*, 1990; Hougs *et al.*, 1993). Furthermore, the numbers reached surprisingly large values in some individuals, especially after a single immunisation. However, any past history of any invasive Hib diseases or any previous immunisations of donors was not mentioned. It is also possible that in peripheral blood non B cells bound to anti-PRP or anti-TT antibodies through Fc receptors could have been mistaken for antigen specific B cells as described in Chapter 3 and by Bell and Grey (Bell and Gray, 2003), and may have produced spots in the ELISPOT assays by eluting the absorbed antibodies, but probably with no diffuse margin and smaller than the spots formed by

true antibody secreting cells. Since the appearance of the counted spots has not been stated, it is possible that the spot formed by the absorbed antibodies were also included in the results.

Subsequent studies conducted by Barington *et al* have demonstrated that when healthy adults were immunised with two doses of PRP conjugate vaccines subcutaneously 4 weeks a part, large numbers of anti-PRP ASC were present in peripheral blood 1 week after the first immunization, whereas the numbers observed after the booster immunisation were very small (median 15-35/10^6 MNC) (Barington *et al.*, 1993; Barington *et al.*, 1994). However, the specificity of the spot formation in the ELISPOT assays was not determined. Furthermore, a similar pattern was seen for the numbers of carrier protein specific ACS in the circulation when adults were pre immunised with a carrier protein and 4 weeks later immunised with PRP conjugated to the same carrier protein (Barington *et al.*, 1993). This has been attributed to the high levels of carrier protein antibodies in the circulation, following recent pre immunisation. Although the values were not stated, according to the graphs in the publication after the second immunisation the numbers of carrier protein specific ASC were marginally greater than the numbers of anti-PRP ASC in the two groups. This finding is similar to the results described in this Chapter where anti-TT ASC numbers were significantly greater than anti-PRP ASC numbers in peripheral blood from mice immunised with two doses of PRP-T conjugate vaccine.

In most of the previous human studies, spot formation was not measured prior to immunisation or from non recently immunised subjects as controls (Munoz and Insel, 1987; Barington *et al.*, 1990; Tarkowski *et al.*, 1990; Barington *et al.*, 1991; Barington *et al.*, 1992; Barington *et al.*, 1993; Barington *et al.*, 1994). Furthermore,

specificity of spot formation in the ELISPOT assays was not tested in the previous murine studies, and in some of the previous human studies (Munoz and Insel, 1987; Barington *et al.*, 1991; Barington *et al.*, 1993; Hougs *et al.*, 1993; Barington *et al.*, 1994).

The inability to detect anti-PRP and TT ASC definitively from mice 5 days after immunisation with a single dose of PRP-T, but their presence in high numbers on day 5 after two doses of immunisation was in agreement with some previous murine studies (Pihlgren *et al.*, 2001; Schallert *et al.*, 2002; Pihlgren *et al.*, 2003a). It is possible that a measurable antibody response was not generated by day 5 post primary immunisation.

PRP or TT specific B cells were not identified by flow cytometric analysis following primary and secondary immunisation. This could be due to the selection of an inappropriate time point. A longitudinal study may have provided useful results (Youngman *et al.*, 2002). It is also important to consider that the two assay systems, flow cytometry and ELISPOT may detect two distinct cell populations and it may not be correct to compare the outcomes of these two techniques. Antigen binding cells identified by flow cytometry are mainly memory B cells and germinal centre cells (McHeyzer-Williams *et al.*, 2000; Youngman *et al.*, 2002). However, some antibody secreting cell precursors expressing surface antigen receptor immunoglobulins could also be detected (Smith *et al.*, 1996; McHeyzer-Williams *et al.*, 2000). In contrast, ASC detected in the ELISPOT assays are plasma cell precursors or mature plasma cells (Smith *et al.*, 1996; McHeyzer-Williams *et al.*, 2000).

Compared with the results of the previous studies, PRP and TT antibody secreting cell numbers in peripheral blood in this present study were very low. It is possible that

significant numbers of PRP and TT antibody secreting cells were not detected in peripheral blood because the time point selected for enumerating ASC was too early. Another possible explanation is that a suppressive effect by the booster immunisation as described Barington *et al* (Barington *et al.*, 1993; Barington *et al.*, 1994) may have decreased the number of ASC in peripheral blood of mice in this study. The human adults who participated in the previous studies may have been immunised with the carrier proteins several times during routine childhood immunisation and had carrier specific T helper cells that may have resulted in a greater immune response when vaccinated with PRP conjugated vaccines. Furthermore, human participants in previous studies may have also been naturally primed by exposure to Hib organisms prior to participation in the experiments. However, a direct comparison cannot be performed due to the difference of species involved in this present study and the previous studies.

ASC and antigen specific B cells are generated in secondary lymphoid organs mainly in the spleen in response to antigen exposure. ASC migrate to lymphoid organs elsewhere including the bone marrow and in mucosa associated lymphoid tissue, while some remain *in situ* (Takahashi *et al.*, 1998; Medina *et al.*, 2002; Ellyard *et al.*, 2003). The memory B cells localise to antigen draining sites in the secondary lymphoid tissues or join the recirculating pool of lymphocytes (Liu *et al.*, 1988). A subpopulation of memory B cells in the circulation is believed to be homing to the bone marrow (Paramithiotis and Cooper, 1997). Thus, the presence of ASC or antigen specific B cells in peripheral blood is transient and may not be detected after a particular time point. Furthermore, the numbers of ASC or antigen specific B cells in the peripheral circulation blood also may not reflect the magnitude of the antibody

response to the antigen. Therefore, studying lymphoid tissues may provide better information about immune responses to antigens.

However, in this present study only spleen tissues were studied in immunised mice. If bone marrow and lymph nodes were analysed more information could have been obtained. It would have been interesting to see the immune response in the cervical lymph nodes as the injections were given subcutaneously at the nape of neck.

For the first time, the presence of anti-PRP ASC in the spleen was studied following immunisation with a PRP conjugate vaccine and compared with that of TT protein antigen. From this study it is concluded that the frequencies of anti-PRP and anti-TT ASC in murine spleen cells are very much higher on day 5 post secondary immunisation than post primary immunisation. Furthermore, the numbers of anti-TT ASC in the splenic tissues were almost double the amounts of anti-PRP ASC. At this given time point the ASC in the blood was extremely low, yet the numbers of anti-TT ASC were significantly higher than those of anti-PRP ASC.

In Chapter 3, PRP antigen specific B cells could not be detected definitively above background. This led to investigation as to whether these cells are confined to lymphoid tissue, using a mouse model in this Chapter. However, PRP specific B cells were again not definitively detected by flow cytometry in splenic tissue from immunised mice.

The ability to detect antigen specific ASC in spleen cell preparations from mice led to the attempted isolation of ASC in lymphoid tissues in humans. Chapter 7 describes the isolation of anti-PRP and anti-TT ASC from tonsil cells from young children.

CHAPTER 7

ISOLATION OF ANTI-PRP AND ANTI-TT ANTIBODY SECRETING CELLS FROM TONSIL TISSUES IN YOUNG CHILDREN

7.1 Introduction

Palatine tonsils located at the entrance of the oropharynx are organised secondary lymphoid organs that contain all cell types and structures necessary to mount humoral and cell mediated immune responses (Bernstein, 1992; Koch and Brodsky, 1993). B cell subsets in the tonsillar tissues generate antibody responses to TI and TD antigens (Dono *et al.*, 1996; Dono *et al.*, 2001). Palatine tonsils are used extensively to study the immune mechanisms in secondary lymphoid organs in humans due to easy accessibility by routine therapeutic surgical intervention.

Since the murine studies described in Chapter 6 showed that PRP ASC could be detected in secondary lymphoid tissues (spleen) but not in blood, further studies were undertaken using cells isolated from tonsillar tissues from children.

ELISPOT assays were used to determine if anti-PRP ASC could be detected in tonsil tissues from children. The detection of anti-PRP ASC was compared with detection of anti-TT ASC. In Australia, children born before 1st May 2000 were immunised against Hib and tetanus at identical time points during the first 4 years of life (see immunisation schedule in Chapter 1).

7.2 Methods

7.2.1 Tonsil collection

Palatine tonsils were collected from three healthy children under 4 years of age who were undergoing tonsillectomy as treatment for recurrent tonsillitis (more than 5 times per year) associated with tonsillar hypertrophy (Table 7.1). At the time of surgery the children were not suffering from acute tonsillitis. All three children had been immunised with Hib conjugate vaccine and TT vaccine at 2, 4, 6, and 18 months of age according to the Australian childhood immunisation schedule. They had no history of invasive Hib disease or any illness suggestive of immune deficiency and were not on any immune suppressive medications.

Table 7.1

Subject information

Age (months)	Sex	Vaccines	Duration since last Hib and TT immunisation (months)
35	Female	PRP-CRM, DTPa	17
41	Male	PRP-CRM, DTPa	23
46	Male	PRP-CRM, DTPa	28

Mean age: 41 months, Mean duration since last immunisation: 23 months

PRP-CRM (HibTITER®), DTPa (Infarix®)

7.2.2 Isolation of MNC from tonsils

Tonsils were collected fresh at the time of surgery and were transported to the laboratory in sterile PBS. The tonsils were placed inside a laminar flow cabinet and were washed extensively in PBS to remove blood and other debris. Fatty and connective tissues around the tonsils were excised. The tonsils were placed on petri dishes containing sterile PBS and were cut into small pieces and minced finely using the plunger of 10 ml plastic syringes, allowing cells to drain into PBS. The suspensions were then filtered through sterile Falcon® 70 μm cell strainers (Becton Dickinson Labware, New Jersey, USA). The resulting single cell suspensions were washed three times with sterile PBS by centrifuging at 1000g for 10 minutes. The MNC were separated from the washed tonsillar cell suspensions by gradient centrifugation on Lymphoprep™ as described in Chapter 2. Viability counts were performed using the trypan blue exclusion method and the MNC counts were adjusted to 1×10^6 viable cells per 1 ml.

7.2.3 ELISPOT assays

PRP and TT ELISPOT assays were performed on tonsillar MNC to detect and enumerate anti-PRP and anti-TT ASC. The assays were performed as described in Chapter 6. Serial dilutions of tonsillar cell suspensions at 25×10^3, 50×10^3, 100×10^3 and 250×10^3cells in 100 μl of RF10 culture medium containing affinity isolated sheep anti human immunoglobulin alkaline phosphatase conjugate (AMRAD Biotech, Victoria, Australia) 1/1000 were added to triplicate wells.

The antigen specificity of the ELISPOT assays was tested by inhibition experiments with excess quantities of free antigen as described in Chapter 6.

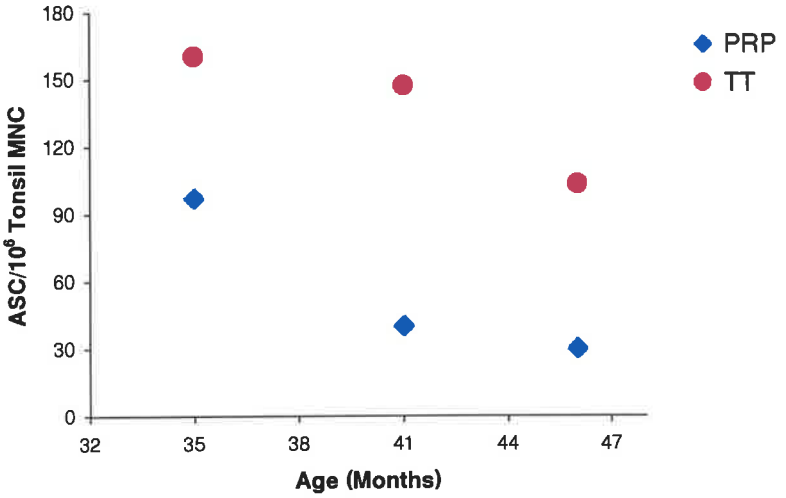
The plates were read under low magnification using a dissecting microscope. A homogeneously stained black single spot with a diffuse periphery was considered to be created by a specific ASC. Two independent observers read each plate blinded to the experimental conditions. The mean numbers of spots were calculated for the triplicate wells. The optimum number of specific ASC spots was observed at 50×10^3 MNC per well. The mean anti-PRP and anti-TT ASC counts for these wells were expressed as per 1×10^6 tonsil MNC.

7.3 Results

Tonsil cells from the youngest child (35 months) produced the highest number of ASC. The anti-PRP ASC count from this child was 97 per 10^6 MNC and anti-TT ASC count was 160 (Fig 7.1 A). Tonsil cells from the child aged 46 months produced the lowest number of ASC; the anti-PRP and anti-TT ASC counts were 30 and 103 per 10^6 MNC respectively. The anti-PRP ASC counts from this child (aged 46 months) was only slightly less than for the child aged 41 months. In contrast there was more gradual decrease with age in the anti-TT ASC counts. For all three individuals the anti-PRP ASC counts were much lower than for the anti-TT ASC. The mean anti-PRP ASC of all three children was 2.5 fold lower than the mean anti-TT ASC count (Fig 7.1 B).

The specificity of the ELISPOT assays was demonstrated by inhibition experiments. The numbers of anti-PRP and anti-TT ASC were inhibited by over 90% and over 95% respectively (Fig. 7.2).

A



B

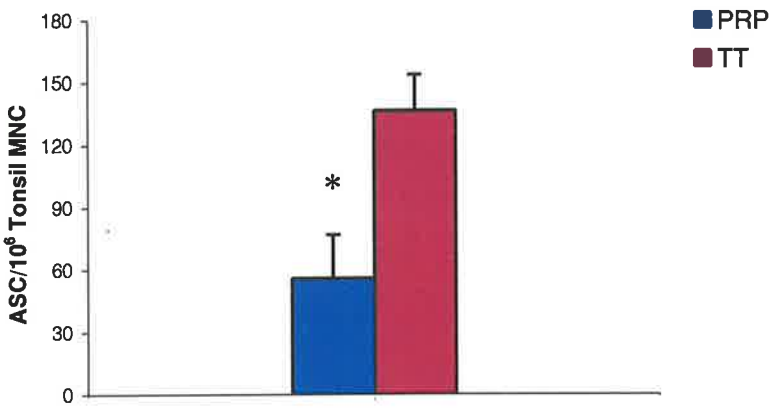


Fig. 7.1. Frequencies of anti-PRP and anti-TT ASC detected by ELISPOT assays in tonsils from three children immunised with 4 doses of Hib conjugate and TT vaccines according to the Australian childhood immunisation schedule. **A.** Anti-PRP and anti-TT secreting cells in the tonsil tissue of each individual child. **B.** The mean anti-PRP and anti-TT ASC in the tonsils of all three children. Error bars denominate SEM. *The difference between the mean number of anti-PRP and anti-TT ASC was statistically significant ($P < 0.05$).

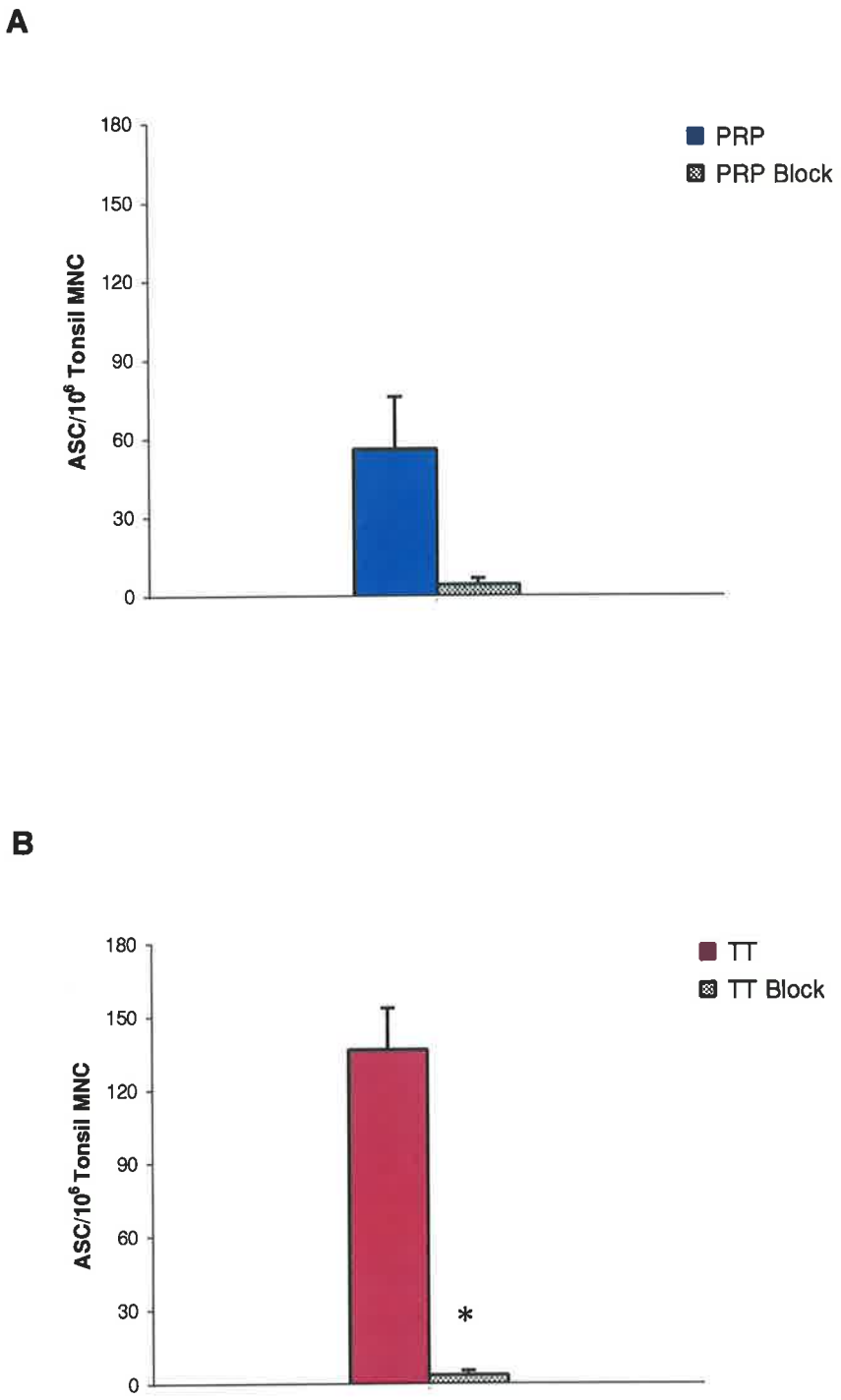


Fig. 7.2. Mean numbers of anti-PRP and anti-TT ASC detected by ELISPOT assays in the tonsils from three children in Fig. 7.1 with and without inhibition. **A.** Mean numbers of anti-PRP ASC. **B.** Anti-TT ASC. Error bars denominate SEM. *The difference between with and without inhibition was statistically significant in **B** (for TT) ($P < 0.05$). Although the inhibition in **A** (for PRP) was markedly less, the difference was not statistically significant ($P = 0.07$).

7.4 Discussion

Anti-PRP and anti-TT ASC were detected in palatine tonsil tissue from children who underwent routine tonsillectomy for recurrent tonsillitis associated with hypertrophy. The greater numbers of anti-PRP and anti-TT ASC were seen in the child who had the shortest PRP and TT post vaccination period. There was a decline in the number of PRP and TT spots with age and increasing time since last immunisation. However, the differences in ASC detected with age could be due to individual variation. Greater subject numbers need to be studied to determine more clearly if there is an inverse relationship between the time from the most recent immunisation / antigen exposure and the number of ASC detected in tonsil cells.

In this study the subjects were restricted to less than 4 years of age as children after 4 years receive TT booster vaccination without Hib vaccination as part of the childhood immunisation program. Since routine tonsillectomy is not usually performed during early childhood the accessibility of tonsils from children less than 4 years of age was limited.

The results of this study show that the numbers of anti-PRP ASC detectable in the tonsils were lower than the numbers of anti-TT ASC at least after approximately 3 years of age. However, the numbers of anti-PRP and anti-TT ASC in the tonsils immediately after immunisation cannot be inferred from these results.

As discussed in Chapter 6, the presence of ASC in the circulation after vaccination is of short duration. Therefore detection of anti-PRP and anti-TT ASC in peripheral blood was not attempted in the studies on these three children.

Although routine childhood Hib immunisation reduces the oropharyngeal Hib carrier state, it does not completely eliminate Hib colonization (Takala *et al.*, 1993; Barbour *et al.*, 1995). Furthermore, certain ethnic populations such as Alaskan natives and Australian Aborigines continue to have high carriage rates in spite of Hib immunisation with conjugate vaccine (Galil *et al.*, 1999; Guthridge *et al.*, 2000). It has been found that approximately 4% of tonsils removed for recurrent tonsillitis were infected with Hib bacteria in the USA (Brook and Shah, 2001). These findings show that tonsil tissues are locally exposed to Hib organisms in addition to systemic Hib immunisation.

Taking this fact into consideration, it is likely that the tonsils used in this present study may have been colonised or infected with Hib bacteria during some time of life or as part of the episodes of recurrent tonsillitis. Therefore, apart from routine immunisation with Hib vaccine it was possible that the immune cells of the tonsillar donors may have been boosted by oropharyngeal exposure to Hib.

Since diseased tonsils have been shown to have increased germinal centre reactions with greater numbers of antigen presenting B cells and T helper cells (Brodsky *et al.*, 1988b; Gorfien *et al.*, 1999) Hib infection of the tonsillar tissues could boost the already vaccine primed immune system to produce a marked local immune response. Furthermore, the persistence of Hib organisms at the specialised epithelial surface of the tonsillar crypts could lead to continuous stimulation of the tonsillar immune system with further B and T cell proliferation (Brodsky *et al.*, 1988a).

If such exposure were to occur, it might have been expected that a greater number of anti-PRP ASC should have been detected in the tonsils, compared to the number of anti-TT ASC in this present study.

No previous studies seem to have been performed to enumerate anti-PRP ASC in tonsillar tissues following immunisation. A study reported by Zanin *et al* in 1994 reported the detection of very small numbers of *Haemophilus influenzae* specific ASC ($10/10^6$ per MNC) in tonsillar tissues by ELISPOT assays from children aged between 3 and 8 years who required tonsillectomy for recurrent tonsillitis (Zanin *et al.*, 1994). However, neither the type specificity of the *H influenzae* species nor the characteristics of the antigen used for detection of ASC were reported. The Hib immunisation status of the study subjects also was not provided, but Hib immunisation with conjugate vaccines was not widely available at the time of the study to children in this age range. Therefore, a direct comparison can not be performed with the results of this present study in which the ASC were detected against the PRP antigen of Hib. Furthermore, the present study was performed on children less than 4 years of age who were immunised with Hib conjugate vaccines during routine childhood immunisation.

Quiding-Jarbrink *et al* studied the frequency of anti-TT ASC from tonsillar tissues from healthy adults 1 week after intra tonsillar (it) or subcutaneous (sc) immunisation with TT (Quiding-Jarbrink *et al.*, 1995). The number of anti-TT secreting cells detected following sc immunisation was negligible, while a small number of antigen specific ASC (mean: $16/10^6$ spleen cells) were detected following it immunisation.

It is not clear why more anti-TT ASC than anti-PRP ASC were seen in the tonsillar tissues in this present study. A few hypotheses could be postulated for this occurrence. As discussed in Chapter 6, studies have shown that ASC generated in secondary lymphoid tissues migrate to distant lymphoid organs such as the bone marrow and mucosa associated lymphoid tissue (Takahashi *et al.*, 1998; Medina *et al.*,

2002). It was recently found that some ASC produced in the spleen remain in situ without homing to distant sites (Ellyard *et al.*, 2003). In this study, the ASC detected more than 17 months after systemic immunisation, could be ASC which have remained in the tonsils without migrating to other sites, similar to the finding in the spleen by Ellyard *et al.* To detect more anti-TT ASC than anti-PRP ASC in tonsil tissues one or several of the following concepts could have happened. (1) The production of anti-TT ASC was greater than that of anti-PRP ASC. (2) The PRP plasma cells generated in the tonsils migrated to distant sites in larger numbers than the migration of TT plasma cells produced in the tonsils. (3) The remaining PRP plasma cells in the tonsils had a shorter life span compared to TT plasma cells.

However, the most likely explanation is that since tonsillar cells do not produce strong immune responses to systemic immunisations (Quiding-Jarbrink *et al.*, 1995; el-Madhun *et al.*, 1998; Brokstad *et al.*, 2002), anti-PRP and anti-TT ASC were generated elsewhere and migrated to mucosal lymphoid tissues including palatine tonsils following parenteral immunisation (Brokstad *et al.*, 1995; Kantele *et al.*, 1997) and remained for a longer duration. If this is so, in contrast to the above statement it is possible that anti-TT ASC migrated to tonsils in greater numbers than anti-PRP ASC. It can be also postulated that anti-TT ASC have a longer life span than anti-PRP ASC as mentioned above.

In Chapter 6, the frequency of anti-TT ASC was much greater than anti-PRP ASC in splenic tissues on day 5 post secondary immunisation. Although the numbers were less, the frequency of anti-TT ASC in peripheral blood was much greater than that of PRP cells. In the tonsil experiment (this Chapter), the numbers of anti-TT ASC were again larger than those of PRP cells even after 17 months post immunisation. Taken

together, it may be that the generation of anti-PRP ASC in the spleen is less compared to the generation of anti-TT ASC. Furthermore, it is likely that more TT cells migrate to distant sites through the circulation than PRP cells. This concept supports the above explanation for detecting greater numbers of anti-TT ASC in the tonsils. If the magnitude of ASC generation correlates with the production of antigen specific memory B cells, it is possible that the failure to detect PRP specific B cells in peripheral blood of humans in Chapter 3 was due to the presence of very low numbers of PRP specific B cells in the circulation and the difficulty of identifying them above background.

CHAPTER 8

GENERAL DISCUSSION

As discussed in Chapter 1, the antibody response to Hib PRP capsular polysaccharide in early childhood is restricted compared to antibody responses to protein antigens. As a result, infants and young children are more susceptible to invasive Hib disease. Development of PRP conjugate vaccines for use in early childhood has been a recent major advance. PRP conjugate vaccines are produced by coupling the PRP polysaccharide to a carrier protein to convert the restricted TI response to PRP to a more effective TD response. These conjugate vaccines provide excellent protection against invasive Hib disease during early childhood. However, it has been shown that the characteristics of the antibody responses to PRP conjugate vaccines are not identical to those for pure protein antigens.

The initial objectives of this project were to develop methods which would enable identification, enumeration and phenotypic characterisation of PRP capsular polysaccharide specific B cells during the development of the immune response to Hib conjugate vaccine in children following immunisation, and compare these responses with those to a pure protein antigen.

No previous studies have investigated these features in the development of antibody responses to PRP conjugated antigen in direct comparison with a protein antigen.

Tetanus toxoid was selected as the protein antigen as immunisation against TT is performed at similar time points to Hib immunisation during the first 4 years of life in children born before 1st May, 2000 in Australia.

To achieve the objectives of this project it was necessary to isolate antigen specific B cells with high purity and good yield. As discussed in Chapter 1, the antigen specific B cell isolation methods reported in the literature had not generally produced results with both high purity and yield. Flow cytometry was used in this project as the isolation technique of choice as it has the advantage of enumerating as well as phenotyping the antigen specific B cells at the same time.

Isolation of PRP specific B cells from peripheral blood from adult volunteers immunised with PRP-OMP conjugate vaccine by flow cytometry following staining of cells with biotinylated antigen was attempted initially in order to develop the methodology for antigen specific B cell isolation (Chapter 3). In these longitudinal experiments PRP specific B cells could not be detected definitively. In contrast, TT binding B cells were identified from peripheral blood from adult volunteers immunised with TT antigen by flow cytometry using biotinylated TT. It is possible that biotinylation of the PRP antigen could have altered the configuration of the antigen preventing it from binding to specific B cells through surface receptors. However, the biotinylated PRP still bound to human serum PRP antibodies when tested by an ELISA. An alternative possibility was that PRP specific B cells were present in very low numbers in peripheral blood and may be present in greater concentrations in lymphoid tissues.

If the failure in detection of PRP specific B cells was due to alteration of the PRP chemical structure during biotinylation, the use of unbiotinylated PRP detected by

PRP monoclonal antibodies in indirect immunofluorescence staining provided an alternative approach for identifying antigen specific B cells by flow cytometry. PRP monoclonal antibodies were produced by the hybridoma technique, fusing spleen cells from PRP-T conjugate vaccine immunised mice with a myeloma cell line (Chapter 4). The PRP monoclonal anybodies were of IgM class and bound PRP antigen specifically. The functional activity of the anti-PRP monoclonal antibodies was demonstrated by bactericidal and chemiluminescence assays.

According to the available literature, only two previous groups have produced murine monoclonal antibodies to PRP capsular polysaccharide. In those studies, the PRP hybridomas were produced using either spleen cells from mice immunised with several injections of formalin killed Hib bacteria (Gigliotti and Insel, 1982) or spleen cells immunised using a combined *in vivo* and *in vitro* immunisation technique with a PRP conjugate vaccine (Bunse and Heinz, 1994).

However, it was shown that the anti-PRP monoclonal antibodies developed could not be used reliably in indirect immunofluorescence assays as they bound "non specifically" to B cells and non B cells, giving rise to false positive results (Chapter 5). As discussed in Chapter 4, other potential applications of the murine anti-PRP monoclonal antibodies developed could be their use in latex agglutination testing, immunochromatography and immunoadsorbant assays for diagnosing Hib infection.

The possibility that PRP specific B cells are more readily identifiable in secondary lymphoid tissues was tested by attempting to identify PRP specific B cells from splenic tissues from immunised mice in Chapter 6. Mice were immunised with PRP-T conjugate vaccine and splenic cells were stained with biotinylated PRP and TT antigens as in Chapter 3. PRP and TT antigen binding B cells were not definitively

detected from the splenic tissues above background by flow cytometry. However, anti-PRP and anti-TT ASC were identified in spleen cells by ELISPOT assays. Anti-PRP and anti-TT ASC were also identifiable in blood, but in much lower numbers. The frequencies of anti-PRP ASC were approximately 2 fold less in the spleen and more than 4 fold less in the blood when compared with those of anti-TT ASC.

The detection of anti-PRP ASC in spleen cells in mice suggested that anti-PRP ASC might also be detected in lymphoid tissues in humans. Since access to splenic tissues in humans was restricted, a pilot study was performed using tonsillar tissues from children undergoing routine tonsillectomy (Chapter 7). Anti-PRP and anti-TT ASC were detected by ELISPOT assays from the tonsillar cells. The total number of anti-PRP ASC in all tested samples was about 2.5 fold lower than the total number of anti-TT ASC in the same samples.

The findings of this project suggest that the generation of PRP specific B cells and anti-PRP ASC following immunisation with a PRP conjugate vaccine is less compared to the generation of protein antigen specific cells under similar conditions. Furthermore, the life span of anti-PRP ASC may be shorter than for ASC for a protein antigen. This is consistent with the conclusions presented in Chapter 1, where a review of the literature shows that although the immune response to PRP antigen is converted to a TD like response by protein conjugation, it still falls short of a true TD response.

As discussed in Chapter 6, several previous studies have enumerated anti-PRP ASC cells from blood from adults immunised with PRP conjugated to TT, DT and CRM_{197} proteins. The frequencies of carrier protein specific antibody secreting cells were also measured. In these previous studies the anti-PRP ASC numbers were higher than

those for the carrier protein specific antibody secreting cell numbers, except in two studies where anti-TT ASC were marginally higher following carrier priming (Barington *et al.*, 1993; Barington *et al.*, 1994). However, the results of the previous studies were highly variable. Specifically, two studies (performed by Barington *et al* and Hougs *et al*) recorded surprisingly large numbers of PRP ASC in some individuals (Barington *et al.*, 1990; Hougs *et al.*, 1993). As detailed in Chapter 6, the antigen specificity of most of the ASC detection assays was not determined by inhibition experiments with excess free antigen, and evidence of active antibody secretion was not demonstrated with a protein inhibitor. None of these previous studies included negative controls using subjects who had not received recent immunisations.

There is a strong correlation between the total number of previous exposures to antigen and the frequency of antigen specific B cells in the immune system (Nanan *et al.*, 2001). Therefore, it is not appropriate to compare the ASC to PRP and TT antigens in adults as the priming of the immune systems of the subjects may not be similar due to previous TT immunisation and natural exposure to Hib organisms throughout life. Ideally, comparisons should be performed between identically or similarly (as much as possible) primed individuals. That is why comparative studies performed during early childhood are useful.

Antigen specific B cell isolation by binding of specific antigens through immunoglobulins or isolation of ASC by spot formation on an antigen coated membrane (ELISPOT) could produce false positive results due to non specific binding. Furthermore, as described by Bell and Grey, adsorbed antigen specific immunoglobulins on cell surfaces through Fc receptors may function as antigen

binding receptors and may result in the isolation of non antigen specific B cells (Bell and Gray, 2003). This was clearly evident in this project where TT binding non B cells were detected by flow cytometry from peripheral blood from immunised donors (Chapter 3). The release of these adsorbed immunoglobulins from non antigen specific cells could produce spot formation in the ELISPOT assays generating false positive results. Therefore, not all antigen binding cells or ASC could be considered as antigen specific cells if the specificity of the detection system is not tested further: it is important to use appropriate experimental techniques to confirm the specificity of the isolated antigen specific B cells or the identified ASC. Techniques to confirm specificity could be the use of negative control experiments, inhibition experiments, development of single cell cultures from isolated cells and testing for in vitro specific antibody production, and the removal of adsorbed immunoglobulins from cells prior to isolation.

However, in the ELISPOT experiments of this project described in Chapter 6, the spots that were counted had diffuse margins and were dark in colour, indicating they were created by ASC rather than by the release of adsorbed immunoglobulin. Number of spots that did not have diffuse peripheries were also present and may have been created by release of antigen specific immunoglobulins bound to Fc receptors on the cells. The studies of Barington *et al* and Hougs *et al* in that detected large numbers of anti-PRP ASC by ELIPOTS have not specifically described the characteristics of the counted spots (Barington *et al.*, 1990; Hougs *et al.*, 1993).

The initial objectives of this project could not be achieved because of the failure to isolate PRP specific B cells in spite of several different approaches. While improvements in cell purification may help, the major difficulties in identifying PRP

specific B cells are the non specific binding phenomenon compounded by the very low frequency of antigen specific B cells. Therefore, alternative approaches to achieving the objectives should be considered.

In this project anti-PRP and anti-TT ASC were identified by ELISPOT assays although PRP antigen specific B cells were not detected in the same samples by flow cytometry. This showed that detecting ASC may be a more practical reality for PRP antigen than attempting to identify PRP antigen specific B cells. This has to be taken into consideration when developing an alternative approach to achieve the initial objective of phenotyping PRP specific B cells and comparing these phenotypes with those of TT specific B cells.

This alternative approach could be developed based on some published techniques. B cells collected from children and adults immunised with Hib conjugate and TT vaccines could be separated initially into different phenotypic subpopulations by flow cytometry and cell sorting (Shi *et al.*, 2003; Sidorova *et al.*, 2003). These isolated phenotypic B cell subpopulations could be stimulated *in vitro* with Hib conjugate vaccine antigen (PRP conjugated to a protein) and TT in separate cultures to proliferate and differentiate into ASC (Breukels *et al.*, 1999). These cultures then could be tested for anti-PRP and anti-TT specific ASC by ELISPOT assays. This will lead to understanding of the different phenotypic B cell subsets involved in the development of antibody response to PRP antigen following immunisation with Hib conjugate vaccine and compare and contrast with that of a protein antigen.

Since B cells in *in vitro* cultures undergo proliferation, comparison of the absolute numbers of antigen specific ASC will not provide accurate data on the total numbers as well as numbers of phenotypic subpopulations of antigen specific B cells in the

immune system following immunisation. However, the frequencies of antigen specific ASC could be expressed as percentages against the respective initial numbers of B cells in the cultures and the percentage ratios could be compared for PRP and TT antigens. Control experiments should be performed with blood from naïve or unimmunised donors for comparison.

Markers such as CD5, CD19, CD20, CD22, CD23, CD27, CD43, CD45, HLD-DR as well as B cell receptor markers for surface IgD, IgM and IgG could be used for phenotypic separation of B cells prior to culture. The phenotypic separation can be performed using a single marker as well as several markers in combination.

It is very important to determine the specificity of the detected ASC in the ELISPOT assays. As discussed before, the assays should be designed to include inhibition experiments with excess free antigen, protein synthesis inhibitors to confirm antibody secretion and methods to elute any absorbed immunoglobulins on cells that could give rise to false positive results.

The B cells could be obtained from blood samples collected from children approximately 4 weeks after routine immunisation (Breukels *et al.*, 1999). A longitudinal study could be performed on these children following each immunisation episode to determine the outcome of the results with increasing age and vaccine antigen exposure.

It would be also be useful to perform separate experiments on B cells obtained from lymphoid tissues such as tonsils. Children and adults could be enrolled who are to undergo tonsillectomy for therapeutic reasons. A Hib conjugate vaccine and a TT vaccine booster could be given to these individuals 1 month prior to the surgical

procedure with appropriate consent. Lymphocytes could be obtained from the tonsils collected from tonsillectomy.

Similarly, it may be possible to obtain B cells from splenic tissues from children and adults who need to undergo elective splenectomy for medical conditions such as thalassaemia , hereditary spherocytosis or chronic congenital thrombocytopaenia.

These studies would make it possible to compare the B cell subpopulation involvement in the development of antibody responses to PRP conjugate antigen and TT antigen in different lymphoid tissues and in the peripheral circulation.

This project could be further expanded to study the B cell involvement in the antibody response to other capsular polysaccharide protein conjugate vaccines such as pneumococcal and meningococcal conjugate vaccines.

In summary, the numbers of PRP specific B cells and anti-PRP ASC in response to Hib conjugate vaccine appear to be lower than seen in response to TT immunisation. Although excellent immune protection is provided by Hib conjugate vaccines, the immune responses appear to differ somewhat from those characteristic of a full TD response in spite of being coupled to a carrier protein. Detection of PRP specific B cells from immunised subjects is a challenging task probably due to the small numbers of such cells in peripheral blood and in lymphoid tissues. Therefore, an alternative approach using an *in vitro* stimulation method combined with spot forming assays is suggested which might allow further characterisation of PRP specific B cells.

APPENDIX

Kodituwakku, A.P., Jessup, C., Zola, H., and Roberton, D.M. (2003) Isolation of antigen-specific B cells.
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