

Persistence of protection against invasive bacteria - memory B cell responses in infants after immunisation

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Thesis submitted in Partial Fulfilment for the Degree of Doctor of Philosophy

Department of Paediatrics,

University of Oxford, Trinity Term 2008



*To my husband, Dominic, and my mother, Chantal,
for all their love and support.*

Abstract

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Rapid waning of antibody and vaccine effectiveness is observed following infant immunisation with protein-polysaccharide conjugate vaccines. This is despite the demonstrable presence of immunological memory. However, disease can develop within a few days of carriage acquisition of encapsulated bacteria. Persistence of functional antibody, therefore, appears to be the key determinant of long-term protection against invasive bacterial diseases. Antibody persistence is thought to depend on the survival of long-lived plasma cells and memory B cells generated in germinal centres (GC).

Using the ELISpot method, the kinetics of the B cell response following a booster dose of MenC conjugate vaccine (MenCV) at one year of age, and following a 2 dose-primary course of a new tetravalent meningococcal vaccine (MenACWY-CRM197) given at 2 and 4 months of age, were determined. It was found that priming with these vaccines induced protective antibody levels in the majority of children but detectable memory B cells only in a subset of children. A strong association was found between the level of polysaccharide-specific antibody and memory B cells produced after priming, and the persistence of functional antibody at one year of age.

The kinetics of a primary B cell response were determined in healthy adults after immunisation with rabies vaccine, and compared to the B cell response following primary immunisation with MenCV in 2 months old infants. The timing of appearance of the B cells in peripheral blood was similar in infants and adults, although the magnitude of the response was slightly lower in infants.

These observations suggest that long-term humoral immunity induced by immunisation with protein-polysaccharide conjugate vaccines in early infancy depends on the production of adequate GCs during priming. The children who generate efficient GCs during priming (identified by higher production of memory B cells, plasma cells and Abs) may best maintain protective antibody levels in the long-term, while those children generating less efficient GCs, have a smaller B cell pool, lower antibody response during priming, and might not maintain protective antibody levels in the long-term.

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List of Abbreviations

Ab: antibody

Ag: antigen

APC: antigen presenting cell

ASC: antibody-secreting cells

BAFF: B cell activating factor

BCR: B cell receptor

CFR: case fatality rate

CpG: cytosine-phosphorothioate-guanine

CRM: cross reactive materials

CRM₁₉₇: mutant peptide related to

diphtheria toxoid

CSF: cerebrospinal fluid

CTL: cytotoxic lymphocytes

DC: DCs

dH₂O: distilled water

DIC: disseminated intravascular

coagulation

ELISA: enzyme linked immunosorbent
assay

ELISpot: enzyme linked immunoSPOT

ET: electrophoretic type

FACS: fluorescence-activated cell sorter

FDCs: follicular DCs

FO: follicular

GC: germinal centre

GMT: geometric mean titre

HBV: hepatitis B vaccine

Hib: *Haemophilus influenzae* type b

HLA: human leucocyte antigen

Ig: immunoglobulin

IL: interleukin

INF: interferon

LDA: limiting dilution assay

LOS: lipooligosaccharides

LPS: lipopolysaccharides

MBCp: memory B cell precursor

MBL: mannose-binding lectin

MenACWY-D: quadrivalent

polysaccharide meningococcal vaccine

conjugated to diphtheria toxoid

MenAC: meningococcal A and C

MenC: meningococcal serogroup C

polysaccharide

MenCV: serogroup C meningococcal

conjugate vaccine

MHC: Major histocompatibility complex

mHSA: methylated human serum albumin

MLEE: multilocus enzyme electrophoresis

MLM: multilevel modelling

MLST: multilocus sequence typing

MZ: marginal zone

NANA: N-acetyl neuraminic acid

OD: optical density

OMP: outer membrane proteins

OMV: outer membrane vesicles

PAMPs: pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PICU: paediatric intensive care unit

PSV-4: quadrivalent plain polysaccharide vaccine

PWM: Pokeweed mitogen

RBC: red blood cell

SAC: *Staphylococcus aureus* Cowan strain

SBA: Serum bactericidal activity (hSBA with human complement; rSBA with rabbit complement)

SNP: single-nucleotide polymorphism

TCR: T cell receptor

TD: T-dependent

TI: T-independent

TLR: Toll-like receptor

TNF: tumor necrosis factor

9vPnC-MenCV: combined 9 valent pneumococcal and meningococcal serogroup C conjugate vaccine

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1 *Neisseria meningitidis* – disease and immunity

1.1 Introduction

Haemophilus influenzae type b, *Neisseria meningitidis* and *Streptococcus pneumoniae* are the principal causes of meningitis and septicaemia in young infants worldwide. These bacteria have a polysaccharide capsule and are common commensals of the human nasopharynx (Broome, 1986; Cartwright et al., 1987). Rarely, these encapsulated bacteria cause invasive disease (meningitis and septicaemia). Despite the availability of effective antibiotics and sophisticated intensive care management, case fatality rates and morbidity among survivors remains very high (Thorburn et al., 2001; Booy et al., 2001; Buysse et al., 2008a; Buysse et al., 2008b; de Greeff et al., 2008). This is because disease can develop and progress very rapidly, whereas early diagnosis is complicated by the non-specific clinical presentation at disease onset (Thompson et al., 2006). The highest rates of invasive bacterial disease are found in young children, reflecting the age-dependent acquisition of anticapsular antibody (Ab) (Edwards, 2001; Goldschneider et al., 1969a). Thus, immunoprophylaxis against the pathogenic strains of these encapsulated bacteria is probably the most powerful strategy towards the elimination of the invasive bacterial diseases of childhood.

Over the last 25 years protein-polysaccharide conjugate vaccines have been developed to protect against *H. influenzae* type b, and various serogroups/serotypes of *N. meningitidis* and *S. pneumoniae* (Makela and Kayhty, 2002). These vaccines are highly immunogenic and have resulted in a huge reduction in the cases of disease caused by these polysaccharide-encapsulated bacteria in the countries that have introduced them in their immunisation schedules (Makela and

Kayhty, 2002). However, it has been reported that infant immunisation is associated with a relatively short duration of protection without booster doses of vaccine (Ramsay et al., 2003b; Trotter et al., 2004). In contrast, at older ages, more sustained protection has been described with just a single dose of a conjugate vaccine (MacLennan et al., 2000; Richmond et al., 1999; Snape et al., 2008a). Recent studies in humans suggest that B cells might be the principal determinant of long-term protection after immunisation with polysaccharide conjugate vaccines through maintenance of Ab and generation of immunological memory.

A key point in infection by *N. meningitidis* is the rapidity of the disease onset, which explains the need for persisting antibody to sustain protection. In this thesis, the basic biology of meningococcus and pathogenesis of meningococcal disease are first described in relation to the cellular basis of long-term immunity and vaccination strategies to combat the disease. Furthermore, this thesis contains specific studies on B cell and Ab responses following immunisation with meningococcal vaccines in infants, and a study comparing different methods of assessment of memory B cells. Finally, a study of the quantitation of the primary B cell response to a novel Ag is also described to improve our understanding of B cell priming.

1.2 Microbiology of *N. meningitidis*

N. meningitidis is an aerobic, Gram-negative coccus, typically arranged in pairs (diplococci). The structure is typical of Gram-negative bacteria, with a thin peptidoglycan layer sandwiched between an inner cytoplasmic membrane and an outer membrane (Figure 1.2-1).

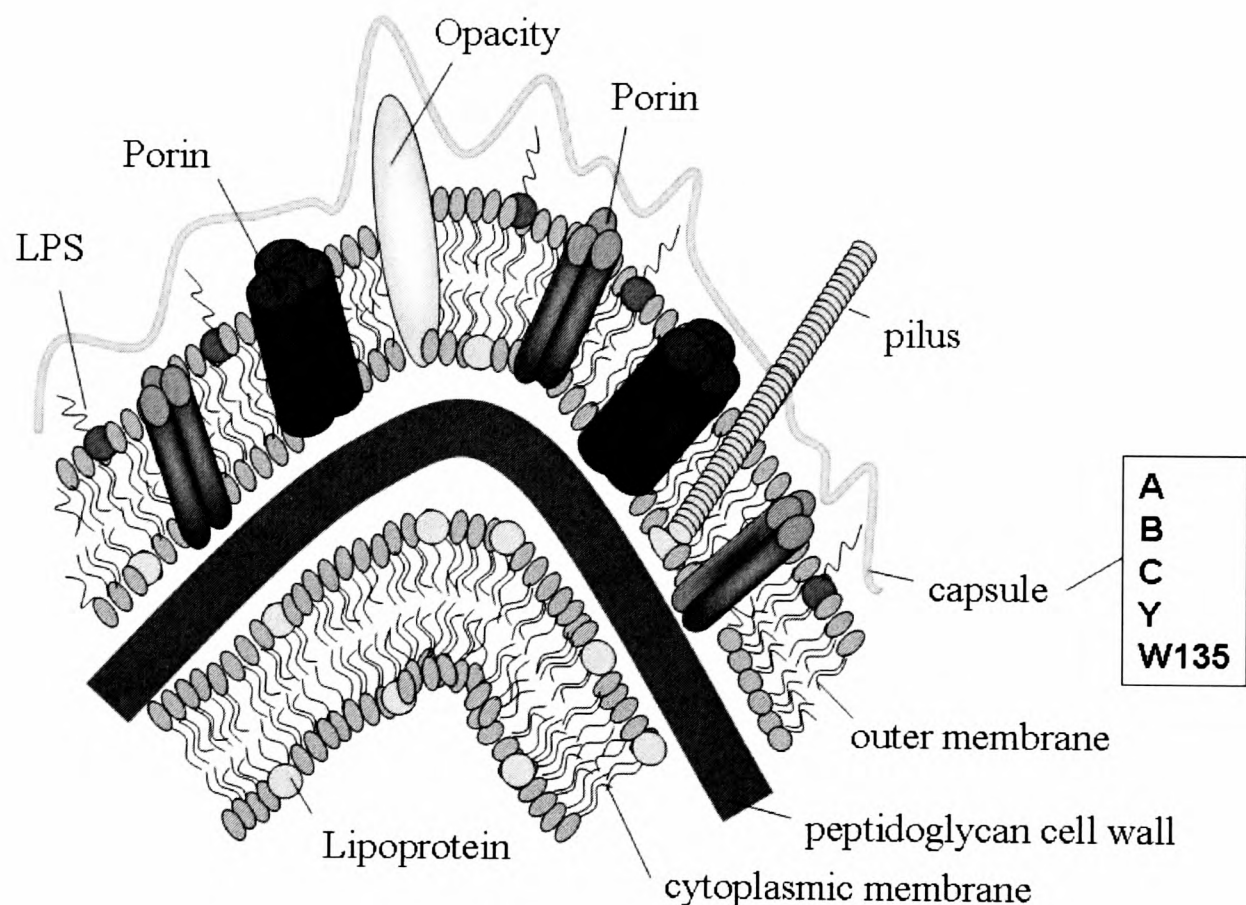


Figure 1.2-1: Diagram of the structure of the cell wall of *N. meningitidis* illustrating the position of the polysaccharide capsule. It should be noted that in reality the capsule is thicker and the OMPs protrude outside (Pollard and Levin, 2000).

The outer membrane is composed of lipids, outer membrane proteins and lipopolysaccharide (LPS). The LPS of *N. meningitidis* contains lipooligosaccharide (LOS), which is composed of lipid A and a core oligosaccharide, but lacks the repeating O-Ag polysaccharide found in most Gram-negative bacteria (Jennings et al., 1980). Pathogenic meningococci are surrounded by a polysaccharide capsule. The specific chemical composition and linkages involved have allowed

the classification of meningococci into serogroups. Thirteen serogroups have been identified to date and the composition of the polysaccharide capsule of the principal serogroups causing disease is summarised in table 1.2-1.

Group	Chemical Composition of Capsular Polysaccharide
A	Partially O-acetylated 2-acetamido-2-deoxy-D-mannose-6-phosphate
B	(2-8)-linked N-acetylneuraminic acid
C	O-acetylated (2-9)-linked N-acetylneuraminic acid
X	2-acetamido-2-deoxy-D-glucose-4-phosphate
Y	Partially O-acetylated alternating sequence of D-glucose and N-acetylneuraminic acid
W135	Alternating sequence of D-galactose and N-acetylneuraminic acid

Table 1.2-1: Chemical composition of the capsular polysaccharide of the principal serogroups causing disease (Bhattacharjee and Jennings, 1976; Poolman et al., 1995).

The surface located or secreted molecules of *N. meningitidis* influence adhesion, invasion and survival of the bacteria in the human host. The capsule is thought to protect the meningococcus from desiccation during transmission between hosts and from Ab-mediated phagocytosis in the bloodstream (Poolman et al., 1995). The pili and Opacity associated proteins mediate adherence to and invasion of host cells (Callaghan et al., 2008; Nassif, 1999). The Porin proteins are outer membrane proteins, encoded by two genes: *porA* and *porB*, which allow the bacteria to acquire nutrients from the host environment by forming pores or channels in the outer membrane (Tomassen et al., 1990). Antigenic variation or changes in any expression of genes encoding outer membrane molecules also mediate meningococcal resistance against host immune defences. *N. meningitidis* has also developed a mechanism to acquire iron from human transferrin through transferrin binding proteins (Larson et al., 2002). Table 1.2-2 summarises the principal virulence factors of *N. meningitidis*.

Component	Function
Capsule	against host defence : complement – dependent bacteriolysis and phagocytosis
Outer membrane proteins (OMP) Porins (PorA/PorB)	form pores or channels for nutrients to pass into the cell and waste products to exit, promote intracellular survival by preventing phagolysosome fusion, resist complement-mediated serum killing
Opacity-associated proteins (Opa/Opc)	adherence to host cells
Lipopolysaccharide (LPS) Lipid A + a core oligosaccharide	lipid A has endotoxin activity; during rapid cell growth, the bacteria release outer membrane blebs, which contain LPS and surface proteins and enhance toxicity and bind endotoxin-mediated protein-directed Abs
Pili	attachment to host cells, transfer of genetic material, motility, and interference with neutrophil killing
IgA protease	Against host defence (degrade IgA)
Transferrin binding protein	mediate acquisition of iron for bacterial metabolism (by direct link to human transferrin)

Table 1.2-2: Table summarising the virulence factors in *N. meningitidis*.

1.3 Meningococcal diversity and classification

Traditionally, meningococcal strains have been classified by serological typing, based on antigenic variation of the major surface components. The serogroup specifies the capsular polysaccharide designation; PorB OMP the serotype; the PorA OMP the serosubtype and the LPS the immunotype (Frasch et al., 1985; Poolman et al., 1995). For example a meningococcus with a serogroup B capsule, a serotype 15 PorB variant, a serosubtype 7,16 PorA variant and expressing LPS with an L3 immunotype would be shown as B:15:P1.7,16:L3. Each of these characteristics was originally determined using polyclonal sera and monoclonal Abs, however lately DNA sequence analysis was also utilised.

N. meningitidis has been divided into 13 serogroups: A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z, based on chemically and antigenically distinct polysaccharide capsules. Strains from patients with invasive disease are encapsulated and five of these serogroups (A, B, C, W135 and Y) cause more than 90% of the invasive disease worldwide (Peltola, 1983). In contrast, approximately 50% of the strains isolated from carriers lack capsules and, therefore, cannot be serogrouped (Claus et al., 2002; McNeil and Virji, 1997). Acapsulate meningococci may possess the genes for capsule synthesis since capsule production in meningococcal strains can switch on and off at high frequencies via a mechanism called phase variation (Hammerschmidt et al., 1994; Swartley et al., 1997). Alternatively, the capsular genes may be absent; capsule null strains do not have the capability to produce a polysaccharide capsule (Claus et al., 2002) but have been still rarely associated with disease (Hoang et al., 2005).

N. meningitidis is highly diverse, both in its surface structures (antigenic diversity) and in the genes encoding metabolic functions. The diversity of the surface antigens (Ags) may be explained by the selection pressure of the host immune system (Maiden, 2006). The surface structures of the meningococcus diversify by random mutation (by substitution, insertion and deletion) and also via genetic recombination following natural transformation with exogenous DNA (Feil et al., 1999; Maiden et al., 1998). The presence of clusters of closely related genotypes in isolate collections examined by multilocus enzyme electrophoresis (MLEE) suggests that recombination is very frequent in *N. meningitidis* (Maiden, 2006). Several studies have also shown that many of the surface structures of *N. meningitidis* are phase-variable allowing adaptation of these Ags in response to changing environmental conditions (Jennings et al., 1999; Klein et al., 1996). These “contingency loci” (Bayliss et al., 2001; Moxon et al., 2006) are genes whose expression is controlled by simple short repetitive sequences of DNA. In *N. meningitidis*, these are usually homopolymeric tracts resulting from slip-strand mispairing during DNA replication. Altered numbers of repeats will thus switch “off” and “on” the expression of the gene or change the level of expression, dependent on whether the repeat sequence is in the open reading frame or the promoter of the gene respectively. These mechanisms allow the bacterial population to diversify and the progeny with a selective advantage in a changing environment will be most favoured. Antigenic variation allows meningococci to switch the type of capsule they express and genetically closely-related meningococci have been shown to express different capsular polysaccharides (Swartley et al., 1997).

Therefore, the serological methods of classification are not ideal because they cannot keep track of antigenic variability and poor expression or masking of surface Ags. They also require a continual need to enlarge the reagent panel of monoclonal Abs used for typing. Molecular methods of classification, such as MLEE; (Selander et al., 1986), pulsed-field gel electrophoresis (PFGE (Popovic et al., 2001)), and multilocus sequence typing (MLST (Maiden et al., 1998)), have been developed to explore the genetic diversity of meningococci. These techniques assess the diversity of housekeeping genes (i.e. genes involved in basic functions, constitutively expressed and under no selective pressures) to find evolutionary relationships among isolates. MLEE assesses the electrophoretic mobility of various, relatively conserved, cytoplasmic enzymes, using starch gel electrophoresis and specific histochemical staining (Selander et al., 1986). PFGE separates long strands of DNA by length and runs the DNA through a flat gel matrix of agarose (Popovic et al., 2001). MLST at a genomic level, assesses fragments of genes that code for cytoplasmic enzymes; the determination of the nucleotide sequence of 7 housekeeping genes allows the separation of the isolates into sequence types (Urwin and Maiden, 2003). Meningococci sharing identical MLEE profiles are identified as an electrophoretic type (ET), those sharing identical PFGE profiles as PFGE-based clonal group, and those sharing identical MLST profiles, a sequence type (ST).

The molecular epidemiology of *N. meningitidis* infection has traditionally been approached by using MLEE (Caugant, 1998; Selander et al., 1986). However, MLEE is labour-intensive, time-consuming, and inconvenient for inter-laboratory comparison. At present, MLST and PFGE are the most widely used methods for tracking global spread of meningococcal clones (Mayer et al., 2002; Nicolas et al., 2001; Popovic et al., 2001). All MLST data are centralised via a publicly available database to detect pathogens that may achieve continental spread. Analysis of MLST

data has revealed that all multilocus genotypes are not equally represented; most of them are observed only once, while a small proportion of ETs/STs are identified repeatedly over a wide temporal and geographical distribution (Maiden, 2006). These frequent ETs/STs form the centre of clonal complexes (or group of related genotypes) which represents groups of clones that are different from one another but are still close enough for a common origin to be recognised.

1.4 Epidemiology

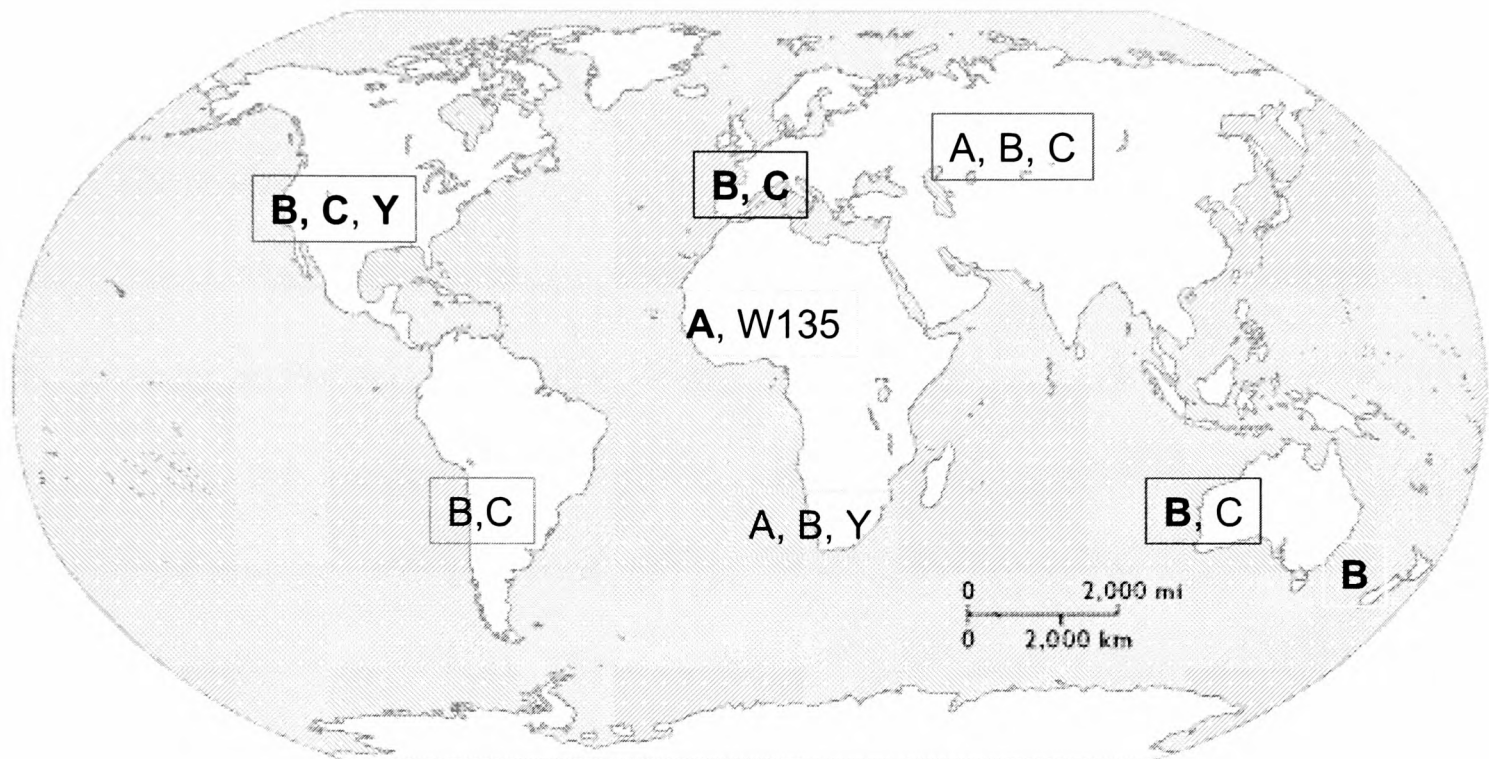


Figure 1.4-1: Worldwide serogroup distribution: Map illustrating the serogroups responsible for the majority of invasive disease caused by *N. meningitidis* in different areas of the world.

N. meningitidis has been responsible for epidemic and endemic meningococcal disease throughout the world for a very long time. The first outbreak of meningococcal disease to be described was by the Swiss physician Vieusseux in Geneva in 1805 (Vieusseux, 1805).

Meningococcal disease is an important cause of morbidity and mortality in developing and industrialised countries. Untreated meningococcal disease is generally fatal, while prompt antibiotic treatment reduces the case fatality rate (CFR) to less than 10% (Booy et al., 2001; Thorburn et al., 2001; de Greeff et al., 2008).

Endemic meningococcal disease occurs worldwide, and epidemics are common in developing countries. Epidemics of meningococcal disease occur after the introduction of a new, virulent strain into an immunologically naïve population. In the absence of epidemics, about 500,000 cases of invasive meningococcal disease are reported each year worldwide, resulting in more than 50,000 deaths (Tikhomirov et al., 1997). Half of these cases occur in the “Meningitis belt” (Lapeyssonnie, 1963), a zone south of the Sahara in Africa comprising 18 countries spreading from Ethiopia in the east to Senegal in the west and including a population of 300 million people. While the incidence of meningococcal disease varies from 1-3/100,000 in most industrialised nations, it may exceed 500 per 100,000 during severe epidemics in sub-Saharan Africa (Schwartz et al., 1989). These different disease rates reflect the various pathogenic properties of *N. meningitidis* strains and diverse socioeconomic, environmental, and climatic conditions.

Of the 12 serogroups, almost all infections are caused by serogroups A, B, C, Y, and W135 (Peltola, 1983; Jacobsson et al., 2008). As shown in Figure 1.4-1, the serogroups of the strains causing disease are not uniformly distributed throughout the world. In most developed countries, the predominant serogroups causing disease are B and C (Caugant, 1998; Jacobsson et al., 2008). Serogroups A and W135 are epidemic in Sub-Saharan Africa (Lapeyssonnie, 1963; Mayer et al., 2002; Traore et al., 2006), whilst serogroup Y is prevalent in the US (Racoosin et al., 1998; Rosenstein et al., 1999; Kaplan et al., 2006). Outbreaks caused by serogroup X have also been described recently in Africa (Djibo et al., 2003).

1.5 Global spread of *Neisseria meningitidis*

Analysis of meningococcal genotypes by MLEE and MLST has demonstrated that relatively few genotypes, named “hyperinvasive lineages”, are responsible for meningococcal disease worldwide (ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, and ST-41/44) (Jolley et al., 2005; Yazdankhah et al., 2004). Hyperinvasive lineages identified as the cause of disease in one country are often found to lead to a subsequent increase in the incidence of disease around the world soon afterwards and may reach a worldwide distribution in the course of a few years (Achtman, 1995; Caugant, 1998).

1.5.1 The ST-1, ST-4 and ST-5 complexes and the group A pandemics

Three clonal complexes (the ST-1, ST-4, ST-5 complexes) are strictly associated with the serogroup A capsular polysaccharide and have caused the majority of meningococcal disease in Africa and Asia in the past 40 years (Wang et al., 1992). ST-1 and ST-5 complexes have also caused outbreaks of meningococcal disease around the world.

At the beginning of the 1960s, ST-1 complex (MLEE designation : subgroup I) was responsible for endemic and epidemic disease in eastern sub-Saharan and northern Africa before spreading to the American continent in the 1970s where this lineage was responsible for outbreaks in Brazil, Pacific north-western USA, and Canada (Caugant, 1998). In the 1980s-1990s, the ST-1 complex was responsible for outbreaks in Australia and New Zealand (Achtman, 1995). It was

also associated with a recent increase of meningococcal disease in Russia.

The ST-5 complex (MLEE designation: subgroup III) has been responsible for three pandemics since the 1960s (Achtman, 1995); the first pandemic wave started in China in the mid-1960s, spread to Eastern Europe, Russia, Scandinavia and then Brazil during the mid-1970s. The second pandemic wave started in the early 1980s in Nepal and India and spread to Saudi Arabia in 1987 during the annual Hajj pilgrimage to Mecca. From there, it was introduced to Europe, the US and Africa by returning pilgrims (Moore et al., 1988). Serogroup A-subgroup III disease was unknown in Africa before 1987, but major subgroup III epidemics have since spread to all countries in the meningitis belt (Achtman, 1994), causing over 150,000 reported cases in 1996.

Meningococci belonging to the ST-4 complex (MLEE designation: subgroup IV-1 and IV-2) were associated with epidemics in West Africa in the 1960s-1980s, and in the USA and the UK during the first and second world wars (Caugant, 1998).

1.5.2 The ST-11 complex

The ST-11 complex (MLEE designation: ET-37 complex) has caused a number of epidemics and pandemics, but unlike the ST-1 and ST-5 complexes, it has been associated with various serogroups (although principally those containing sialic acid), corresponding to serogroup B, C, Y and W135 (Wang et al., 1993). The first recorded ST-11 outbreaks occurred in US army recruit camps in the 1960s and triggered the development of the first effective meningococcal vaccines (see section 1.11). In 1967, the predominant strains switched from serogroup B,

against which a plain polysaccharide is ineffective, to a serogroup C epidemic, which could be interrupted with polysaccharide vaccines. During the 1990s, a new ST-11 complex variant (first identified in Canada and designated using the MLEE terminology as the ET-15 variant of the ET-37 complex (Whalen et al., 1995)) spread throughout the world and was responsible for outbreaks in the USA, Israel, Scandinavia in the early 1990s, the Czech republic in 1993, Australia in 1994 and the UK in 1995. These outbreaks of ST-11 complex displaying serogroup C capsule prompted the development and licensure of serogroup C conjugate vaccines. Several countries, including the UK (Miller et al., 2001), Spain (Larrauri et al., 2005) and the Netherlands (Welte et al., 2005) introduced these vaccines into national immunisation programmes. In 2000 and 2001, another variant of this ST-11 clonal complex was responsible for outbreaks of serogroup W135 disease in Saudi Arabia, again associated with the Hajj pilgrimage. This variant spread to Europe and the USA was because of the returning pilgrims and their close contacts (Taha et al., 2000). In 2002, the first large epidemic caused by W135 occurred in Burkina Faso, resulting in more than 12,000 cases (Decosas and Koama, 2002). However, most outbreaks in the meningitis belt since 2002 have been again caused by serogroup A strains of the ST-5 complex.

1.5.3 The ST-8 complex

The ST-8 complex (MLEE designation: cluster A4) contains both serogroup B and C organisms which were first isolated in western Europe in the 1960s and which have been associated with several epidemics across the world (Caugant, 1998).

1.5.4 The ST-32 complex

ST-32 complex (MLEE designation: ET-5 complex), frequently expressing the serogroup B capsule, has spread throughout Europe and the Americas since 1970s (Caugant et al., 1986, 1987). Since then, ST-32 has achieved a global distribution and many subclones of this lineage have been identified which exhibit distinct serological reactivities. These have been isolated from the UK, Spain, Chile, the USA, South Africa, and Asia (Caugant, 1998; Cruz et al., 1990). Epidemics associated with serosubtypes of the ST-32 complex in Norway and Cuba have led to the development of outer membrane vesicle vaccines targeting the specific outbreak strains (Bjune et al., 1991; Sierra et al., 1991). These vaccines were effective against the epidemic strains, but a lack of cross-protection against other members of the same clonal complex was observed (Perkins et al., 1998)

1.5.5 The ST-41/44 complex

During the 1990s, members of the ST-41/44 complex (MLEE designation: lineage III) have replaced ST-32 complex meningococci as the major cause of serogroup B meningococcal disease in many European countries. This is a highly diverse clonal complex. A particular variant of this complex was responsible for the epidemic in New Zealand (Martin et al., 1998) and stimulated the development of another OMV vaccine against this particular strain (the New Zealand meningococcal B vaccine MeNZB). Mass vaccination of the New Zealand population was initiated in 2004 (Oster et al., 2005) and various studies have shown that the MeNZB vaccine was very effective and immunogenic in children older than 16 months of age (Kelly et al., 2007; Wong et al., 2007).

1.6 Nasopharyngeal carriage

Humans are the only natural carriers of *N. meningitidis*. Exposure to *N. meningitidis* occurs from aerosol spread and mostly results in asymptomatic nasopharyngeal carriage (Figure 1.6-1). Invasive meningococcal disease is a rare consequence of infection with *N. meningitidis*. The incidence of meningococcal disease is higher in young children, with a secondary peak in teenagers, and declines with age. In contrast, asymptomatic nasopharyngeal carriage is common, with a population prevalence of around 10% (Cartwright et al., 1987). Carriage prevalence varies with age, being highest in teenagers (24-37%) and lowest in young children (<3% in children younger than 4 years) (Cartwright et al., 1987; Caugant et al., 1994; Caugant et al., 1988; Claus et al., 2005) (Figure 1.6-2). Carriage rates then decrease to less than 10% in older age groups.

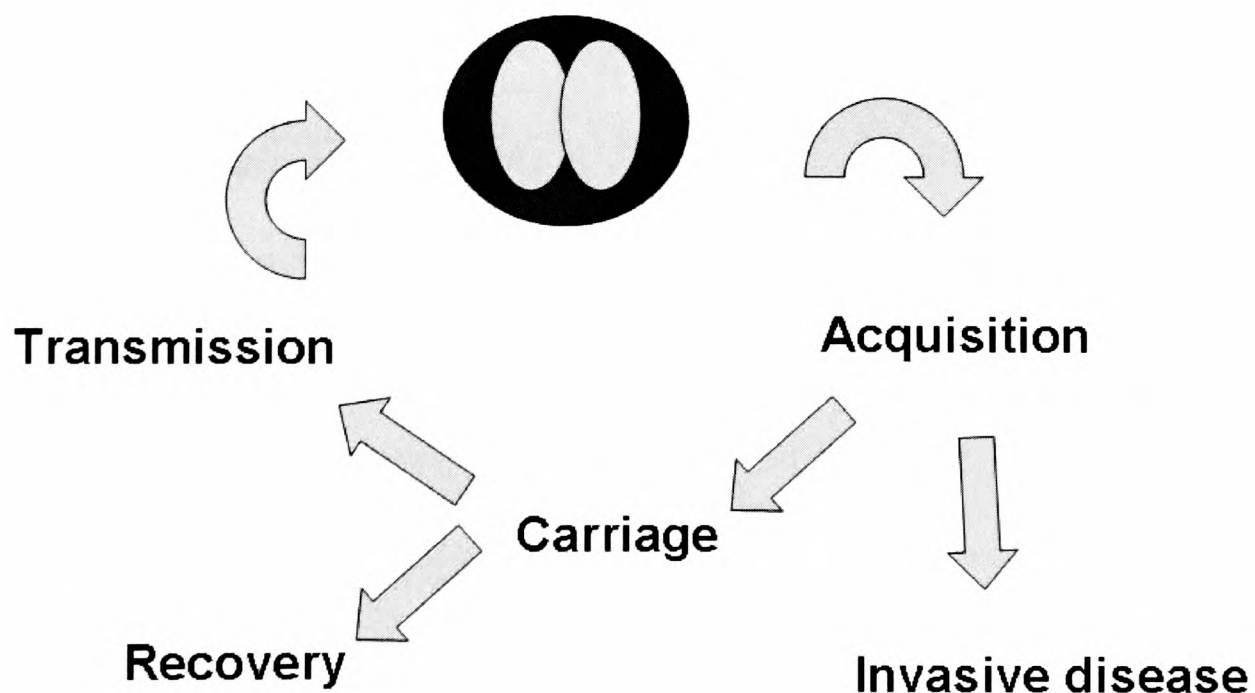


Figure 1.6-1: The meningococcal colonisation cycle. Acquisition of meningococci usually leads to asymptomatic carriage and further transmission to a susceptible individual. Rarely, acquisition may lead to invasive disease.

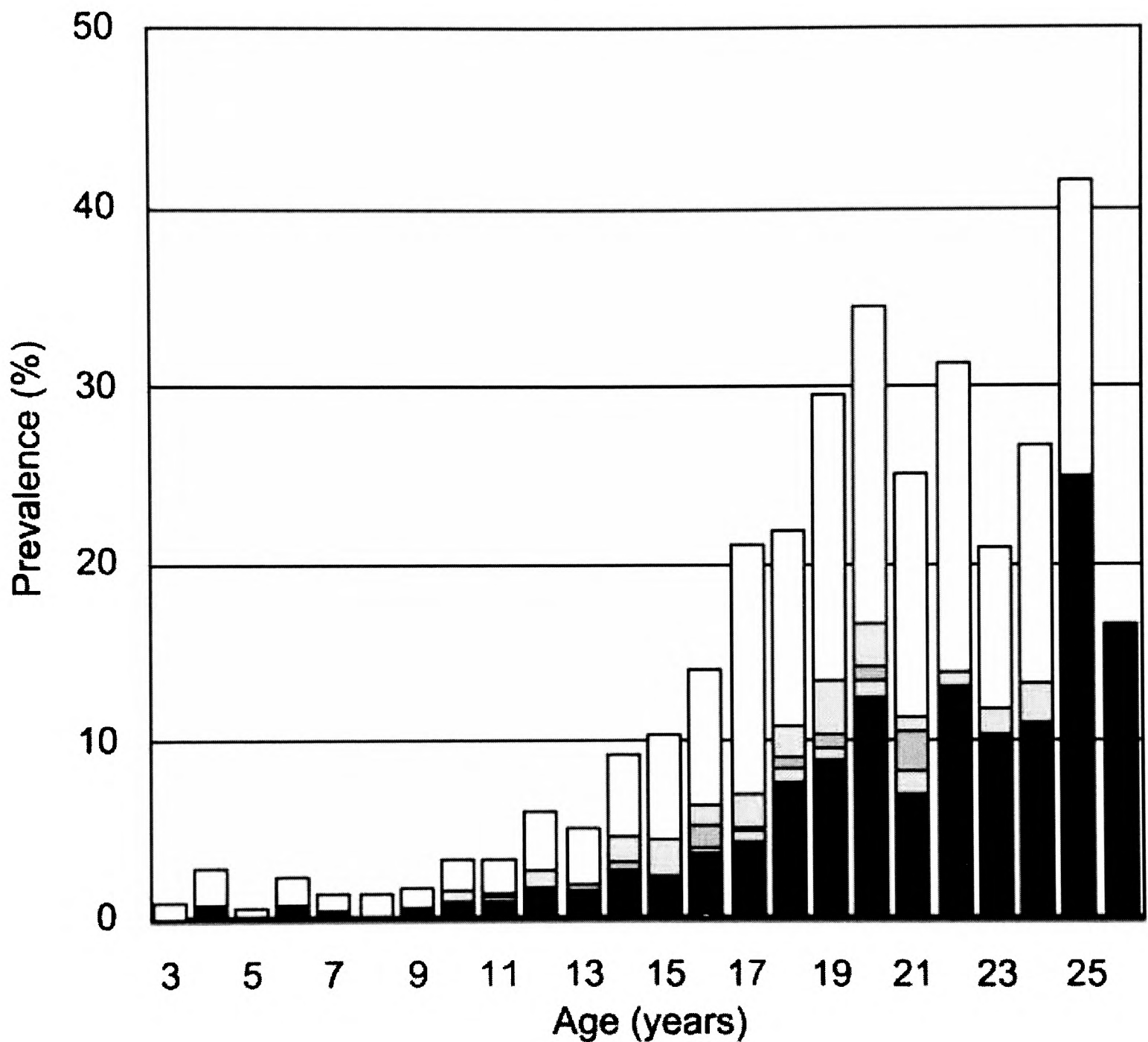


Figure 1.6-2: Age-specific meningococcal carriage rates by serogroup in one particular population in Germany (black, serogroup B; light grey, serogroup C; dark grey, serogroup W135; mid grey, serogroup Y; white, all other not groupable serogroups) (Claus et al., 2005).

Infants more commonly carry *Neisseria lactamica*, a related but non-pathogenic organism. The rates of carriage of *N.lactamica* increase from 3 to 18 months of age (21%) and then decline by 14-17 years of age (1.8%) (Gold et al., 1978). Carriage of *N. lactamica*, commensal bacteria (e.g. *Moraxella catarrhalis*) and other pathogenic and non-pathogenic strains of *N. meningitidis*

presumably contribute to the development of natural immunity to meningococcal infection. Indeed, all these organisms share highly homologous structures (Braun et al., 2004), and therefore, can induce cross-reactive Abs to *N. meningitidis* (Gold et al., 1978; Goldschneider et al., 1969b). The absence of capsule in *N. lactamica* suggests that OMPs and LPS are the major sources of this immunological activity (Sanchez et al., 2002).

The majority of people will, on one or on multiple occasions in life, carry meningococci asymptomatically in the throat (Broome, 1986; Stephens, 1999; Ala'Aldeen et al., 2000). According to modelling studies by Trotter *et al.*, an individual might experience during their lifetime four episodes of carriage of serogroup B meningococci, seven episodes of carriage of other meningococci, six episodes of carriage of *N. lactamica*, and 0.5 episodes of carriage of serogroup C meningococci (Figure 1.6-3) (Trotter et al., 2006). The duration of carriage varies; it may be chronic or sporadic (Broome, 1986) and may be strain specific. Carriage is typically transient, with clearance occurring after specific Abs develop. It is unclear whether carriage primes the immune cells. However, this question needs to be kept in mind when designing studies to assess the cellular basis of the immune response to vaccination.

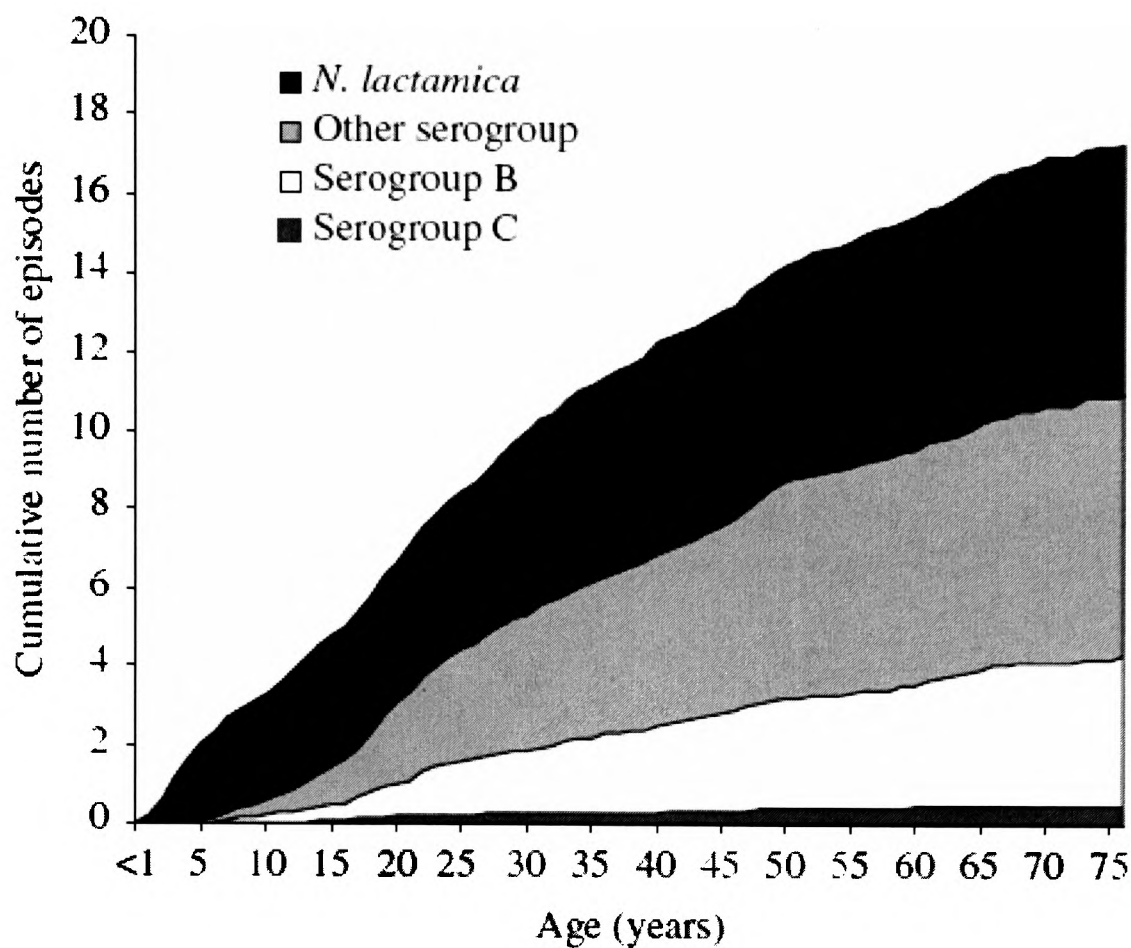


Figure 1.6-3: Estimated average cumulative episodes of carriage by age (Trotter et al., 2006).

Studies of the serogroups and genotypes of *N. meningitidis* in asymptomatic carriers and in patients with meningococcal diseases have shown that carried meningococci are more diverse (Caugant et al., 1988; Jolley et al., 2000) than disease-associated meningococci (Maiden et al., 1998) (see Table 1.6-1). Carried meningococci may be either encapsulated, expressing 1 of the 13 capsular polysaccharides, or acapsulate, because of genetic down-regulation of capsule expression (Swartley et al., 1996), by inactivation of genes in the capsule gene cluster, or by the absence of capsule gene cluster as in capsule null meningococci (Claus et al., 2002).

Clone complex	No. of isolates		Disease association odds ratio (95% confidence interval)	No. of isolates of serogroup:				
	Disease	Carriage		B	C	Y	W135	Other or not serogroupable
ST-11	93	10	52 (20–135)	11	83		6	3
ST-23	7	32	0.2 (0.1–0.7)	4		22	1	12
ST-32	54	18	0.9 (0.4–2.2)	59	4	2		7
ST-35	6	18	0.3 (0.1–1.1)	12		1		11 ^a
ST-162	16	17	0.8 (0.4–1.18)	27			2	4 ^b
ST-269	15	7	6.1 (0.5–72.8)	19		1		2
ST-41/44	44	42	1.1 (0.5–2.3)	67	2	2		15

Table 1.6-1: Some principal clonal complexes of *N. meningitidis* and their association with disease (odds ratio of disease to carriage prevalence with 95% CI) and serogroup among disease isolates (Yazdankhah et al., 2004).

Rates of transmission and carriage increase in closed and semi-closed populations, such as among military recruits (Caugant et al., 1992; Jones et al., 1998; Tyski et al., 2001), university students (Neal et al., 2000), and in the household contacts of a case of meningococcal disease (Olcen et al., 1981; Brooks et al., 1995; Cardenosa et al., 2001). In addition, several risk factors have been shown to be associated with meningococcal carriage: individuals with respiratory tract infections (Stephens, 1999); low socio-economic status; social behaviour, such as attendance at pubs/clubs, intimate kissing, and smoking (Davies et al., 1996; Coen et al., 2006; MacLennan et al., 2006);. As *N. meningitidis* is transmitted primarily by asymptomatic carriers, an increase in meningococcal disease incidence is therefore, likely to be caused by changes in the meningococcal clones carried in the population (Goldschneider et al., 1969b). The cause of progression from carriage to invasive disease in some individuals is unclear but it may depend on both the host and the organism.

1.7 Pathogenesis

For most individuals, carriage of meningococci is an immunising process, resulting in a systemic protective Ab response (Stephens, 1999). However, in a small proportion of the population, *N. meningitidis* penetrates the mucosa and gains access to the bloodstream, causing systemic disease (Aycock and Mueller, 1950).

There are many conditions necessary for the development of invasive meningococcal disease; exposure to a pathogenic strain, colonisation of the naso-oropharyngeal mucosa, passage through that mucosa, and survival of the meningococcus in the bloodstream (Schwartz et al., 1989). These processes are influenced by bacterial properties, climatic and social conditions, preceding or concomitant viral infections, and the immune status of the patient (Goldschneider et al., 1969a, b; Haneberg et al., 1983; Moore et al., 1990; Cartwright et al., 1991; Fischer et al., 1997).

Meningococci are transferred from one person to another by direct contact or via droplets (Nelson, 1996). The transmission rate of virulent clones is higher and invasive disease often develops within the first week after acquisition (Edwards et al., 1977; Marks et al., 1979; Ala'Aldeen et al., 2000). The development of meningococcal disease depends on the chance that a person will encounter and acquire a virulent bacterium (Nelson, 1996). The risk is increased in households where a case of meningococcal disease has occurred (De Wals et al., 1981); overcrowding (Baker et al., 2000; Moodley et al., 1999); exposure to tobacco smoke (Fischer et al., 1997; Haneberg et al., 1983); and concurrent viral infection of the upper respiratory tract

(Cartwright et al., 1991; Haneberg et al., 1983; Moore et al., 1990). The two latter increase the formation and spread of respiratory droplets or diminish the functional and mechanical integrity of the respiratory mucosa as a barrier to invasion (Fischer et al., 1997; Moore et al., 1990). Colonisation occurs on the exterior surface of the mucosal cell and subsequently, intra- or subepithelially (Stephens and Farley, 1991). Damage to the nasopharyngeal mucosa, such as by preceding viral infections (Moore et al., 1990), and active or passive smoking (Fischer et al., 1997), promotes colonisation (Rayner et al., 1995) and possibly invasion.

Pili are the major adhesins that permit the attachment of the bacteria to nonciliated epithelial cells. A membrane cofactor (CD46), which is expressed on all human cells except erythrocytes, has been identified as a receptor for *Neisseria* type IV pili (Johansson et al., 2003). Adhesion of meningococci to host cells leads to a transient up-regulation of PilC production, down-regulation of capsule synthesis (Deghmane et al., 2000) and removal of sialic acid from LPS, which seems essential for meningococcal interaction with host cells (Yazdankhah and Caugant, 2004). After initial pilus-mediated attachment, closer binding to the host cell occurs via the OMPs Opa and Opc. Opa binds to carcino-embryonic Ag (CD66) receptors (Callaghan et al., 2008; Virji et al., 1996). Interactions with this receptor on phagocytic and endothelial cells mediate the phagocytosis of meningococcus by epithelial cells and cytokine production (Griffiths et al., 2007; Meyer, 1999), meningococci then traverse the cell in membrane-bound vacuoles (Stephens and Farley, 1991).

Once through the epithelium, meningococci may gain entry into the bloodstream (Stephens et al., 1993). They can survive and proliferate in the bloodstream via their virulence factors or

because of a deficit of the host defence. The major mechanism inhibiting the multiplication of meningococci in the blood is complement mediated bactericidal activity in human sera (Goldschneider et al., 1969a). Host factors that decrease bactericidal activity increase the risk of developing invasive disease. These factors include the absence of bactericidal Abs against meningococci (Goldschneider et al., 1969a, b), deficiencies in the complement cascade (Figueroa et al., 1993), and the presence of blocking immunoglobulin A Abs that inhibit the bactericidal activity of human sera (Griffiss, 1975). The latter suggests that mucosal immunisation may not be appropriate to induce protection against *N. meningitidis*. Meningococci have also developed mechanisms that protect them from the bactericidal activity of human sera. Both the sialic acid residues in the capsule of serogroups B and C meningococci and the sialylation of LPS inhibit complement activation, probably by enhancing the affinity of the alternative pathway inhibitor factor H to C3b (Horstmann, 1992; Mandrell et al., 1991). Furthermore, *N. meningitidis*, like *H. influenzae* and *S. pneumoniae*, possesses the ability to produce extracellular IgA1 proteases that can inactivate specific IgA1 Abs (Lomholt et al., 1992; Vitovski et al., 1999).

During growth and lysis of meningococci, endotoxin is released as vesicular outer membrane structures (blebs) (Figure 1.7-1). These consist principally of LPS, but also OMPs, lipids, and outer membrane fragments (Andersen, 1989; Devoe and Gilchrist, 1973). In response to endotoxin, there is production of complement activating products and different level of cytokines and chemokines such as tumor necrosis factor (TNF) α and interleukin (IL) 1β (Emonts et al., 2003), which varies among individuals. Studies by Brandtzaeg *et al.* have clearly demonstrated that the severity of meningococcal septicaemia is directly related to levels of circulating endotoxin (Brandtzaeg et al., 1989b). The systemic inflammatory response to *N.*

meningitidis can overreact and provoke serious tissue damage to the host (see below).

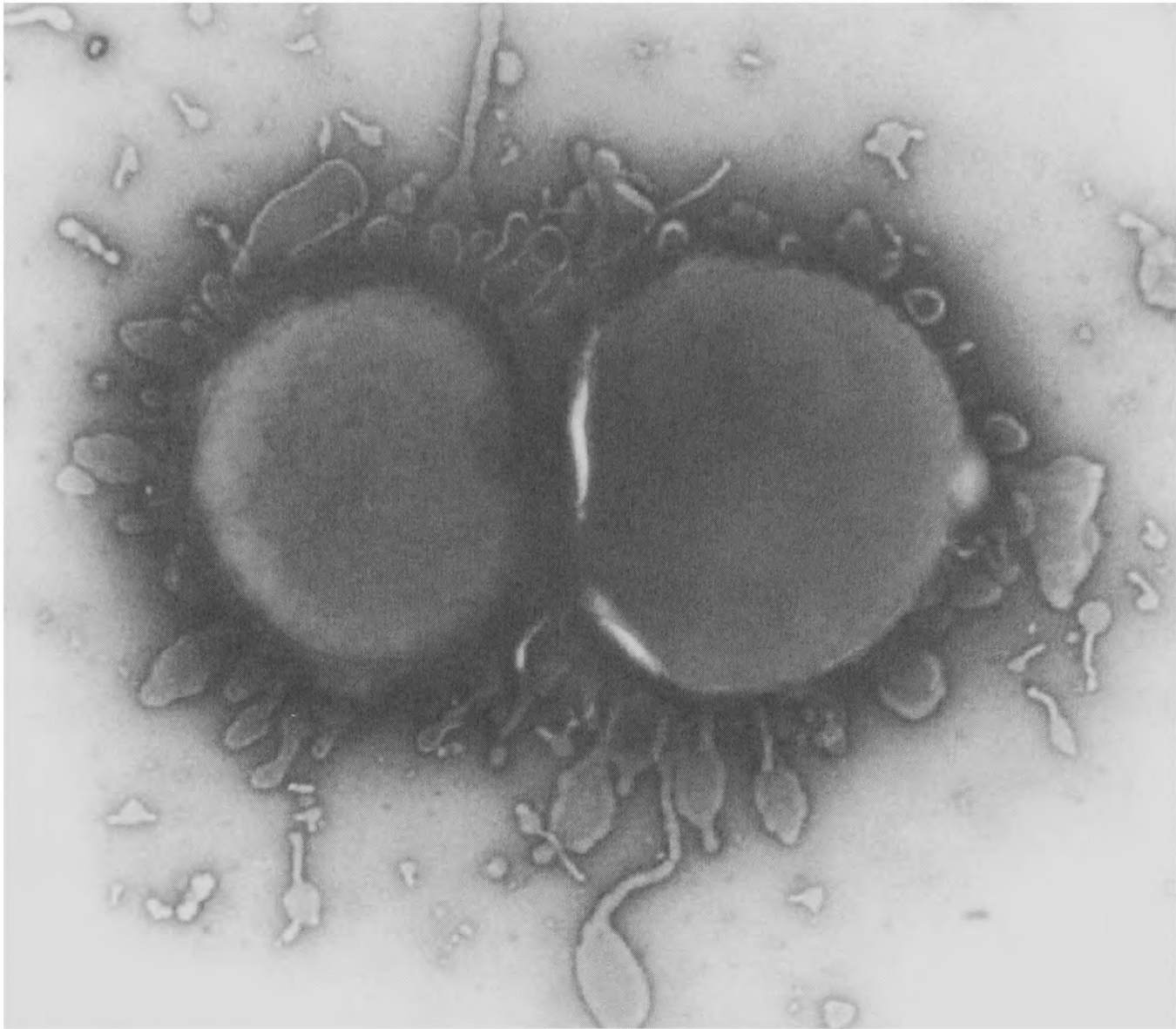


Figure 1.7-1: Electron microscopy of a pair of meningococci surrounded by “blebs” of membrane, which contain endotoxin, reproduced by (Kroll et al., 1997).

1.8 Meningococcal disease

1.8.1 Clinical manifestations

Classically, patients with meningococcal disease present with fever and a non-blanching rash with features of meningitis and /or septicaemia. However, this classical presentation usually develops later on in disease progression and early manifestations only include less specific symptoms of sepsis, such as leg pain, cold hands and feet, and abnormal skin colour (Thompson et al., 2006). Of children who progress to invasive meningococcal disease, 30-50% develop meningitis alone, 7-10% septicaemia alone, and 20-40% a mixed picture of meningitis and septicaemia (Havens et al., 1989; Kirsch et al., 1996).

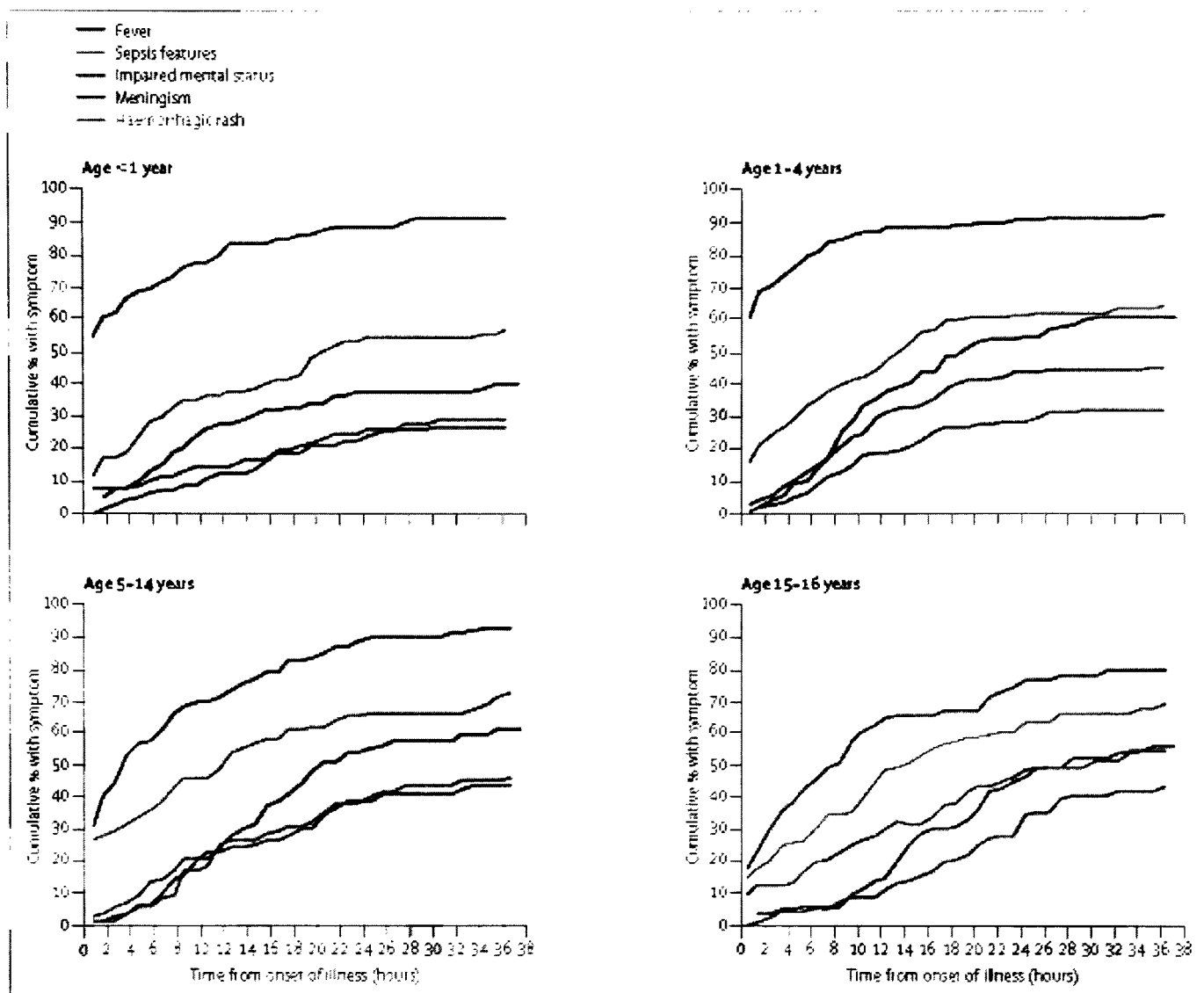


Figure 1.8-1: Time of onset of clinical features of meningococcal disease (Thompson et al., 2006). Sepsis features = abnormal skin colour, cold hands and feet, or leg pain. Impaired mental state = unconsciousness, confusion or delirium, or seizure. Meningism = neck stiffness or photophobia).

1.8.1.1 Rash

Most patients with acute meningococcal infection have a skin rash, which may present as a maculopapular rash, a petechial rash, or a purpuric rash (Baxter and Priestley, 1988; Marzouk et al., 1991; Riordan et al., 1996; Schildkamp et al., 1996; Thompson et al., 2006). It often presents as a non-specific maculopapular rash and progresses over time to a petechial or purpuric rash (Figure 1.8-2).

The lesions of the maculopapular rash are pink, raised toward the centre, 2-15 mm in diameter, and with indistinct borders. They are not confluent and they blanch on pressure. They are often localised on the trunk and the extremities. Petechiae can present in the centre of the macules. A maculopapular rash may change within minutes into a haemorrhagic rash, with general deterioration (Marzouk et al., 1991; Riordan et al., 1996).

The lesions of the petechial rash are small, about 1-2 mm in diameter, non-blanching and localise mainly on the trunk and lower limbs. However, they may also be seen on the face, the palate and the conjunctivae. They may progress to large purpuric lesions with black necrotic centres or may become confluent and develop into haemorrhagic bullae (described as purpura fulminans).

The presence of a haemorrhagic rash is characteristic of meningococcal disease and reflects coagulopathy. Both pro-coagulant and anticoagulant pathways of haemostasis are dysregulated as a consequence of activation of the inflammatory and coagulation cascades, in addition to endothelial dysfunction (Pathan et al., 2003). The defect of the coagulation mechanism in meningococcal sepsis might result from a combination of the loss of anticoagulant proteins such as proteins C and S from the plasma, and the failure of anticoagulant mechanisms on the endothelial surface. The endothelial receptors required for protein C activation (endothelial protein C receptor and thrombomodulin) are down-regulated on the endothelium of patients with meningococcal septicaemia (Faust et al., 2001). In addition, there is a reduction in the levels of circulating activated protein C and antithrombin III and suppression of the normal fibrinolytic mechanisms. This results in intravascular clot formation, with suppression of the normal mechanisms, which degrade intravascular thrombi, and the clinical syndromes of

disseminated intravascular coagulopathy and purpura fulminans (Esmon, 2005).

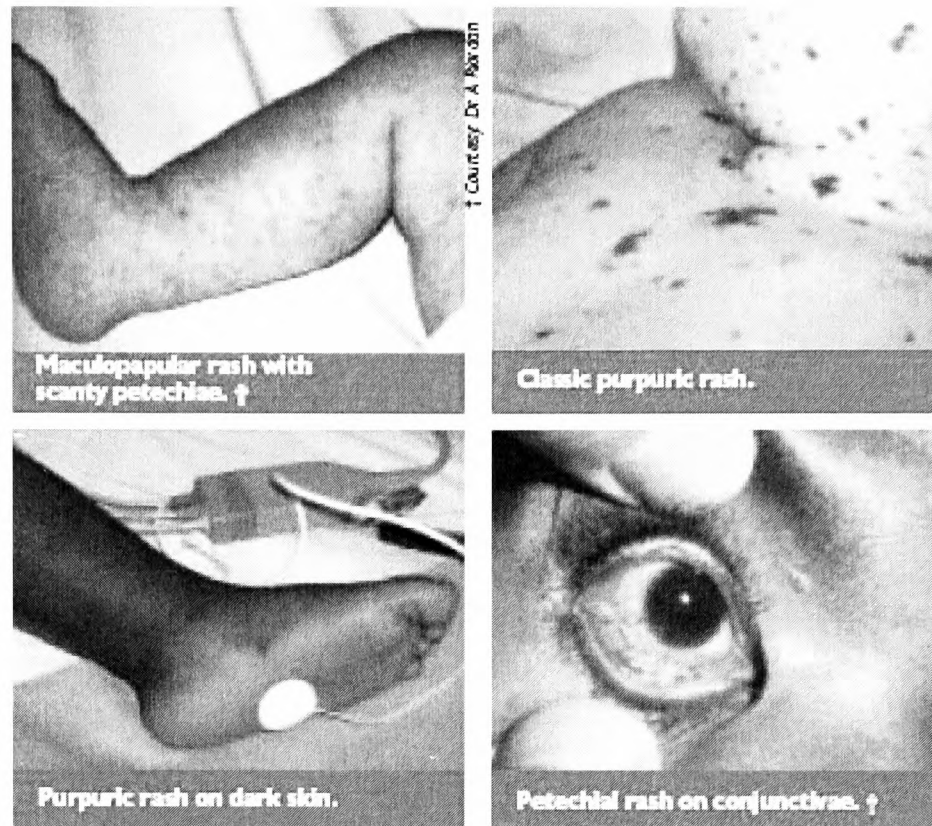


Figure 1.8-2: Possible rash in meningococcal disease (Pathan et al., 2003).

1.8.1.2 Shock/septicaemia

Septicaemia in meningococcal disease is multi-factorial and results from a combination of increased vascular permeability, myocardial dysfunction and disseminated intravascular coagulation (Mercier et al., 1988; Nadel, 1995; Ninis, 2007). The increased vascular permeability results from the inflammatory response to the release of endotoxin from the bacteria (Ninis, 2007). This induces endothelial cell injury, which causes leakage of water and plasma proteins out of the intravascular compartment. The patient becomes hypovolaemic due to the reduction in circulating volume, thus reducing the cardiac output (Ninis, 2007). Myocardial dysfunction arises from the release of endotoxin and inflammatory mediators (e.g. IL6), which reduce myocardial contractility (Kumar et al., 1996; Parrillo, 1993; Pathan et al., 2004). The hypovolaemia and myocardial dysfunction leads to progression of shock with tissue

hypoxia. The clinical features of shock arise because perfusion of the vital organs is maintained at the expense of perfusion of non-vital organs (e.g. skin, kidneys and gut). In the early phases of shock, these processes compensate for hypovolaemia and maintain the central circulating blood volume and cardiac output (Nadel and Kroll, 2007). As a result, patients may present with tachycardia, cold peripheries, prolonged capillary refill time, oliguria, tachypnoea/hypoxia, a decreased level of consciousness (reflecting central nervous system hypoperfusion and/or hypoxia), and metabolic acidosis (reflecting tissue hypoperfusion) (Pollard et al., 1999a).

It should be noted that hypotension appears very late in children because compensatory mechanisms are maintained until the myocardium fails (Pollard et al., 1999a).

Disseminated intravascular coagulation develops in response to the release of endotoxin and inflammatory mediators. This leads to the activation of the coagulation cascade and down-regulation of anticoagulant and fibrinolytic pathways, contributing to multiple organ failure and purpura fulminans through microvascular thrombosis (Esmon, 2005; Faust et al., 2001; Pathan et al., 2003).

A third of all patients with invasive meningococcal disease may develop fulminant meningococcal septicaemia within a few hours, before any signs of meningitis become apparent (van Deuren and Brandtzaeg, 2000). It is caused by exponential growth of meningococci in the bloodstream, with high concentrations of endotoxin and cytokines in plasma (Brandtzaeg et al., 1989a; Ovstebo et al., 2004). It is characterised by shock and disseminated intravascular coagulation. Clinical deterioration is rapid and half of the patients who die will do so within 24 hours after the first clinical manifestation (van Deuren et al., 2000). However, the mortality rate

of fulminant meningococcal septicaemia has recently dropped below 4% in the UK because of the centralisation of care in a paediatric intensive care unit specialised in meningococcal disease, the dissemination of information on initial recognition and management of meningococcal disease and the use of mobile intensive care service (Booy et al., 2001).

1.8.1.3 Meningitis

The mechanisms by which meningococci invade the meninges and pass across the blood-brain barrier are unclear. Once in the subarachnoid space, meningococci proliferate in an uncontrolled way (Brandtzaeg et al., 1992), because the immune system is absent (Simberkoff et al., 1980; Zwahlen et al., 1982). The liberation of endotoxin induces the activation of pro-inflammatory cytokines (e.g. TNF and IL1), which increase the permeability of the blood-brain barrier and contribute to the development of the clinical manifestation of meningitis (Quagliarello et al., 1991; Sharief et al., 1992).

In older children with meningococcal meningitis, the following symptoms and signs predominate: headache, fever, vomiting, photophobia, neck stiffness, positive Kernig's and Brudzinski's signs, and lethargy; in younger children: poor feeding, irritability, a high-pitched cry and bulging fontanelle. Seizures and focal neurological signs occur less frequently (Nadel and Kroll, 2007).

The major difference between sepsis and meningitis is that in meningitis the inflammatory response is localised in an extravascular compartment where factors of the complement and coagulation systems are absent. While meningococcal sepsis is the most devastating form of

sepsis, meningococcal meningitis has a low rate of mortality and neurological sequelae compared to other types of bacterial meningitis (Schuchat et al., 1997). Around 5% of survivors suffer from neurological sequelae, varying from sensorineural deafness, mental retardation, spasticity, and/or seizures to concentration disturbances (Oostenbrink et al., 2002; Buysse et al., 2008a; Buysse et al., 2008b).

1.8.1.4 Raised intracranial pressure

Raised intracranial pressure (ICP) is caused by inflammation of the meninges and capillary leak leading to cerebral oedema (Tunkel and Scheld, 1993) but is uncommon in meningococcal meningitis (Odio et al., 1991). Signs of raised ICP include a declining level of consciousness, focal neurological signs including unequal, dilated or poorly responsive pupils, hypertension and relative bradycardia. Papilloedema is a late finding in acutely raised intracranial pressure (Pollard et al., 1999a).

1.8.1.5 Other clinical manifestations

Much less frequently, other syndromes are associated with meningococcal disease, including conjunctivitis (Barquet et al., 1990), otitis media, sinusitis, epiglottitis, arthritis (Schaad, 1980), urethritis, pneumonia and pericarditis (Rosenstein et al., 1999). A rare syndrome, chronic meningococemia, may present as fever, rash, joint pain and headache over several weeks (Tzeng and Stephens, 2000). In patients with a low level of bacteraemia, meningococci are cleared spontaneously. This transient meningococemia is characterised by a short febrile “flu”-like episode (Munro, 2002).

1.8.2 Diagnosis

The initial diagnosis is clinical, and management should not be delayed while waiting for the results of laboratory investigations, because there is often no laboratory evidence of the disease early in the course of the disease (Pollard et al., 1997).

1.8.2.1 Microscopy

Gram stain of cerebrospinal fluid (CSF) is sensitive and specific, but microscopy is of limited value for blood specimens because most patients have few organisms in their blood. Nowadays this diagnostic tool has been replaced by the PCR.

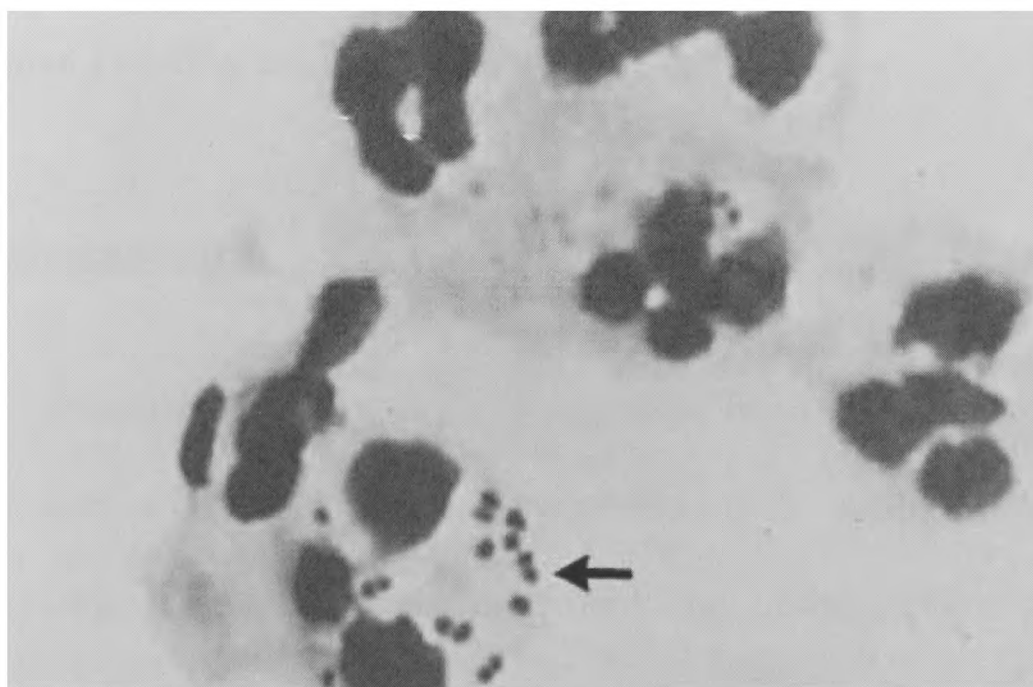


Figure 1.8-3: Gram stain of CSF showing *N. meningitidis* (Rosenstein et al., 2001).

1.8.2.2 Culture methods

N. meningitidis is generally present in CSF, blood, and sputum. Culture is definitive, but the organism is difficult to grow since it dies rapidly when exposed to cold or dry conditions. Cultures become positive after 12 to 24 hours. Definitive identification of *N. meningitidis* from the different *Neisseria* species is based on the mode of oxidation of carbohydrates. The different members of *Neisseria* produce acid from various carbohydrates (glucose, maltose, lactose, and sucrose). The production of acid from oxidation of glucose and maltose allows the identification of *N. meningitidis* (Gray and Fedorko, 1992; van Deuren et al., 1993; WHO, 1999)

1.8.2.3 Oxidase test

The oxidase test determines the presence of cytochrome oxidase in the organism. This test is rapid but has a lower specificity, as other members of the genus *Neisseria* or unrelated bacterial species may also give a positive reaction (WHO, 1999).

1.8.2.4 Latex agglutination test

Antisera for the principal disease-causing serogroups of *N. meningitidis* are available. Agglutination will occur with the serogroup corresponding to the antiserum. A negative control is performed with saline. Absence of agglutination with any of the antisera or the saline would define the strain as non-groupable. Several commercial diagnostic kits are available for rapid latex agglutination serology assays. These tests may be less sensitive and require many specimens to give reliable results (Perkins et al., 1995; WHO, 1999).

1.8.2.5 Nucleic acid amplification

Polymerase chain reaction (PCR) assays to detect meningococcal DNA in blood or CSF are becoming very useful but their costs remain higher than traditional culture methods. PCR assays are rapid, simple, and unaffected by prior antibiotic administration, as only the presence of genomic DNA is necessary for detection (Cartwright and Kroll, 1997). Sensitivity and specificity of PCR assays are high (91%) (Kaczmarek et al., 1998); however, false-positive results can occur due to contamination, and false negative results can be seen with presence of inhibitors in the CSF. PCR assays allowing determination of capsular status are also available (Failace et al., 2005; Olcen et al., 1995).

1.8.3 Monitoring and treatment

There is consensus that children with meningococcal disease should be recognised early and start parenteral antibiotic therapy (which should not be delayed by diagnostic procedures) promptly and at the same time as good supportive care particularly of cardiovascular and respiratory systems. Management priorities include the evaluation of airway, breathing and circulation, fluid resuscitation if indicated; and initiation of third generation cephalosporin therapy (ceftriaxone or cefotaxime). The initial assessment should focus on detection of early signs of shock and raised intracranial pressure (Pollard et al., 1999a). An algorithm has been developed to guide early management of meningococcal disease in children, by Pollard *et al.* 1999 at St Mary's PICU and is published by the Meningitis Research Foundation (www.meningitis.uk) (Figure 1.8-4).

Cefotaxime or ceftriaxone rather than penicillin is normally used as the initial treatment of patients with clinical diagnosis of meningococcal disease. This is because, until microbiological information is available, the possibility of penicillin resistance or an alternative bacterial aetiology cannot be excluded (Pollard et al., 1999a).

Shock should be treated with volume resuscitation (initially with 20 ml/kg colloid) to restore the intravascular compartment and maintain tissue perfusion and oxygenation. Early fluid resuscitation is associated with improved survival (Carcillo et al., 1991). When signs of shock persist after 40-60 ml/kg, elective intubation and ventilation in a specialist paediatric intensive care unit should be initiated because of the risk of pulmonary oedema (Carcillo and Fields, 2002). Myocardial support with inotropes should also be started (Pollard et al., 1999a). Monitoring of the adequacy of volume replacement includes heart rate, blood pressure, central venous pressure, urine output, metabolic status, and peripheral perfusion (Pollard et al., 1999a).

Children with meningococcal sepsis often have abnormalities in metabolism, including anaemia, hypoglycaemia, coagulopathy, metabolic acidosis, and electrolyte abnormalities (e.g. hypokalaemia, hypocalcaemia, hypomagnesaemia) (Khilnani, 1992). Repeated biochemical monitoring should be undertaken at hourly intervals during resuscitation and derangements corrected rapidly (Figure 1.8-4) (Pollard et al., 1999a). In a child with raised intracranial pressure concomitant with shock, the priority of management is to correct the shock because an adequate blood pressure is necessary to perfuse the brain in this case (Pollard et al., 1999a). Then, the child should be intubated and ventilated to control CO₂ in the low normal range, because a rise in CO₂ would cause an increase in cerebral blood flow and a further rise in

intracranial pressure (Sarnaik and Lieh-Lai, 1993). Lumbar puncture should be avoided in the context of shock due to sepsis, rash suggestive of meningococcal disease or clinical features of raised ICP (Advanced Life Support Group, 2004).

It is recommended to give systemic dexamethasone for suspected bacterial meningitis to reduce neuronal damage (Schaad et al., 1995; van de Beek et al., 2007). Other adjunctive treatments to reduce morbidity and mortality have been investigated (e.g. glucocorticoid, fresh-frozen plasma, plasma exchange, and other immunomodulating); however, these have not shown additional improvements in outcome (Cathie et al., 2008).

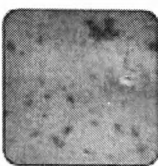
Antibiotic chemoprophylaxis is recommended for those who have been in close contact with diseased patients (Bilukha and Rosenstein, 2005).

1.8.4 Prognosis

Meningococcal disease remains an important cause of mortality and morbidity for children and young adults in both industrialised and developing countries. The overall mortality rate is 10% (Thorburn et al., 2001; Goldacre et al., 2003; Jensen et al., 2003; de Greeff et al., 2008; Jacobsson et al., 2008). The mortality rate is around 5% for those with meningitis alone and 5-40% for those with septicaemia alone (Kirsch et al., 1996; Thorburn et al., 2001; Booy et al., 2001). Around 10% of survivors develop permanent sequelae, such as deafness, mental retardation, and amputation (Oostenbrink et al., 2002; Buysse et al., 2008a; Buysse et al., 2008b).

Early Management of Meningococcal Disease in Children*

5th Edition



RECOGNITION

May present with predominant SEPTICAEMIA (with shock), MENINGITIS (with raised ICP) or both. Purpuric/petechial non-blanching rash. Rash may be atypical or absent in some cases.

- Call consultant in A&E, Paediatrics, Anaesthesia or Intensive Care
- DO NOT ATTEMPT LUMBAR PUNCTURE
- IV Cefotaxime (50mg/kg) or Ceftriaxone (80mg/kg)

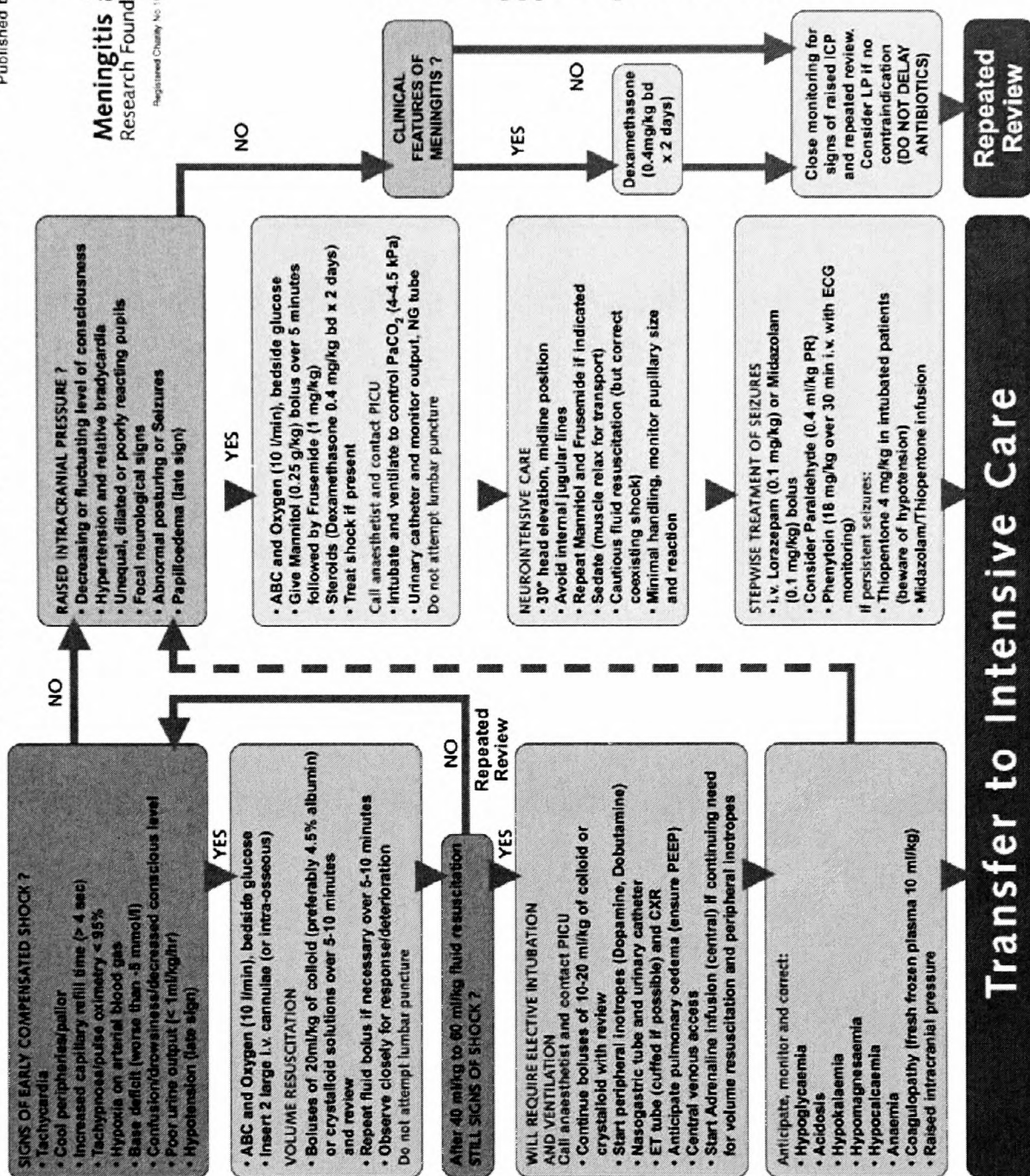
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Department of Paediatrics, Imperial College School of Medicine, St Mary's Hospital, London W2
1Arch Dis Child; March 1999; 80: 290-296

Conscious Level	Age	Normal Values
Alert	<1	Respiratory Rate/min 30-40
Responds to Voice	1-2	110-160
Unresponsive	2-5	25-35
	5-12	100-150
	>12	95-140
		80-120
		60-100

Estimate of child's weight (1-10 years)
Weight (kg) = 2 x (age in years + 4)

Normal systolic blood pressure = 80 + (age in years x 2)

N.B. Low BP is a pre-terminal sign in children

Observe HR, RR, BP, Perfusion, Conscious Level
Cardiac monitor and pulse oximetry. Take blood for Glucose, FBC, Clothing, U&E, Ca⁺⁺, Mg⁺⁺, PO₂, Blood cultures, Blood Gas (base, base deficit), Cross-match

Inotropes
Dopamine or Dobutamine at 10-20 mcg/kg/min. Make up 3 x weight (kg) mg in 50 ml 5% dextrose and run at 10 ml/hr = 10 mcg/kg/min.
(These dilute solutions can be used via a peripheral vein.)
Start Adrenaline via a central line only at 0.1 mcg/kg/min. Make up 300 mcg/kg in 50 ml of normal saline at 1 ml/hour = 0.1 mcg/kg/min.

Intubation (call anaesthetist)
Atropine 20 mcg/kg (max 600 mcg) AND Thiopentone 3-5 mg/kg AND
Suxamethonium 2 mg/kg (caution, high potassium) ET size = age/4 + 4. ETT
length (oral) = age/2 + 12 (use cuffed ET tube if possible). Then: morphine
(180 mcg/kg) and midazolam (100 mcg/kg) every 30 mins.

Hypoglycaemia (Glucose < 3 mmol/l)
5ml/kg 10% dextrose bolus i.v. and then dextrose infusion at 80% of maintenance
requirements over 24 hours.

Correction of metabolic acidosis pH < 7.2
Give half correction NaHCO₃ i.v.
Volume (ml) to give = (0.3 x weight in kg x base deficit +2) or 8.4%NaHCO₃ over
20 mins, or in neonates, volume (ml) to give
= (0.3 x weight in kg x base deficit) of 4.2% NaHCO₃.

If K⁺ < 3.5 mmol/l
Give 0.25 mmol/kg over 30 mins i.v. with ECG monitoring.
Caution if anuric.

If total Calcium < 2 mmol/l or ionized Ca⁺⁺ < 1.0
Give 0.1 ml/kg 10% CaCl₂ (0.7 mmol/ml) over 30 mins i.v. (max 10 ml) or
0.3 ml/kg 10% Ca Gluconate (0.22 mmol/ml) over 30 mins (max 20 ml).

If Mg⁺⁺ < 0.75 mmol/l
Give 0.2 ml/kg of 50% MgSO₄ over 30 mins i.v. (max 10 ml).

Prophylaxis of household contacts
Inform Public Health. Give Rifampicin (bd for 2 days)
< 1yr 5 mg/kg • 1-12yrs 10 mg/kg • > 12yrs 600 mg
or Ceftriaxone (single im dose)
< 12yrs 125 mg • > 12yrs 250 mg
or Ciprofloxacin as single 500 mg dose (not in children <2 or in pregnancy)
< 12yrs 250 mg • > 12yrs 500 mg

Diagnosis
LP may be important if the diagnosis or aetiology is in doubt, i.e. when meningococcal
symptoms predominate and where no rash is present, or in infants with fever
without a focus. It must not be performed when there are contraindications (e.g.
RCP, shock, coagulopathy). LP should never delay treatment.
Blood Cultures, throat swab, whole blood (EDTA specimen) for PCR. CSF (if
available) for culture and PCR. Rapid latex antigen test and aspirational scrapings
from skin showing haemorrhagic rash (if locally useful).

Serology
For suspected cases with no isolate or where PCR does not identify serogroup,
clotted blood sample to reference laboratory (acute within 72 hrs and
convalescent 16-28 days after presenting symptoms).

Isolates and PCR samples from hospitals in England, Wales and Northern Ireland
(local protocols for PCR services may apply)
HPA Meningococcal Reference Unit
Tel: 0161 276 6757 Fax: 0161 276 5744

Isolates and PCR samples from hospitals in Scotland
Scottish Meningococcal and Pneumococcal Reference Laboratory
Tel: 0141 201 3536

Figure 1.8-4: Algorithm for early management of meningococcal disease in children (from the Meningitis Research Foundation, www.meningitis.org).

1.9 Immunity to *Neisseria meningitidis*

Defence against *N. meningitidis* depends on multiple components of both the innate and acquired immune systems, including complement proteins, dendritic cells (DCs), macrophages, neutrophils, Abs, B cells, and T cells.

Defence against *N. meningitidis* starts at the nasopharyngeal mucosa, with the epithelial barrier, which already contains several specialised cell types of the immune system, such as DCs, macrophages and plasma cells secreting IgA (Cheroutre, 2004; Pollard and Frasch, 2001). Once in the circulation, invading meningococci are neutralised by phagocytes, complement proteins, and Ab.

1.9.1 Cells of the innate immune system

DCs have two roles in controlling meningococcal infections; they eliminate meningococci by internalisation and they present neisserial Ags to T cells. DCs are found on the nasopharyngeal mucosa where they phagocytose invading microorganisms and shuttle them from the epithelium to regional lymph nodes through the upregulation of the chemotactic receptor CCR7. Once in secondary lymphoid tissues they present Ag fragments in major histocompatibility complex (MHC) molecules and induce T cell activation through the upregulation of costimulatory signals (e.g. CD80, CD86 and CD40) (Banchereau and Steinman, 1998). DCs, like other cells of the innate immune system, recognise highly conserved structures of microbes, so-called pathogen associated molecular patterns (PAMPs), through pattern recognition receptors

(Janeway and Medzhitov, 2002). Toll-like receptors (TLRs) are one of the best characterised pattern recognition receptors (Barton and Medzhitov, 2002; Takeda and Akira, 2005). Among the members of the human TLRs family, TLR4 recognises the LPS of *N. meningitidis* (Miller et al., 2005) and TLR2 the PorB OMP (Singleton et al., 2005).

Macrophages, like DCs, link innate and adaptive immunity, are associated with the nasopharyngeal epithelium, and express a variety of pattern recognition receptors. They are also found in the blood-brain barrier (Williams et al., 2001). They phagocytose microorganisms and make them accessible for recognition by the adaptive immune system by MHC-mediated presentation (Kurzai, 2006). As with DCs, the uptake of meningococci by macrophages is independent of Ab opsonisation. However, macrophages can also phagocytose bacteria opsonised by Ab (Kurzai, 2006). Once internalised, the bacteria are rapidly inactivated in phagolysosomal compartments whether or not they are encapsulated. However, presence of the polysaccharide capsule seems to delay the phagolysosomal fusion (Read et al., 1996).

Neutrophils are the major leukocyte type in peripheral blood and are the predominant type of cells found in the CSF of patients suffering from meningococcal meningitis (Kurzai, 2006). Neutrophils are recruited to the sites of infection upon the release of chemotactic factors, such as C5a (Kurzai, 2006). Once at the site of infection, they internalise *N. meningitidis*, through both opsonophagocytosis and non-opsonic phagocytosis (Mayer-Scholl et al., 2004). Phagocytosis of opsonised meningococci (by IgG or C3b) by neutrophils requires the expression of Fc receptors or complement receptors on the surface of phagocytes (van de Winkel and Capel, 1993). Non-opsonic phagocytosis by neutrophils occurs through pattern recognition receptors (e.g. TLR2, TLR4 and CD14) (Kurt-Jones et al., 2002). Members of the

carcinoembryonic Ag-related cell adhesion molecule family (CAECAM, CD66) have been characterised as host cell receptors on the neutrophil surface that interact with bacterial Opa and Opc proteins (Virji et al., 1996).

Several bacterial virulence factors may impair the function of the cells of the innate immune system; the polysaccharide capsule of *N. meningitidis* prevents recognition and phagocytosis of the bacteria by DCs (Kolb-Maurer et al., 2001; Unkmeir et al., 2002) and macrophages (Read et al., 1996). The sialylation of the LPS reduces the recognition of *N. meningitidis* by DCs (Unkmeir et al., 2002).

1.9.2 The complement system

The complement system is a system of plasma proteins that can be activated directly by pathogens (alternative pathway) or indirectly by pathogen-bound Ab (classical pathway). Alternatively, mannose binding lectin (MBL) binds to surface structures on bacteria, such as PorB and Opa of meningococci, and can activate the complement system via associated serine proteases (MASP-1 and MASP-2) (MBL pathway) (Estabrook et al., 2004; Ikeda et al., 1987; Kawasaki et al., 1978). It leads to a cascade of reactions occurring on the surface of pathogens and generating various effector functions. Complement activation induces the formation of a membrane attack complex that can directly lyse the bacteria. Deposition of complement on the cell surface (opsonisation) also induces phagocytosis by specialised immune cells.

The early events of all three pathways of complement activation consist of a cascade of

reactions that lead to the generation of a C3 convertase, which cleaves complement component C3 into C3b and C3a. C3b binds covalently to the bacterial cell membrane and opsonises the bacteria, helping phagocytes to internalise them. C3a is a peptide mediator of local inflammation. C5a and C5b are generated by the cleavage of C5 by a C5 convertase formed by C3b bound to the C3 convertase. C5a is also a mediator of inflammation. C5b triggers the late events in which the terminal components of complement assemble into a membrane-attack complex that can damage the membrane of certain pathogens (Schneider et al., 2007) (Figure 1.9-1).

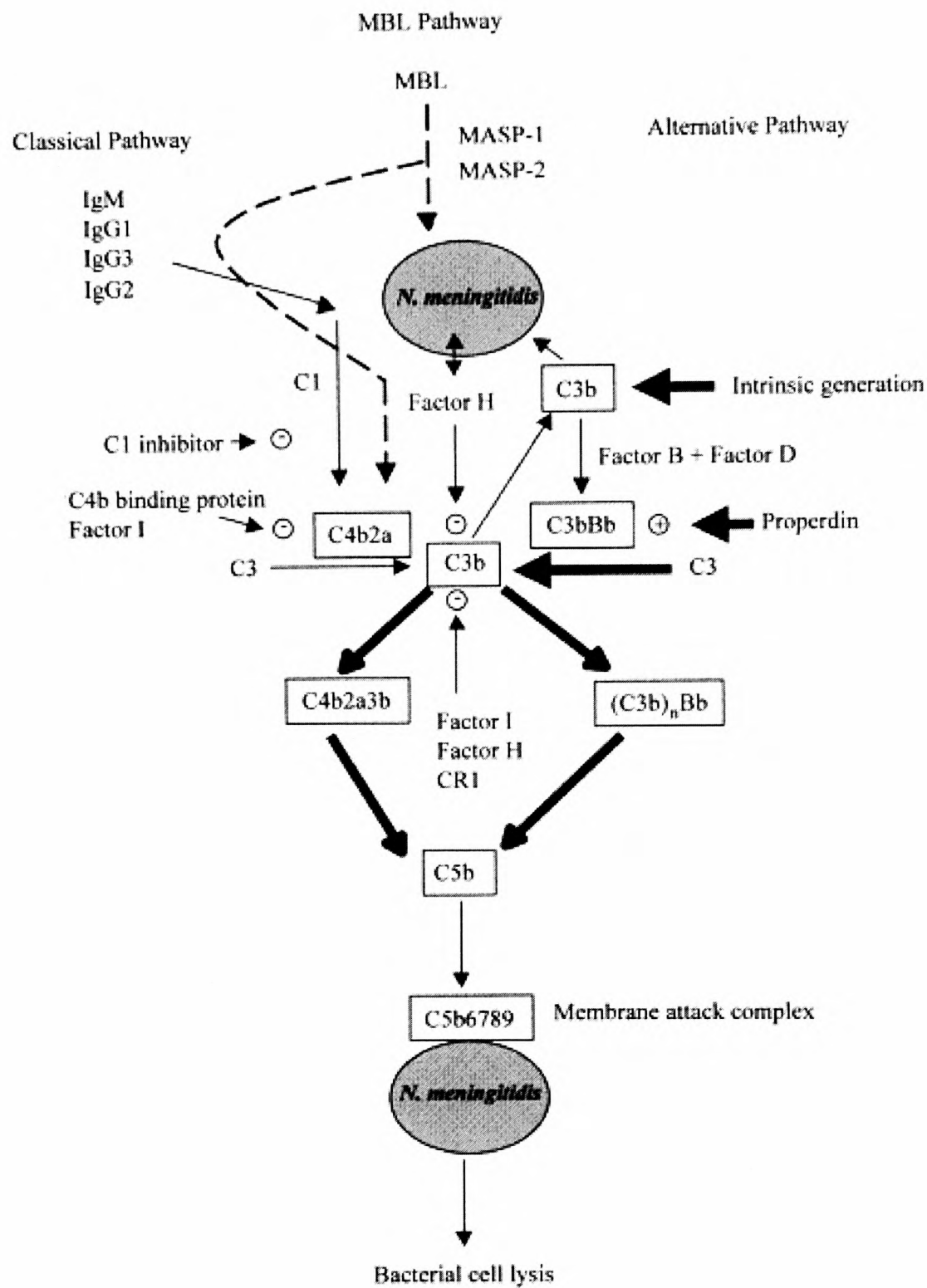


Figure 1.9-1: Components and effector actions of the complement system (Pollard and Frasch, 2001).

In the immune individual, classical pathway complement activation occurs following binding of specific Ab to OMPs and polysaccharide Ags on the surface of meningococci. In the non-immune individual the alternative complement pathway is activated following the binding of C3b to the bacterial cell surface (LPS and capsule) (Pollard and Frasch, 2001) or the binding of MBL to carbohydrate surface structures of *N. meningitidis* through the MBL pathway (Estabrook et al., 2004; Ikeda et al., 1987; Kawasaki et al., 1978). Killing is mediated by the terminal lytic complement pathway or by increased phagocytosis (Kawasaki et al., 1989; Kuhlman et al., 1989). Levels of MBL in the blood peak at 6-18 months of age (Summerfield et al., 1995; Super et al., 1989); however, there is a relative deficiency of late complement components in infancy (Lassiter et al., 1992).

Meningococci have developed mechanisms to resist the attack by complement (named serum resistance) (Hammerschmidt et al., 1994); it has been shown that strains of *N. meningitidis* that have sialic acid in their capsular polysaccharide (serogroup B, C, W, Y) were able to inhibit the complement activation. These strains have the capacity to synthesise 5'-cytidinemonophospho-N-acetylneuraminic acid (CMP-NANA), which is used as a source of sialic acid for the synthesis of the polysaccharide capsule but can also be used to sialylate the LPS (Mandrell et al., 1991). As LPS has been identified as the target for C3b binding on *Neisseria*, it is believed that the sialylation of LPS might obscure the complement target and therefore confer serum resistance (Edwards and Apicella, 2002; Horstmann, 1992; Mandrell et al., 1991).

1.9.3 Antibody (Ab)

1.9.3.1 Disease incidence and the central role of Ab

The central role of Ab in protection against meningococcal disease has been demonstrated indirectly by the inverse relationship between anti-meningococcal Ab titers or bactericidal activity of serum and the age-related incidence of meningococcal disease (Goldschneider et al., 1969a, b; Riedo et al., 1995) (Figure 1.9-2). The highest incidence of disease is from 6 months, after which maternal Abs have waned, to two years of age (Goldschneider et al., 1969a; Shepard et al., 2003; Health Protection Agency, 2008a). After this age, the incidence of meningococcal disease becomes progressively less common throughout childhood, reflecting the importance of age-dependent acquisition of Ab. There is a secondary peak of meningococcal disease during early adulthood, which is attributed to the increase in crowding and transmission in this age group (Balmer and Miller, 2002; Kvalsvig and Unsworth, 2003).

The direct correlation between susceptibility to meningococcal disease and the absence of bactericidal Ab was further demonstrated by the finding that military recruits who had detectable bactericidal Ab frequently became carriers but did not contract the disease (Goldschneider et al., 1969a; Griffiss, 1982).

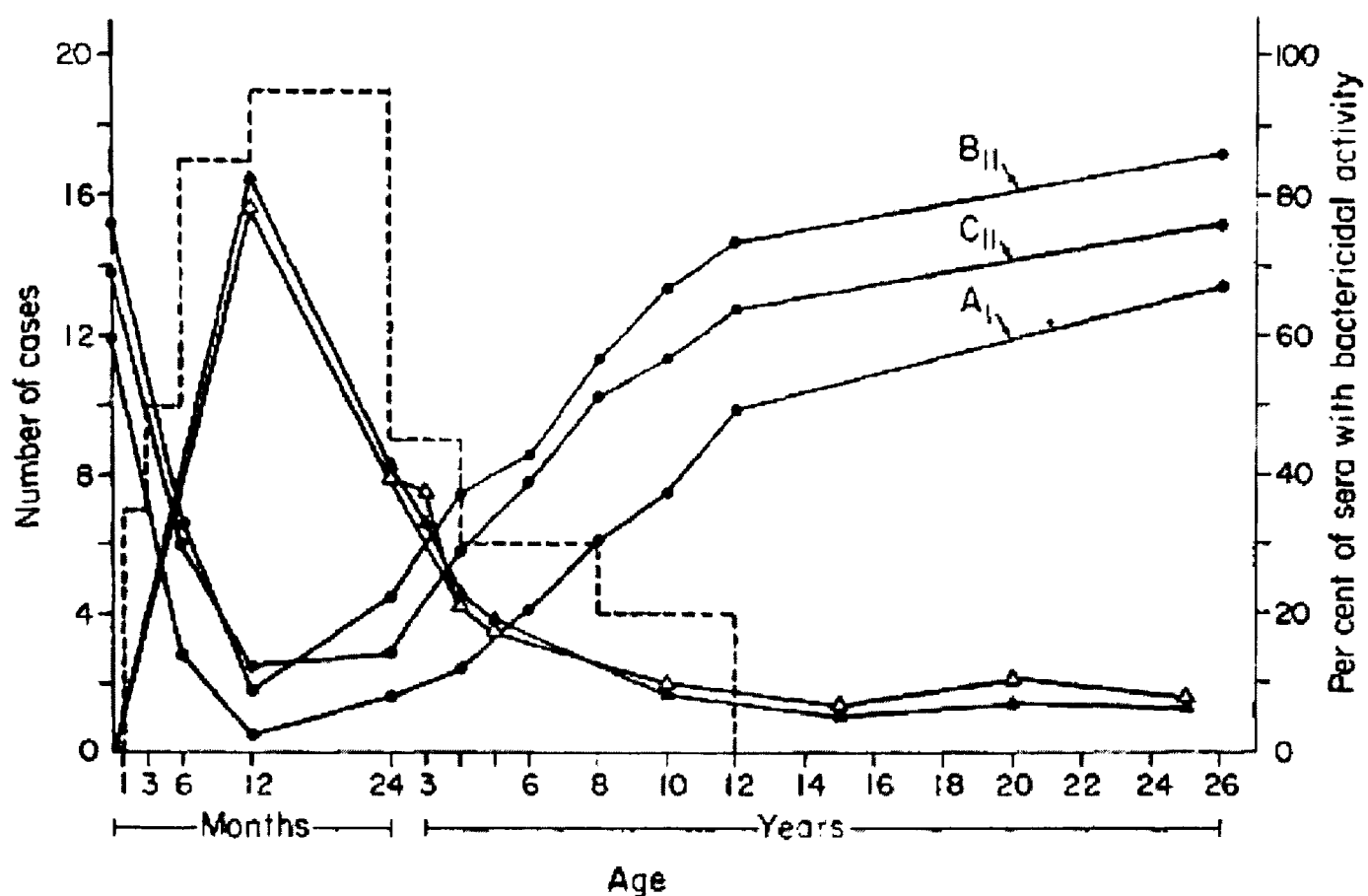


Figure 1.9-2: The inverse relation between proportion of sera with serum bactericidal titer \geq 1:4 with human complement against serogroup A, B and C *N. meningitidis* and the age-related incidence of meningococcal disease of serogroups A, B and C in the US (Goldschneider et al., 1969a).

1.9.3.2 Acquisition of anti-meningococcal Ab

During the first months of life, infants are protected from meningococcal disease by the presence of maternal Ab, which is obtained passively during gestation (Goldschneider et al., 1969a). Maternal Ab is then replaced by acquired immunity during childhood. Exposure to non-pathogenic *Neisseria*, such as *Neisseria lactamica*, and other cross-reacting species in the nasopharynx increases the level of specific Ab during childhood (Sanchez et al., 2002; Troncoso et al., 2000). It has been shown that *N. lactamica* and *N. meningitidis* share highly homologous structures (Braun et al., 2004; Troncoso et al., 2000). The absence of the capsule in *N. lactamica* indicates that the acquisition of protective Ab against *N. meningitidis* through the carriage of *N. lactamica* might be directed to the OMPs and LPS of *N. meningitidis* (Sanchez et

al., 2002), rather than to the capsule. However, several enteric bacteria have a capsule that is structurally and immunologically identical to the capsular polysaccharide of meningococci (e.g. the capsule from *E. Coli K1* is identical to the serogroup B capsule), suggesting that the carriage of cross-reacting bacteria also induce Ab directed against the capsule of *N. meningitidis* (Kasper et al., 1973). Asymptomatic carriage of non-virulent strains of *N. meningitidis* also contributes to immunity against invasive meningococcal disease, by inducing an increased concentration of IgA in saliva (Robinson et al., 2002) and of bactericidal Ab response (Jones et al., 1998). These Abs might be directed against the OMPs (particularly PorA, PorB and Opa), LPS (Jones et al., 1998), or the capsular polysaccharide. The slow acquisition of these Abs explains the vulnerability of young children.

1.9.3.3 Ab classes

Systemic infection by *N. meningitidis* induces IgG, IgA and IgM Abs (Flaegstad et al., 1990; Rosenqvist et al., 1988). In children, the IgG-Abs produced are predominantly of the IgG1 and IgG3 subclasses (Pollard et al., 1999b; Sjursen et al., 1990). IgG2 is very low in young children and reaches about 20% of adult levels at the age of 1 year, and 50% at the age of 5 years (Lee et al., 1986; Plebani et al., 1989). There is no increase in IgG4 Ab (Pollard et al., 1999b; Sjursen et al., 1990).

The activity of the different immunoglobulin isotypes in immune defence against *N. meningitidis* has been studied previously (Lowell et al., 1980b; Vidarsson et al., 2001). Abs use two principal mechanisms to neutralise the invading bacteria: opsonisation followed by phagocytosis and the activation of the classical complement pathway followed by direct killing

or opsonisation followed by phagocytosis (Pollard and Frasch, 2001). IgG use both mechanisms, although IgG1 and IgG3 are the most efficient activators of complement, but also the predominant subclasses for opsonophagocytosis, as the major phagocyte Fc γ receptor, Fc γ RI, has a high affinity for IgG1 and IgG3 (Aase and Michaelsen, 1994; Vidarsson et al., 2001). IgM is extremely potent in activating the complement (Griffiss and Bertram, 1977) but does not interact with phagocytic cells (Lowell et al., 1980b). However, it has been shown that IgA-Ab, induced by enteric exposure to other bacteria expressing cross-reacting Ags, blocks complement mediated lysis of meningococci by IgG or IgM (Griffiss, 1975; Griffiss and Bertram, 1977; Griffiss and Goroff, 1983). Other studies have demonstrated the capacity of IgA to initiate opsonophagocytosis (Lowell et al., 1980a) or to activate the alternative complement pathway (Hiemstra et al., 1987).

1.9.4 Cells of the adaptive immune system

In contrast to the innate immune system, which recognises pathogens through conserved, pathogen-associated pattern molecules, the adaptive immune response relies on complex mechanisms of gene rearrangement to allow identification of a broad spectrum of epitopes. The antigenic specificity of B cells is determined by genetic rearrangement of the variable (V) immunoglobulin genes and the specificity of T cells is determined by genetic rearrangement of the T cell receptor (TCR) genes (Kurzai, 2006).

B cells produce Ab and, therefore, the B cell recognition of *N. meningitidis* is the principal determinant of the Ab response to meningococcal infection. B cells recognise different meningococcal Ags, such as the polysaccharide capsule, which activates B cells polyclonally

(see below). Furthermore, the various OMPs in the bacterial outer membrane of *N. meningitidis* can also activate specifically individual clones of B cells (Delvig et al., 1996; van der Voort et al., 1997), although it has been observed that some OMPs could also activate B cells polyclonally (Vordermeier et al., 1987). OMP Ags can be proteolytically digested by Ag presenting cells (APC) and B cells. The derived peptides are then expressed on the surface of these cells in association with a MHCII molecule for presentation to T helper cells (Wiertz et al., 1996). The MHCII restricted cognate interaction between B cells and T cells induces the development of the germinal centre (GC) with the associated isotype class switching and somatic hypermutation of immunoglobulins and the development of long-lived plasma cells and memory B cells (MacLennan, 1994) (see section 1.10.3.2).

In contrast, the polysaccharide capsule cannot be processed and bound to the MHCII molecule for presentation to T helper cells. They are T-independent (TI) Ags. TI Ags can be subgrouped into type 1 and type 2; TI-1 Ags can trigger an Ag-independent polyclonal activation of naïve and mature B cells but TI-2 Ags cannot activate B cells by themselves (Weintraub, 2003). Meningococcal capsule is a TI-2 Ag. It is composed of multiple identical antigenic epitopes (polymers of mono- or disaccharide units) in close proximity to each other that cross-link multiple membrane immunoglobulin molecules on a B cell. This allows the activation of B cells without the help of T cells (Ada and Isaacs, 2003; Lesinski and Westerink, 2001; Mond et al., 1995a). However, because of the lack of T cell help, there is no induction of high avidity Ab and immunological memory. The ability to respond to TI Ags appears to be restricted to a limited set of B cells (see section 1.10). It was reported that co-stimulatory signals may be required for the activation of B cells by bacterial capsular polysaccharides (Snapper et al., 1997; Vos et al., 2000). These signals might be provided by the interaction of the CD19/CD21

complement receptor complex on B cells with complement component C3 attached to capsule polysaccharide (Fearon and Carroll, 2000) and through ligands for TLR, that are expressed on B cells simultaneously with the Ag-specific receptor (Iwasaki and Medzhitov, 2004) and through different cytokines secreted by T cells activated by epitopes present on the bacterial surface (Rijkers et al., 1998). It was reported that TLR2 might recognise the meningococcal protein PorB (Massari et al., 2002), and TLR9 CpG DNA motives (Vos et al., 2000). However, there is reduced expression of CD21/CR2 complement receptor on infant B cells (Griffioen et al., 1993) and human neonates have low levels of C3, which only attain adult levels after one year of life (Pedraz et al., 1980). These observations might impair B cell activation by polysaccharide Ags.

It is unclear whether the natural Ab response to the capsular polysaccharide of *N. meningitidis* is entirely TI or whether the presence of OMPs, processed in conjunction with the meningococcal polysaccharides by APCs, may convert the Ab response into a T cell dependent one (Donnelly et al., 1990).

1.10 Cellular basis of humoral immunity

Diverse subsets of B cells respond to T-dependent (TD) and TI Ags. Mature B cells can be divided into follicular (FO), marginal zone (MZ), and B1 cells (Martin and Kearney, 2001). The FO B cells and MZ B cells are referred to as B2 cells. The B1 and MZ B cells participate in the earliest Ab response to TI Ag (Alugupalli et al., 2004; Martin and Kearney, 2001). FO B cells participate in TD responses.

The peripheral blood B cell pool in humans is composed of cells that express CD27 and possess somatically mutated immunoglobulin V-regions (memory B cells), and IgM+IgD+CD27- cells that express unmutated V genes. CD27 is the cell surface Ag marker for memory and almost all CD27+ B cells possess somatically mutated immunoglobulin V-region genes (Agematsu et al., 2000; Klein et al., 1998; Tangye et al., 1998). Somatic mutated immunoglobulin V-region genes are a defining property of memory B cells resulting from somatic hypermutation in the germinal centre (GC) (section 1.10.3.2) and result in Ab of high affinity against the original triggering Ag. Only CD27+ B cells undergo immunoglobulin gene rearrangement with consequent class switching to IgG, IgA or IgE isotypes. The CD27- B cells are further distinguished into CD5+ and CD5- cells. The CD5+ B cells do not participate in TD immune responses and represent the B1 cells. The CD5- cells represent the precursors of GC B cells in TD immune responses and are termed the naïve B cells (Klein et al., 1998) (Figure 1.10-1). In adults naïve B cells constitute around 50% of the circulating B cell population (Klein et al., 1998). This proportion is higher in children (Agematsu et al., 1997).

The CD27+ B cells constitute an increasing percentage of the peripheral blood B cells with age

(around 5% in cord blood increasing to 20-40% in adults) (Agematsu et al., 1997). Human memory B cells can be subdivided into 40% class switched, 20% IgM only, and 40% IgM+IgD+CD27+ cells (Klein et al., 1998) (Figure 1.10-1).

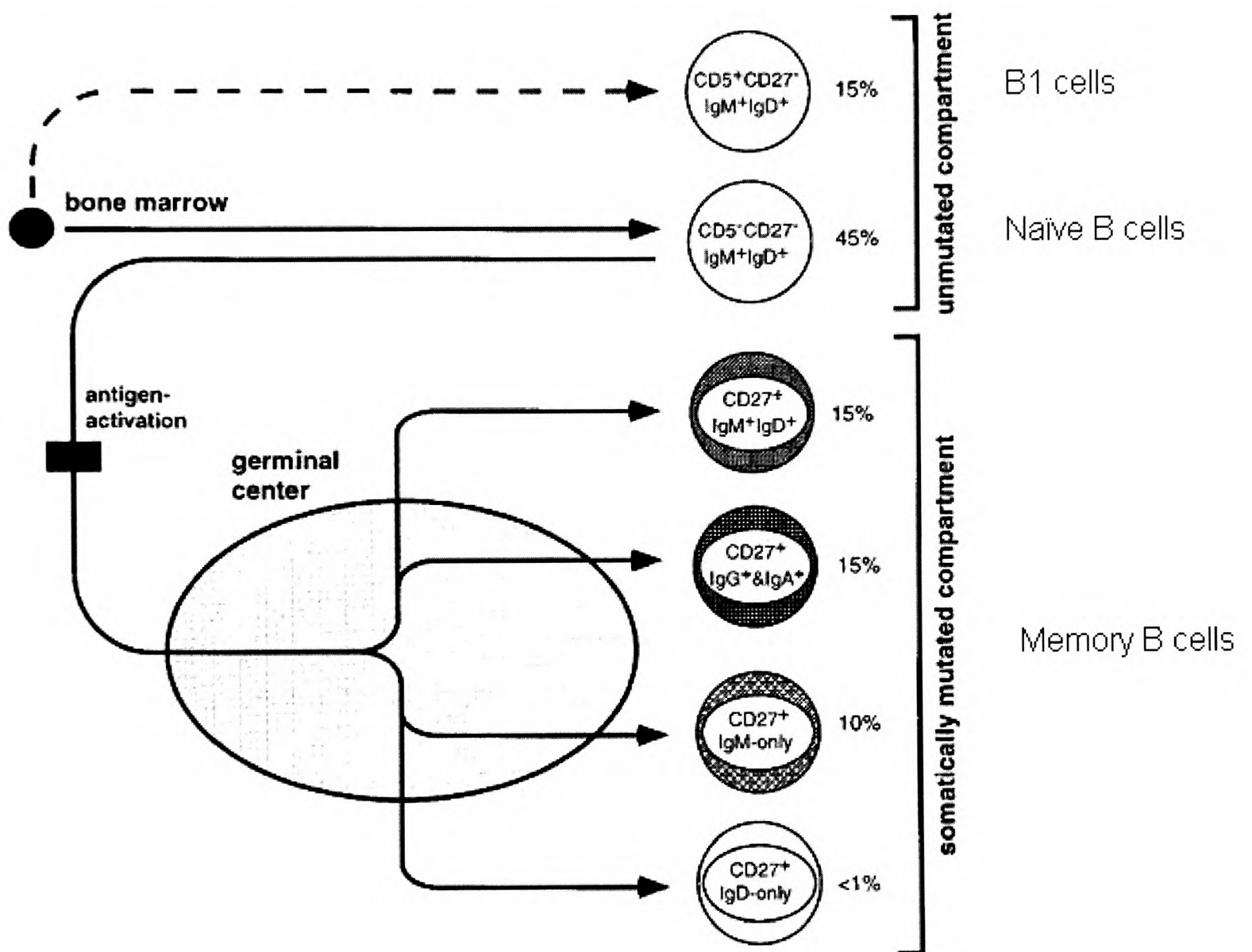


Figure 1.10-1: The peripheral blood B cell compartment in humans (Klein et al., 1998).

1.10.1 B1 B cells

B1 cells are a CD5⁺ subpopulation of B cells, which express IgM and are restricted to the peritoneal and pleural cavities in mice (Martin and Kearney, 2001). B1 B cells produce natural Abs (Martin and Kearney, 2001), a component of innate immunity. They are made in response to TI-Ags and, apparently, their production does not require exposure to Ags. Most natural Abs are low affinity anti-carbohydrate Abs that are produced by MZ B cells or B1 cells (Merino and Gruppi, 2006). Two mechanisms are thought to drive B1 lymphocytes to differentiate into plasma cells: stimulation by microbial Ags or mitogens of the gut flora and stimulation by autoantigens (Merino and Gruppi, 2006). Plasma cells of the B1 lineage produce Abs that bind with low affinities to multiple Ags, often microbial structures shared by a variety of pathogens (Boes, 2000). The continuous activation of B1 cells by bacterial Ags, mitogens and autoantigens implies that plasma cells of the B1 lineage should be short-lived and constantly replaced by newly formed plasma cells (Manz et al., 2005).

These cells constitute a major proportion of the B cell population of children but only a minor proportion in adults (Baumgarth et al., 2005). B1 cells develop earlier than B2 cells, already present during foetal and neonatal life, whereas B2 cells develop from late neonatal life onwards (Baumgarth et al., 2005).

B1 cells are maintained throughout life (Boes, 2000) and are responsible for producing short-term, low-affinity Ab responses that provide a first line of defence against pathogen invasion, while B2 cells respond more slowly but produce much more efficient (high affinity) Ab and

generate memory B cells that provide lasting protection against invasion (Baumgarth et al., 2005).

1.10.2 Marginal zone (MZ) B cells

MZ B cells are a distinct subset of B cells that respond mainly to TI Ags, such as the polysaccharide capsule of bacterial pathogens, and produce IgM. Their phenotype is $IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo}CD1^{hi}$ (Spencer et al., 1998), they express CD27+ and have somatically mutated V regions. The high expression of CD21 helps them to attach to complement coated polysaccharides (Oliver et al., 1997). MZ B cells are localised in the MZ of the spleen, which is a splenic structure between the T-cell rich peri-arteriolar lymphoid sheath (PALS) and the red pulp (Martin and Kearney, 2002) (Figure 1.10-2). This structure is composed of MZ B cells, specialised macrophages and reticular cells. The macrophages located in the MZ of the spleen are particularly efficient at trapping polysaccharides, which persist for prolonged periods on their surface and are recognized by MZ B cells (Martin and Kearney, 2002; Pillai et al., 2005). The location of MZ B cells permits them to respond rapidly to blood-borne pathogens filtered through the spleen (Pillai et al., 2005). MZ B cells are rapidly activated upon Ag encounter, producing plasma cells in 1-3 days (Oliver et al., 1999). These characteristics make MZ B cells important cells for the defence against invasive bacteria expressing TI Ags (Martin and Kearney, 2001).

However, it has been reported that MZ B cells requires the spleen for their generation and/or survival. (Kruetzmann et al., 2003). For this reason, loss of spleen, splenic hypofunction or congenital asplenia results in higher rates of disease caused by encapsulated bacteria (Mond et

al., 1995b). Infants under the age of two years are also more susceptible to encapsulated bacterial infections. This is because before this age, children have an immature splenic MZ structure that is unable to support MZ B cells (Kruschinski et al., 2004; Martin and Kearney, 2002). In infants younger than two years, the structure of the MZ is disorganised, MZ macrophages are immature and infant MZ B cells are characterised by the absence of CD21 expression (Timens et al., 1989). Therefore, although MZ B cells may be present at low frequency in early infancy, they are still incapable to mount TI responses.

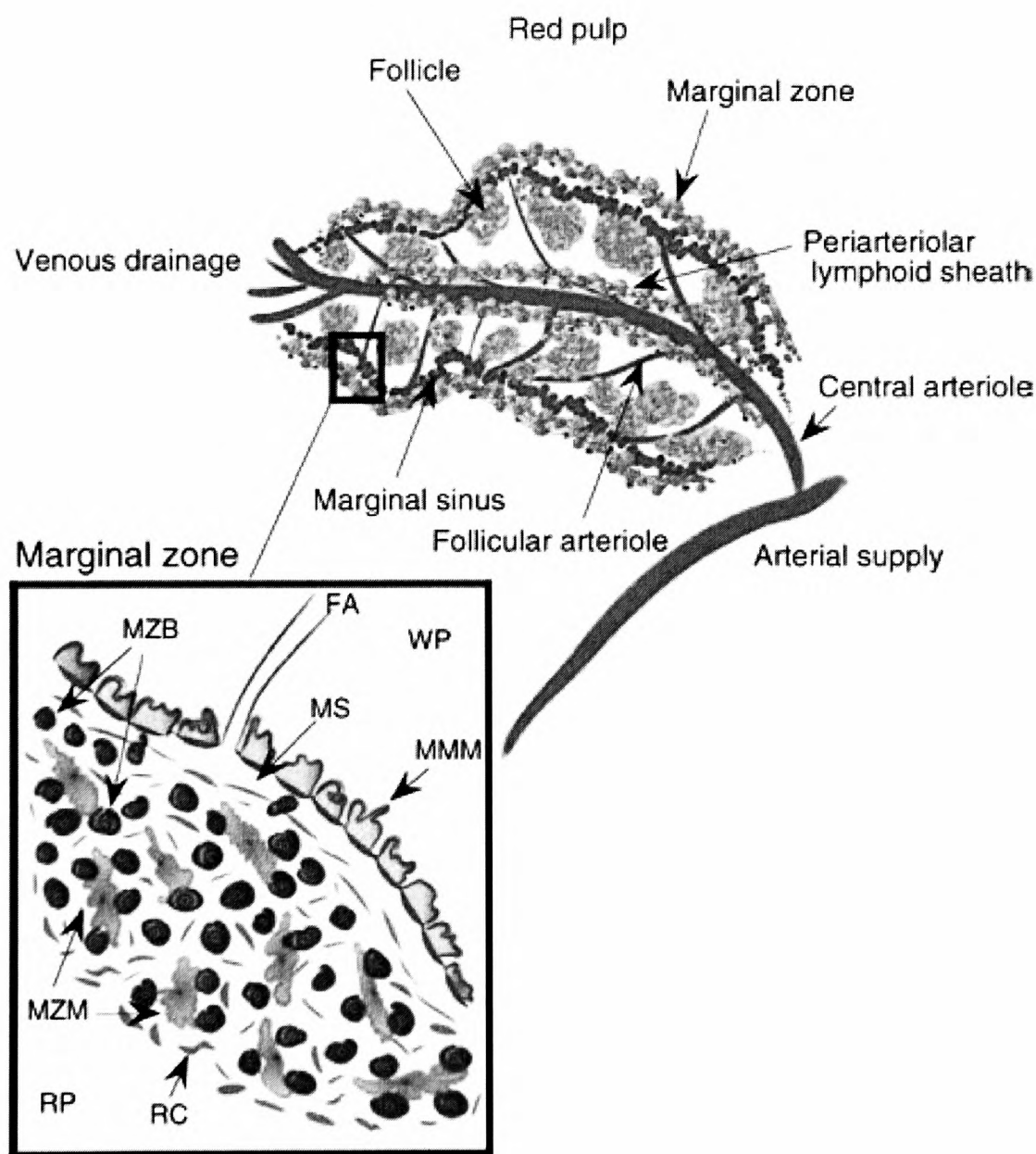


Figure 1.10-2: Morphology of the spleen. Central arterioles are surrounded by the periarteriolar lymphoid sheath (or T cell zone) and by the B cell follicles. These arterioles branch into follicular arterioles (FA), which feed into the marginal sinus (MS). The marginal zone lies between the sinus and the red pulp (RP) of the spleen. RC = reticular cell, MMM = marginal metallophilic macrophage, MZB= marginal zone B cell, MZM = marginal zone macrophage, WP = white pulp (Pillai et al., 2005).

In contrast to murine MZ B cells, human MZ B cells recirculate, are found in many anatomical sites other than the spleen, and appear to be identical to somatically mutated, circulating IgM+ memory B cells (Tierens et al., 1999; Weller et al., 2004). The somatic mutations observed in both cells seem to be similar and extrafollicular (Weller et al., 2004; Weller et al., 2001). Along with this hypothesis, patients with X-linked hyper-IgM syndrome, who lack functional CD40 or CD40 L protein and, therefore, cannot develop GCs with the associated isotype class switching of immunoglobulin, somatic hypermutation, affinity maturation and production of memory B cells, still have IgM+IgD+CD27+ B cells with a mutated immunoglobulin receptor (Weller et al., 2004; Weller et al., 2001). Additionally, the mutations are already present in very young infants, suggesting that this phenomenon does not require exposure to the Ag (Weller et al., 2004). It has been postulated, therefore, that human MZ B cells appear first as naïve non-mutated B cells, but then rapidly diversify by somatic hypermutation, either in response to environmental Ags or by a spontaneous developmental process (Pillai et al., 2005; Weller et al., 2005a). These cells, then carrying somatic mutations, continue to reside in the MZ or in other secondary lymphoid organs (Weller et al., 2004).

The role of MZ B cells in TD immune responses is unclear. It has been proposed by Weller et al. (2005) that, before two years of age, the immune response to polysaccharide conjugate vaccines is GC-dependent and induces the production of memory B cells and affinity maturation (Weller et al., 2005b). However, they suggested that beyond two years of age, because the splenic MZ is mature, immune response to plain polysaccharide or polysaccharide conjugate vaccines would only be of the MZ type and extra-germinal because the MZ sequesters the polysaccharide (Weller et al., 2005b). A study by Kelly et al. (2006), comparing

the immune response to the serogroup C plain polysaccharide vaccine with the serogroup C meningococcal conjugate vaccine (MenCV), observed that only the latter was able to induce a persistent production of memory B cells (Kelly et al., 2006).

1.10.3 Follicular (FO) B cells

1.10.3.1 Naïve B cells

Naïve B cells (CD5-CD27-IgM+IgD+) participate in TD immune responses. These cells die in a short period of time in the absence of B cell receptor (BCR) activation (Lam et al., 1997). Naïve B cells are activated through BCR binding by Ag and MHCII-peptide restricted cognate interaction with T cells. In addition, a third signal is required which may be either from cytokines produced by T cells, macrophages or DCs or signalling by Ag through TLRs, that are expressed on activated B cells simultaneously with Ag-specific receptors (Bernasconi et al., 2003; Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2005; Ruprecht and Lanzavecchia, 2006).

Upon Ag encounter, naïve B cells differentiate first in extrafollicular foci into plasma cells that produce Ab of low affinity and of IgM isotypes (Jacob et al., 1991a). These cells seem to be short-lived with a half life of 1-10 days (Nossal and Makela, 1962; Ochsenbein et al., 2000; Schooley, 1961). Naïve B cells can also migrate to the follicular area of secondary lymphoid organs to initiate a GC reaction. In the course of the GC reaction, somatically mutated class-switched, IgM-only, IgM+IgD+, IgD-only cells, all of which express CD27, are generated

(Klein et al., 1998), and are known as FO B cells. After selection, they leave the GC as memory B cells and mediate a secondary immune response upon re-challenge.

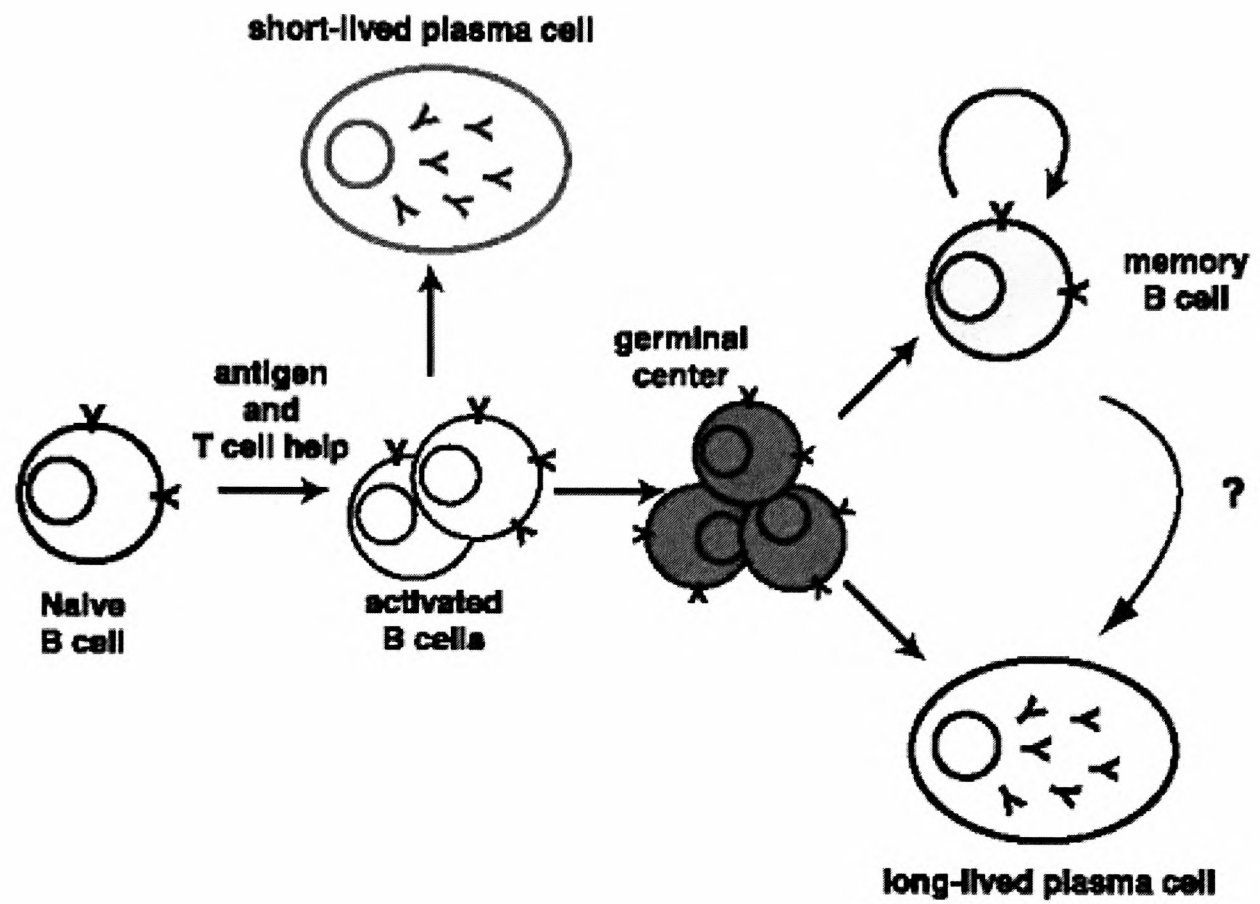


Figure 1.10-3: Activation of naïve B cells and memory B cell and plasma cell differentiation (Crotty and Ahmed, 2004).

During a TD immune response, the activation of B cells progresses in a cascade of cellular events that include 5 phases (McHeyzer-Williams and McHeyzer-Williams, 2005) (Figure 1.10-4).

- Phase I: The presentation of the Ag by MHCII complexes on activated APCs is critical for the selection and clonal expansion of the effector T helper cells.
- Phase II: The interactions between cognate effector T helper cells and naïve B cells promote the development of either short-lived plasma cells or GCs.
- Phase III: These GCs expand, diversify, and select high-affinity Ag-specific B cells for entry into the long-lived memory B cell compartment.
- Phase IV: Upon Ag re-challenge, memory B cells rapidly proliferate and differentiate into plasma cells under the cognate control of memory T helper cells.

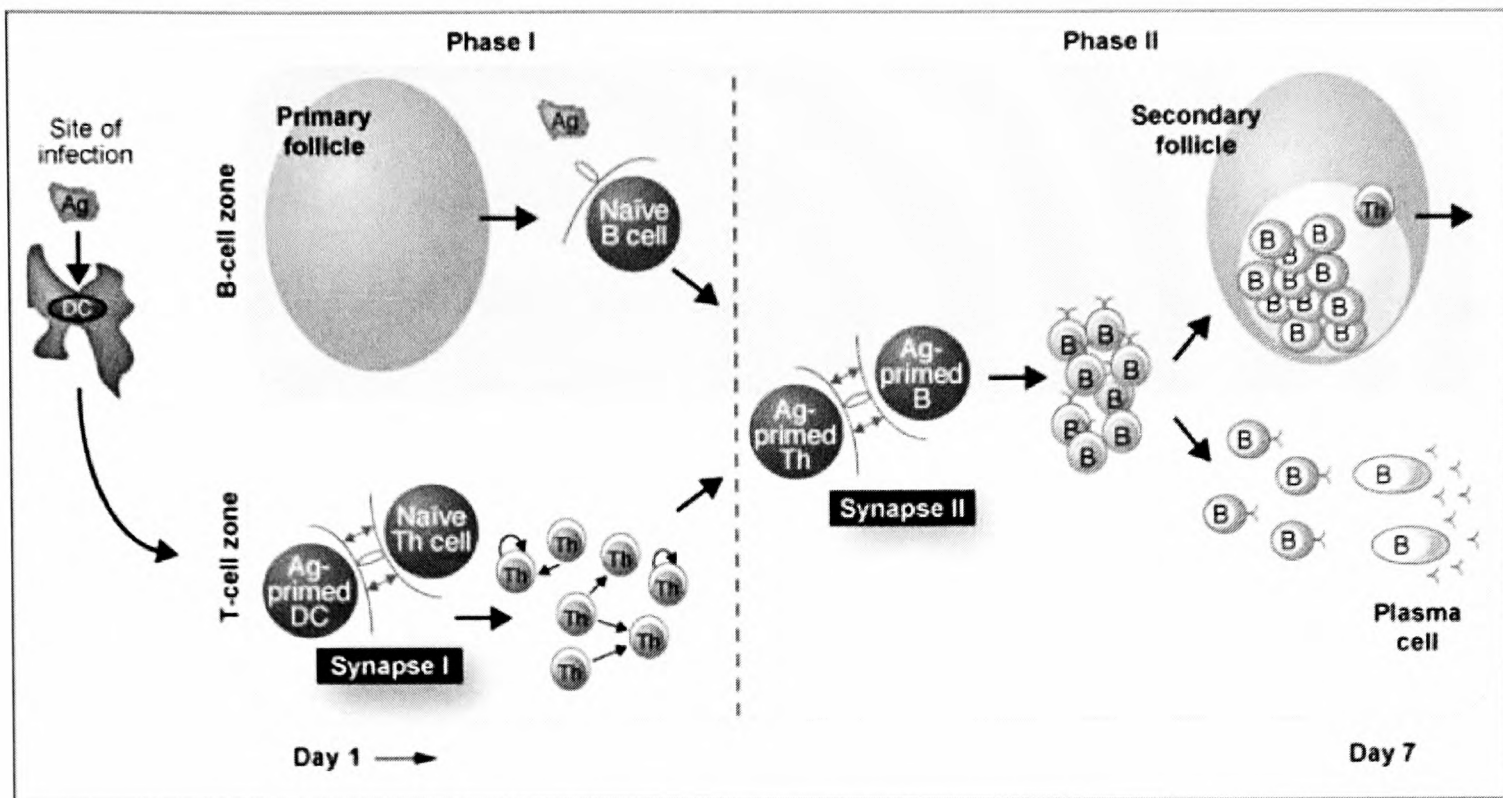
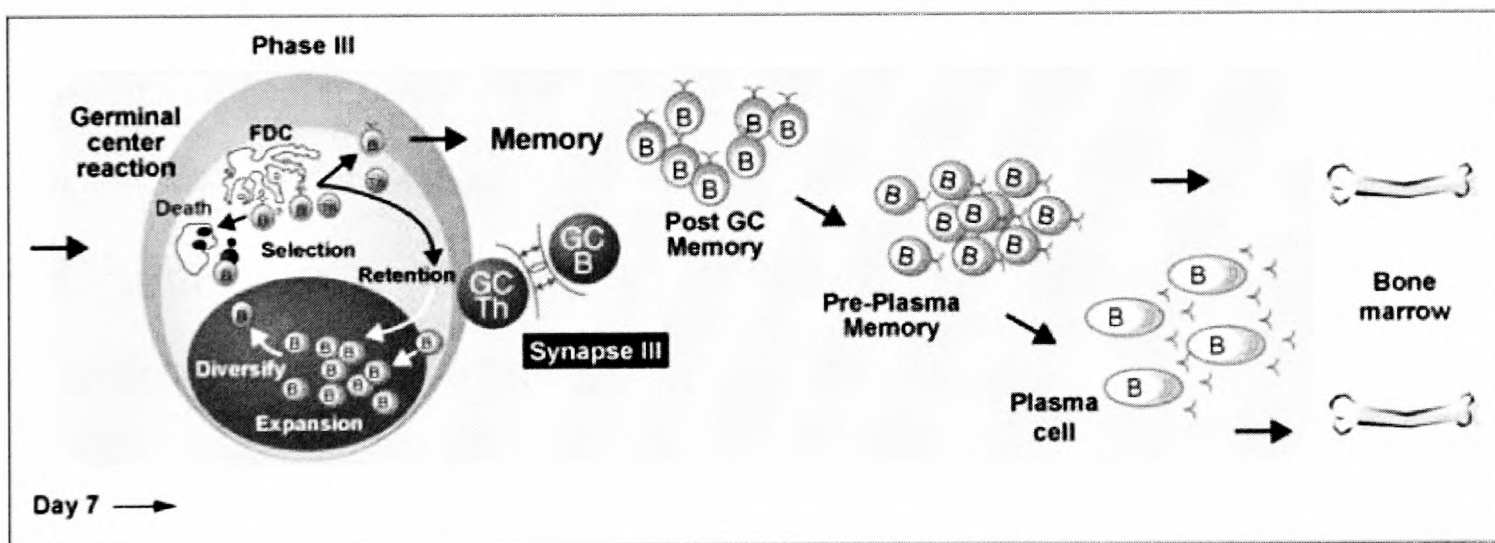
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Figure 1.10-4: Activation of B cell in a TD immune response. (A) Cellular interactions leading to the activation of effector T helper cells (phase I) and effector B cells (phase II) in secondary lymphoid organs. (B) Cellular activity in the GC reaction that leads to the development of Ag-specific memory B cells (phase III), which can then differentiate into long-lived plasma cells, which migrate in the bone marrow (McHeyzer-Williams and McHeyzer-Williams, 2005).

1.10.3.2 Germinal centre (GC)

The formation of a GC requires the presence in secondary lymphoid organs of follicular DCs, Ag-specific B cells and T cells. Chemokines appear to be central for GC development and B cell trafficking within the GC; CXCL12 and its receptor CXCR4, and CXCL13 and its receptor CXCR5 are responsible in humans for the follicular organisation and homing of B and T cells (Allen et al., 2004). Molecules of the TNF family and B-cell activating factor (BAFF) might also play key roles in the induction and maintenance of GCs (Vora et al., 2003; Wolniak et al., 2004). The compartmentalisation of the different cells within the GC might also depend on chemokines (Cozine et al., 2005). Studies of human tonsils have observed that the apical region of the GC “light zone” is populated with follicular DCs (FDC), centrocytes and some T helper cells, and that the basal region “dark zone” is formed of centroblasts (see Figure 1.10-5). It has been suggested that B cells proliferate and hypermutate in the “dark zone” and that selection and differentiation occurs in the light zone (Liu et al., 1992).

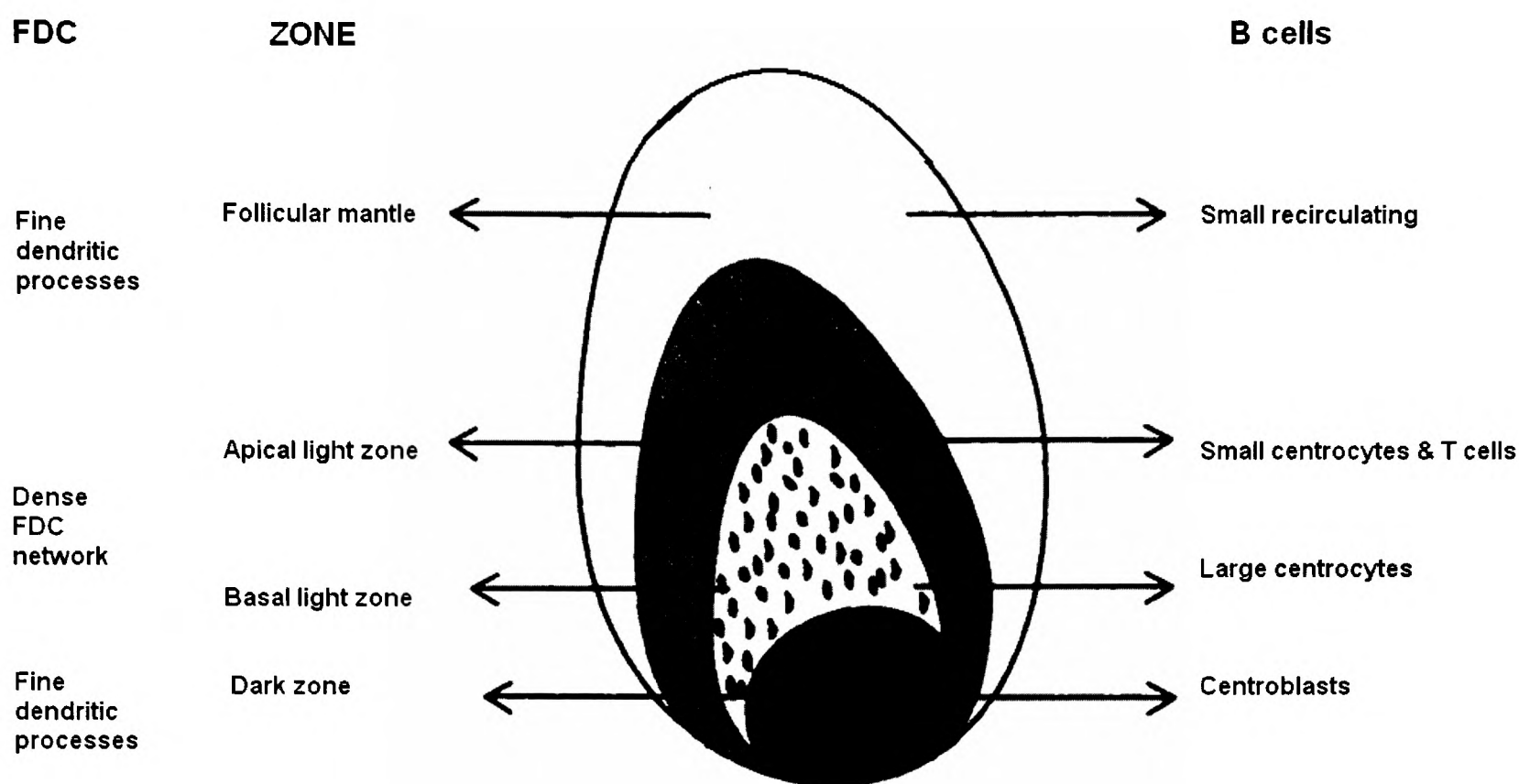


Figure 1.10-5: Schematic presentation of a GC as seen in human tonsils (Thorbecke et al., 1994).

In the “dark zone” of the GC B cells proliferate, mutate their immunoglobulin V region by somatic hypermutation, and thus generate a diversity of clones that contain cells with differing affinities for the Ag (depending on the rearranged immunoglobulin variable (V) region genes) (Berek et al., 1991; Jacob et al., 1991b). During the phase of clonal expansion, the BCR might be downregulated (Liu et al., 1992). The B cells then move to the “light zone” where they encounter Ag presented on FDCs (Liu et al., 1992). Upon some unidentified stimulus from FDC, the B cells start to expose their immunoglobulin on their surface and in this stage are referred to as centrocytes. The B cells that bind Ag with the highest affinity avoid apoptosis and survive (Smith et al., 1997) to re-enter the “dark zone” and begin the cycle again, or they receive differentiation signals to become plasma cells (through CD23 and IL1 α) or memory B cells (through interaction with T helper cells and CD40 ligands) (Arpin et al., 1995; Liu et al., 1992; Smith et al., 1997).

In early life, studies in mice have demonstrated a limited induction of the GC, associated with a slow maturation of FDC networks (Pihlgren et al., 2003), and the B cells are unable to induce T cell activation (Marshall-Clarke et al., 2000). Furthermore, macrophages and DCs are rare in the spleen and do not acquire Ag processing and presentation capacity until later in life (Clerici et al., 1993).

1.10.3.3 Plasma cells

Plasma cells are terminally differentiated non-dividing cells, which constitutively secrete Ab (Ahmed and Gray, 1996). Plasma cells downregulate expression of surface immunoglobulin, MHCII and CD20, and upregulate the expression of CD38 (Bernasconi et al., 2002). Conventional models postulate that plasma cells are short-lived (lifespan of several days to weeks) (Nossal and Makela, 1962; Schooley, 1961) and are continuously generated de novo from memory B cells (Crotty and Ahmed, 2004). Studies in mice demonstrated, however, that a fraction of plasma cells are long-lived and can secrete Ab for extended periods of time in the absence of memory B cells, with a lifespan from 3 months to more than a year (Manz et al., 1997; Slifka et al., 1998; Slifka et al., 1995). Their survival appears to depend on signals provided by stromal cells in a limited number of niches situated in the bone marrow, and it is believed that these signals protect plasma cells from apoptosis (Cassese et al., 2003; DiLillo et al., 2008; Fairfax et al., 2008). The number of these survival niches seems to be limited because the frequency of plasma cells in bone marrow is constant throughout life and in different species (Brieva et al., 1991; DiLillo et al., 2008; Fairfax et al., 2008; Manz et al., 2005). However, the existence of long-lived plasma cells in humans remains controversial, although

recent studies in humans depleted of CD20+ provide support for their existence in humans (Cambridge et al., 2003; DiLillo et al., 2008; Edwards et al., 2004).

At a steady state, plasma cells of particular Ag specificity are not detectable in peripheral blood. However, following immunisation with a variety of vaccine Ags, plasma cells appeared rapidly in blood; in primed individuals plasma cells are first seen by day 4 with a peak by day 6/7, and are not detectable anymore in peripheral blood by day 12 (Clutterbuck et al., 2006; Kelly et al., 2006; Traggiai et al., 2003). The transient appearance of the plasma cells in peripheral blood following immunisation is thought to represent their translocation from secondary lymphoid tissue, where they are formed, to the bone marrow via the blood stream (Manz et al., 2005). It is also possible that these cells appearing at day 6 post-immunisation come from long-lived plasma cells residing within the bone marrow. These may then be subsequently activated to escape the bone marrow by an unknown signal after new exposure to the Ag. Long-term plasma cell survival in bone marrow niches is regulated by signals produced by bone marrow stromal cells, which express adhesion molecules including CXCL-12, V-CAM-1 and BAFF and secrete different cytokines (Shapiro-Shelef and Calame, 2005). However, some intrinsic properties of plasma cells might determine their survival in bone marrow niches, and perhaps newly formed plasma cells are superior to older plasma cells in their capacity to respond to chemokines secreted by DCs in bone marrow niches.

Traggiai et al. (Traggiai et al., 2003), assessing the plasma cell response to a booster dose of tetanus toxoid vaccine in adult volunteers, observed that total IgG- plasma cells increased with the same kinetics as tetanus toxoid-specific plasma cells. They also observed that plasma cells specific to Ags, unrelated to the vaccine but to which the donor was immune, also increased

after tetanus toxoid vaccine. However, the frequency of these plasma cells specific to Ags, unrelated to the vaccine, were seen at a frequency several orders of magnitude lower than the tetanus toxoid-specific plasma cells. Traggiai et al. (Traggiai et al., 2003) explained their observations through the polyclonal activation of memory B cells (see section 1.10.3.4), i.e. at a steady state, memory B cells of all specificity respond to environmental Ags and continuously differentiate into plasma cells maintaining a constant level of plasma cells throughout life. Another possible reason for the appearance of plasma cells of Ag specificity unrelated to the vaccine, following immunisation, is the competition for bone marrow survival niches between newly generated plasma cells and old plasma cells. These cells could be long-lived plasma cells of the bone marrow, dislocated from their niches by newly formed Ag-specific plasma cells (Manz et al., 2005). There must be a process of regulation of the number of spaces available for plasma cells to prevent an excessive number of cells filling the bone marrow. It has been observed that plasma cell numbers in adult bone marrow are relatively constant and remain around 1-2% of bone marrow cells (0.2-0.4% of bone marrow mononucleated cells) (Brieva et al., 1991; Terstappen et al., 1990). Similarly, a study of adults immunised with tetanus toxoid showed a significant increase in tetanus toxoid-specific plasma cells following immunisation, with a phenotype characteristic of plasmablasts (CD27^{hi}, CD138⁺, HLA-DR^{hi}, CD38^{hi}, CXCR4^{lo}). However, at the same time, they observed an increase in plasma cells of specificity unrelated to the vaccine and with a more mature phenotype (CD27^{hi}, CD138⁺, HLA-DR^{lo}, CD38⁺, CXCR4^{hi}) (Odendahl et al., 2005). CXCR4 is a ligand for CXCL12 (Kunkel and Butcher, 2003), which is expressed by bone marrow stromal cells. In another study, no significant variation in plasma cells specific to Ags unrelated to the vaccine was observed following immunisation (Clutterbuck et al., 2006).

However, studies in murine models suggest that bone marrow homing of long-lived plasma cells is lower in early life (Pihlgren et al., 2001). It has been observed that plasma cells are produced in early life and exit the lymph node to enter the bone marrow, but that, once in the bone marrow, they die rapidly because of limited functionality of the bone marrow survival niches (Pihlgren et al., 2006; Pihlgren et al., 2001). This is a possible explanation for the low persistence of Ab in infants following immunisation.

1.10.3.4 Memory B cells

Memory B cells are FO B cells generated through the GC reaction following TD Ag exposure. They are characterised by having somatically mutated immunoglobulin genes and the CD27 marker on their surface (Agematsu et al., 2000; Klein et al., 1998; Tangye et al., 1998). In the absence of Ag, memory B cells reside in secondary lymphoid organs, such as the lymph nodes and spleen (Maruyama et al., 2000; Rajewsky, 1996) or in the bone marrow (Osmond, 1986), and mediate secondary immune responses upon re-challenge.

It is also believed that memory B cells continuously re-circulate between secondary lymphoid organs through the blood for years after immunisation, allowing them to encounter and react with Ags at these sites (Nanan et al., 2001; Salmi and Jalkanen, 1997). It has, therefore, been suggested that memory B cells found in the circulation should be representative of the entire B cell pool (Nanan et al., 2001). However, in several studies of previously primed subjects (Clutterbuck et al., 2008; Clutterbuck et al., 2006; Kelly et al., 2006), the frequency of memory B cells specific to the vaccine Ag increased consistently and rapidly following immunisation. If memory B cell frequency prior to immunisation is representative of the size of the entire pool of

these cells, the increase in their frequency following immunisation should account for newly generated memory B cells or rapid proliferation of previously formed memory B cells. Alternatively, the memory B cells, which appear in the first week following immunisation in primed subjects, might be re-circulating memory B cells from previous priming (Vitetta et al., 1991). The newly formed memory B cells might appear later after newly stimulated naïve B cells have proliferated in GCs and differentiated into memory B cells.

In a study by Kelly et al. (Kelly et al., 2006), involving teenagers primed with MenCV and then vaccinated with either the plain polysaccharide vaccine or an other dose of MenCV, it was found that only the conjugate vaccine was able to induce a significant increase of the frequency of serogroup C meningococcal polysaccharide (MenC) specific-memory B cells after immunisation, and these cells appeared in the blood 6-7 days after the immunisation. According to these observations, it appears that MenCV but not plain polysaccharide vaccine induces the appearance of memory B cells in the blood of primed subjects rapidly after immunisation. It can, therefore, be tentatively concluded that the conjugate vaccine might induce a GC reaction and that the memory B cells that appear rapidly after immunisation are newly formed. However, it could also be that conjugate vaccines induce the proliferation of previously formed memory B cells and their escape from lymph nodes, induced by an unknown signal, after new exposure to the Ag. Human studies have shown that memory B cells divide more rapidly than naïve B cells (Macallan et al., 2005; Tangye et al., 2003).

Crotty et al. (Crotty et al., 2003a) have described the kinetics of memory B cell responses after smallpox vaccination as follows: specific memory B cells initially decline post-immunisation,

but then reach a plateau ~10-fold lower than the peak and are maintained for >50years after vaccination at a frequency of ~0.1% of total circulating IgG+ B cells (Figure 1.10-6). Other studies (Clutterbuck et al., 2006; Kelly et al., 2006; Nanan et al., 2001) have observed that the frequency of specific IgG-memory B cells after a booster immunisation increases from day 0 to 12 and then gradually declines.

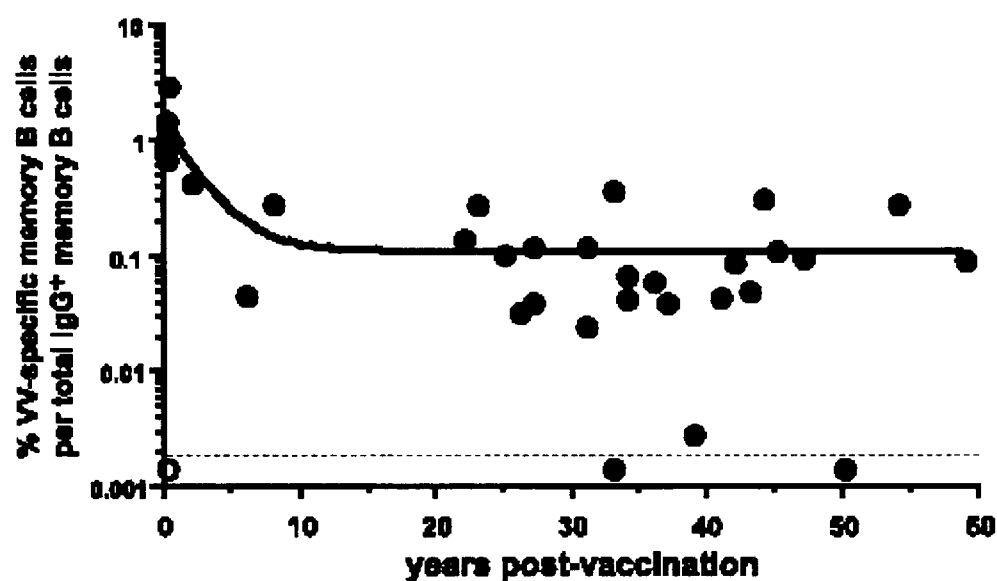


Figure 1.10-6: Longevity of smallpox vaccine-specific B cell memory according to Crotty et al. (Crotty et al., 2003a).

These observations suggest that, following immunisation, memory B cells migrate out of the circulation and home to secondary lymphoid organs, ready to mediate secondary immune responses upon rechallenge (Maruyama et al., 2000; Nanan et al., 2001; Osmond, 1986). The time over which memory B cells disappear from the peripheral blood varies with regard to the Ag under investigation and the age of the subjects (Crotty et al., 2003a; Kelly et al., 2006; Nanan et al., 2001). It appears that memory cells are kept in circulation for a short period following Ag exposure, perhaps for the function of protecting against endemic disease (Crotty et al., 2003a) or by cellular competition in secondary lymphoid organs (Crotty et al., 2003a).

It has been suggested that there are two main categories of mechanisms involved in persisting activation of memory B cells: Ag-dependent and Ag-independent. In the Ag-dependent model, the Ag can persist on FDCs, or can be constantly generated from a proliferating pathogen or periodic re-exposure to a pathogen (Crotty and Ahmed, 2004). In the Ag-independent model, memory B cells proliferate and differentiate into plasma cells *in vitro* in response to polyclonal stimuli of two types (Bernasconi et al., 2002). One type of stimulus is derived from microbial products, such as LPS or unmethylated single-stranded DNA motifs (CpG oligonucleotides), which stimulate B cells via TLR4 and TLR9 (Krieg, 2002; Poltorak et al., 1998). The other type of stimulus is T cell activation by unrelated Ags, which stimulate B cells through the cytokines secreted and CD40 ligand (Banchereau et al., 1994; Lanzavecchia, 1983). This is referred to as bystander T cell help (Bernasconi et al., 2002). Therefore, during an Ag-specific response, the increased availability of activated T cells results in increased production of all plasma cells (Bernasconi et al., 2002). However, in several studies there were no observed changes in the frequency of plasma cells or memory B cells specific to recall Ags following immunisation with a specific-Ag (Clutterbuck et al., 2006; Nanan et al., 2001).

1.10.3.5 Ab

Traggiai et al. (Traggiai et al., 2003) have observed that, following immunisation in primed individuals, serum IgG increases from days 5 to 8, and reaches a plateau level that persists for 1 month before declining with a half life of 40 days, and reaches pre-boost levels after 250 days. It has been suggested that there is “short-term serological memory” which is Ag-dependent and lasts for a few months, and “long-term serological memory” resulting from Ag-independent

polyclonal activation of memory B cells (Bernasconi et al., 2002). The kinetics of the Ab response has been explained in accordance with this hypothesis (Figure 1.10-7); following Ag exposure, memory B cells proliferate and differentiate massively to short-lived plasma cells, but this response is transient because of the negative feedback induced by the high level of Abs generated. Some plasma cells generated in this way might become long-lived, survive in bone marrow niches and continue to produce Abs for a few months. In the long-term, all memory B cells respond to environmental Ags and continuously proliferate and differentiate into plasma cells, maintaining a constant level of serum Abs and plasma cells throughout life.

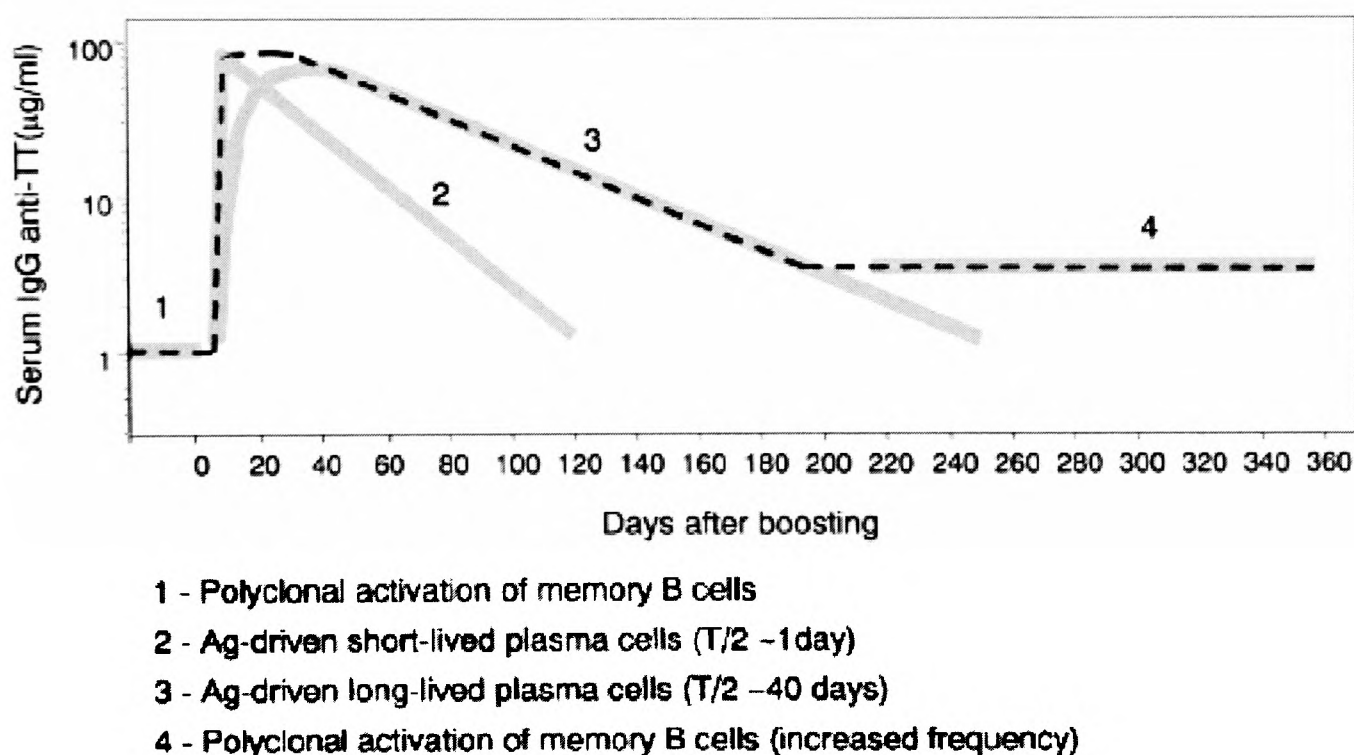


Figure 1.10-7: Mechanisms that sustain serum Ab levels following boosting, according to Traggiai et al. (Traggiai et al., 2003).

Immunological memory is the ability of the immune system to respond more rapidly with high avidity switched Abs, to a secondary exposure with the Ag. Primary responses result from the activation of naïve B cells, whereas secondary responses are due to stimulation of naïve B cells and expanded clones of memory B cells. In humans, Abs are produced within 10-14 days after

vaccination in the primary immune response (Brinkman et al., 2003), and within 4-6 days in the secondary immune response (Bernasconi et al., 2002; Snape et al., 2006).

1.10.4 CD4 T cells

As mentioned earlier in section 1.9.4, T helper cells have a major role in the induction of long-term immunity to meningococcal infection. Following the first exposure to the bacteria TD-B cell responses are induced, which lead to the formation of high avidity long-lived plasma cells and memory B cells in GC.

1.10.4.1 Effector T cells

It is believed that natural exposure to *N. meningitidis* induces a TD immune response, through the recognition of OMP derived peptides presented on MHC molecules by APCs to the naïve T cells (through TCR-CD3 complex and CD4 molecules). However, costimulatory signals are required for the complete activation of naïve T cells, which are provided by the interaction between CD28 (on T cells) with CD80/CD86 (on APCs), and CD40 ligand (on T cells) with CD40 (on APCs) (Lenschow et al., 1996). Naïve T cells can be distinguished by the expression of CD45RA, the lymph node homing receptors CD62L and CCR7 and the costimulatory receptors CD28 and CD27 (Appay and Rowland-Jones, 2004). Continuous Ag exposure leads to the proliferation and differentiation of the naive T helper cell progenitors to short-lived effector T helper cells or memory T helper cells (Reiner et al., 2007).

The proliferating T helper cells can differentiate into two major subtypes of effector T cells: Th1 or Th2 cells, depending of the effect of the cytokines (such as IL12 and IL4) produced in the early events of the immune response (Mosmann et al., 1986). It has been suggested that high levels of co-stimulation promote Th2 differentiation through an increase in IL4 production, which has an autocrine effect. In contrast, Th1 differentiation depends on the production of IL12 by APCs (Murphy et al., 1994). These two subsets of T helper cells have different roles in B cell activation. Th1 cells produce IFN γ and induce the switching of B cells to several IgG isotypes, that can bind to high affinity Fc γ receptors and complement proteins (e.g. IgG1 and IgG3) (Stevens et al., 1988). In contrast, Th2 cells produce IL4 and IL5 and stimulate the production of IgM and non complement fixing IgG isotypes (e.g. IgG4) (Coffman et al., 1993).

1.10.4.2 Memory T cells

Some of the T cells activated during primary exposure to the Ag will persist as memory T cells. In comparison to naïve T cells, memory T cells can respond and proliferate more rapidly following an antigenic stimulation, having a low threshold for activation and being less dependent on costimulation (Ahmed and Gray, 1996; Sprent, 1994). Furthermore, they upregulate the CD40 ligand, which helps in the activation of B cells (Sallusto et al., 2004).

These memory T cells can be differentiated into central memory T cells and effector memory T cells, through the different chemokine receptors and other markers expressed on their surface. Central memory T cells express CD27, CD28, CD45RO⁺, CD62L and CCR7 (Sallusto et al., 2004). The two last receptors are important for homing in lymphoid tissues. Central memory T cells correspond to the memory B cells; they are a reserve of long surviving clones, which re-

circulate between the blood and secondary lymphoid organs, responding to Ag re-exposure by rapid proliferation and differentiation into effector T helper cells (Lanzavecchia and Sallusto, 2000).

By contrast, effector memory T cells provide immediate protection against re-infection; they proliferate less well, but can produce cytokines very rapidly. They lose their constitutive expression of CCR7 but can still express CD62L (Sallusto et al., 1999), and express other chemokine receptors important for homing in inflamed tissues. They correspond to the plasma cells, migrating to the inflamed peripheral tissues and produce IFN γ , IL4 and IL5 within hours following Ag exposure (Sallusto et al., 2004).

In blood, the central memory T cells are dominant, however, the effector memory T cells predominate in peripheral tissues (e.g. lung, liver and gut), while the central memory T cells predominate in the lymph nodes and tonsils (Campbell et al., 2001).

The T cell response peaks by 1-2 weeks after Ag exposure, and is then followed by apoptosis of most effector T cells. As for the B cells, the duration of the memory T cell immunity depends on the continuous turn over of memory T cells clones, which might be Ag-dependent (TCR signalling) but also depends on cytokines (e.g. IL7) (Seddon et al., 2003). Effector and memory T cells have been shown to persist for up to 10 years following Ag-exposure and their frequencies increase in response to booster immunisations (Sallusto et al., 1999). It was observed that memory T cell responses decline with a half life of 8-15 years (Hammarlund et al., 2003).

It has been proposed that the fate of T cell differentiation depends on the strength of the signals delivered by TCR and cytokine receptors, which in turn depends on the concentration of Ag and costimulatory molecules, and also on the duration of the interaction between T cells and APCs (Iezzi et al., 1998; Valitutti et al., 1995). A weak stimulus induces naïve T cells to proliferate but not to differentiate into effector Th1 or Th2 cells, whereas a strong signal would promote massive proliferation of the T cells and their differentiation into effector T cells, some of which would persist as effector memory T cell. In contrast, central memory T cells emerge from the proliferation of non effector T cells (Iezzi et al., 2001) (Figure 1.10-8). Therefore, the magnitude of the primary immune response might determine the duration of memory (Hou et al., 1994). In consequence, it was proposed that the duration of Ag exposure and co-stimulatory signals could be manipulated during priming in order to optimise the response to immunisation (Beverley, 2008).

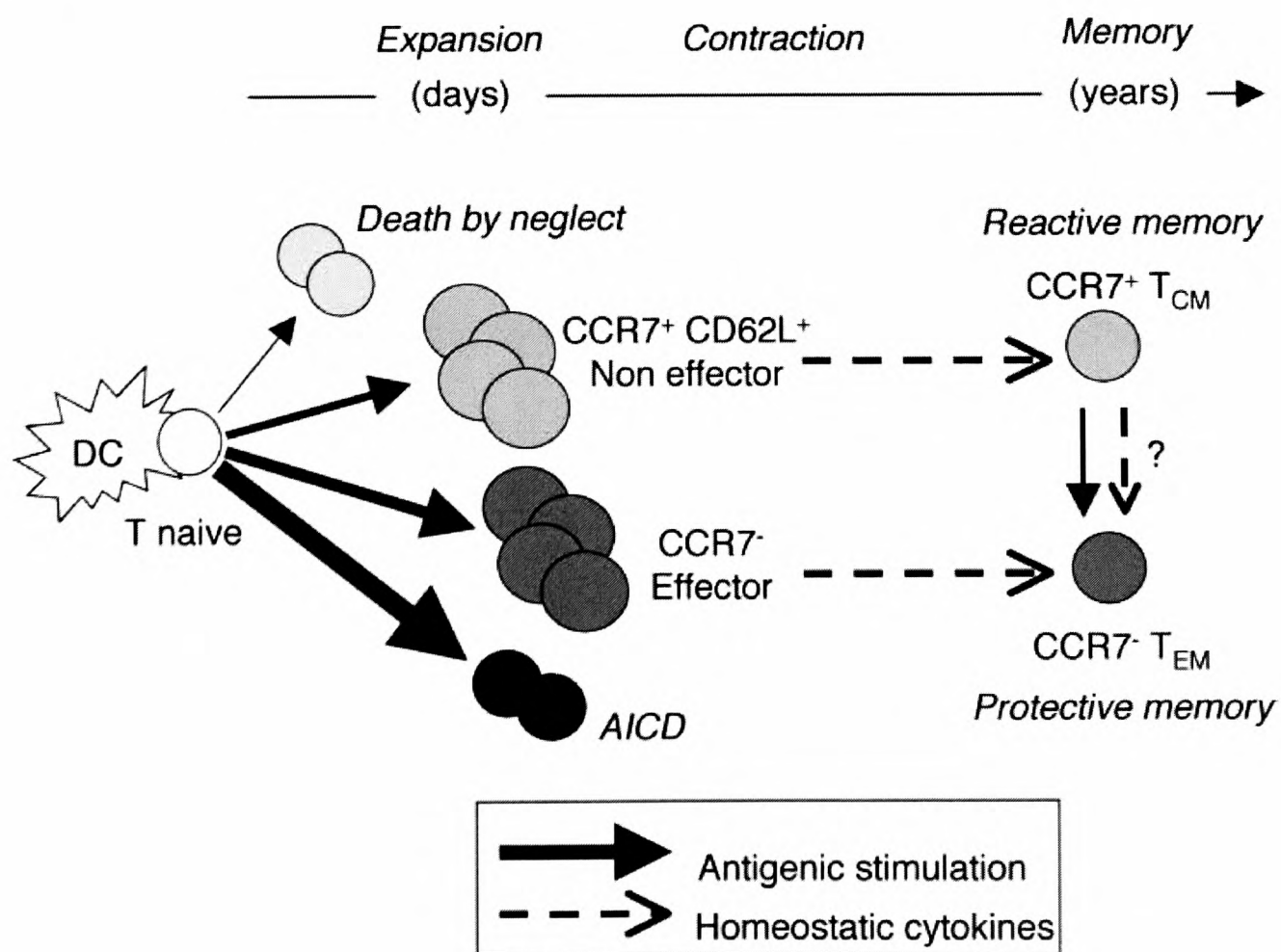


Figure 1.10-8: The fate of the T cell differentiation may depend on the strength of the antigenic stimulation (indicated by the length and thickness of the solid arrows) or of Ag-independent events (indicated by the dotted lines). AICD = activation-induced cell death.

1.11 Meningococcal vaccines

1.11.1 Plain polysaccharide vaccines

Of the 12 serogroups of *N. meningitidis* only five (A, B, C, W135, Y) are associated with invasive disease. This observation, together with the evidence that bactericidal anticapsular Abs confer protection against meningococcal disease (Goldschneider et al., 1969a, b), makes serogroup specific polysaccharides the principal targets for vaccine development.

Gotschlich and co-workers developed the first efficacious meningococcal vaccines during the 1960s; they purified high molecular weight capsular polysaccharide and showed that it was immunogenic in adult volunteers (Gotschlich et al., 1969a; Gotschlich et al., 1969b). Bivalent (A, C) and tetravalent (A, C, W135, Y) plain polysaccharide meningococcal vaccines have been available since the 1970s (Granoff and Harris, 2004). These vaccines have been widely used over the past three decades to interrupt outbreaks of meningococcal disease and to protect individuals with a high risk of contracting meningococcal disease. However, because of their TI nature, polysaccharide vaccines have several disadvantages that restrain their use in immunisation programs (Morley and Pollard, 2001); they are poorly immunogenic in infants less than 2 years old, and they do not induce immunological memory and herd immunity (Granoff et al., 2004; Lepow et al., 1977). Furthermore, repeated doses of polysaccharide vaccines have been associated with a decrease in subsequent Ab levels (hyporesponsiveness) (Borrow et al., 2000b; Granoff et al., 1998; Granoff and Pollard, 2007; MacLennan et al., 1999).

1.11.2 Protein polysaccharide conjugate vaccines

The problems associated with the TI nature of plain polysaccharide vaccines can be overcome by conversion of the polysaccharide into a TD Ag (Avery, 1931; Makela and Kayhty, 2002; Weintraub, 2003). Protein Ags are usually TD Ags and polysaccharides can be made TD by chemical conjugation of the polysaccharide Ag to a carrier protein (Avery, 1931). This allows the uptake and processing of both the polysaccharide and protein carrier by APCs. The derived peptides are then presented to T helper cells in MHCII molecules on the surface of APCs (McCool et al., 1999; Parker, 1993). The MHCII restricted cognate interaction between B cells and T cells provides the necessary co-stimulatory signals to the B cell to begin the process of GC reaction with the induction of isotype switching of immunoglobulin, somatic hypermutation, selection for high avidity Ab and the development of long-lived plasma and memory B cells (MacLennan, 1994).

These vaccines have many properties that contribute to their effectiveness, including the ability to elicit protective immunity in infants, induce immunological memory, provide herd immunity and overcome hyporesponsiveness.

Meningococcal conjugate vaccines were developed throughout the 1980s and the first human trials were conducted in 1991 (Costantino et al., 1992) using meningococcal A and C capsular oligosaccharides conjugated to the mutant diphtheria toxoid protein, CRM₁₉₇. This conjugate vaccine generated TD immune responses and was, therefore, able to induce isotype switching, affinity maturation and immunological memory (Borrow et al., 2000a; MacLennan et al., 2001).

In the early 1990s the emergence of the ST11 clone bearing the C serogroup capsule (see section 1.5.2) brought about an increased incidence of meningococcal diseases caused by serogroup C meningococci in some industrialised countries (North America, Canada, Spain, UK, etc.) and concentrated efforts on the development of a MenCV.

A monovalent MenCV was developed and, in 1999, the UK was the first country to introduce MenCV into its immunisation schedule where it was offered to all people younger than 18 years (Miller et al., 2001). The UK used a three-dose regimen in infants with the primary immunisation schedule at 2, 3, and 4 months, a two-dose regimen in older infants (5-11 months age group) and a single dose in older children and adolescents (1-18 years) (Snape and Pollard, 2005). The MenCV had a vaccine coverage of around 90% for routine infant immunisation and around 85% in the catch up campaign (Miller et al., 2001; Trotter et al., 2004). The national MenCV immunisation programme resulted in a rapid decline in meningococcal disease cases caused by serogroup C meningococci in the age groups targeted by the vaccine (Miller et al., 2001), with a decline in group C carriage (Maiden et al., 2008; Maiden and Stuart, 2002) (Figure 1.11-1). Furthermore, it was reported that there was high vaccine effectiveness and induction of herd immunity (Maiden et al., 2008; Miller et al., 2001; Ramsay et al., 2001; Ramsay et al., 2003a). However, questions about the long-term effectiveness of the MenCV were raised (see section 1.12.7).

In 2006 the MenCV schedule was modified as follows: 2 doses at 2 and 4 months of age followed by a booster dose at 12 months of age to sustain vaccine protection in the younger age groups (www.advisorybodies.doh.gov.uk/jcvi/chilhoodimmunisationoc05.pdf).

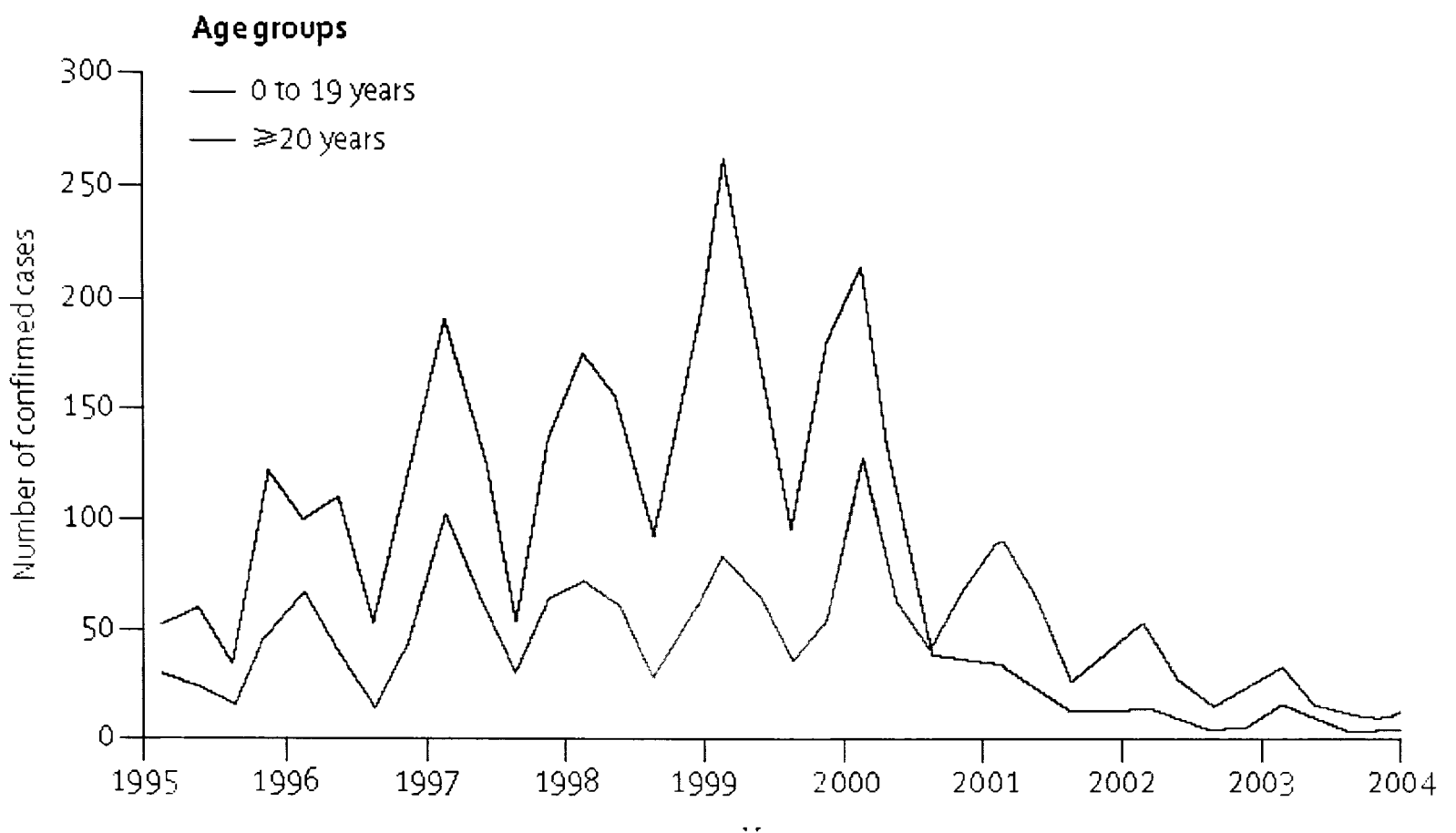


Figure 1.11-1: Cases of serogroup C meningococcal disease by age group from 1995 to 2004 (Trotter et al., 2004).

There are currently three different licensed MenCV, manufactured by Novartis (Menjugate), Baxter (Neisvac-C) and Wyeth (Meningitec) (see Table 1.11-1). These vaccines are conjugated to different carriers and are variably immunogenic.

Tetravalent meningococcal A, C, Y and W135 conjugate vaccines are being developed by various manufacturers. A tetravalent vaccine conjugated to the diphtheria toxoid prepared by Sanofi Pasteur (MenACWY-D; Menactra) was licensed in the US in January 2005 for use in adolescents and adults (11-55 year olds) (Bilukha and Rosenstein, 2005) and in May 2006 in Canada for 2-11 year old children (Pace and Pollard, 2007; Snape et al., 2008b). Rennels et al. (2004) demonstrated that the MenACWY-D vaccine was poorly immunogenic in infants (Rennels et al., 2004). Other quadrivalent conjugate vaccines are currently being developed by

Novartis vaccines and GlaxoSmithKline biologicals. The Novartis MenACWY is conjugated to CRM₁₉₇ (MenACWY-CRM₁₉₇), in contrast to the MenACWY-D licensed in the US and Canada. Furthermore, the MenACWY-CRM₁₉₇ uses aluminium phosphate adjuvant and the quantity and lengths of the saccharide chain selected is different (Snape et al., 2008b). New results from phase II studies with the MenACWY-CRM₁₉₇ suggest that this vaccine is highly immunogenic in young children (Snape et al., 2008b).

	Proprietary name	Manufacturer	Carrier protein	Adjuvants	Date first licensed
Monovalent (serogroup C) (MenCV)	Meningitec	Wyeth	CRM ₁₉₇	AlPO ₄	UK, October 1999
	Menjugate	Novartis	CRM ₁₉₇	Al (OH) ₃	UK, March 2000
	NeisVac-C	Baxter	Tetanus toxoid	Al (OH) ₃	UK, July 2000
Tetravalent (A, C, W135, Y) (MenACWY)	Menactra (MenACWY-D)	Sanofi Pasteur	Diphtheria toxoid	None	USA, January 2005 for 11-55 year old Canada, May 2006 for 2-11 year old

Table 1.11-1: Licensed meningococcal conjugate vaccines.

1.11.3 Vaccines against serogroup B *N. meningitidis*

Serogroup B *N. meningitidis* causes around 50% of meningococcal disease cases worldwide (Griffiss et al., 1991), and is the only serogroup whose infection cannot be prevented by capsular polysaccharide-based vaccines. Serogroup B capsular polysaccharide is a polymer of α (2-8)-linked N-acetyl-neuraminic acid, which is also present in human tissues, such as the

neural cell adhesion molecule involved in cell-to-cell adhesion (Griffiss et al., 1991). A conjugate vaccine using group B capsular polysaccharide was developed by replacing the N-acetyl groups of the sialic acid residues with N-propionyl groups. However, the Abs induced by this vaccine had no functional activity (Bruge et al., 2004). Therefore, because of the risk of induction of autoimmunity (Finne et al., 1983) and because of the lack of immunogenicity, commercial development of polysaccharide-based group B vaccines was abandoned.

Alternative approaches have focused on other Ags, such as OMPs and LPS. It was found that bactericidal immune responses could best be induced using the natural outer membrane environment as a delivery vehicle. Native outer membrane vesicles (OMVs) consist of an intact outer membrane and contain OMPs and LPS. One of the OMPs, PorA, was identified as a main target for serum bactericidal activity in an animal model (Saukkonen et al., 1989). This protein is expressed by almost all meningococci; however, there are a large number of PorA proteins which elicit variant specific Abs that do not confer protection against meningococci with heterologous PorA variants (Girard et al., 2006). Other OMPs, such as the transferrin-binding protein (Danve et al., 1993), PorB, Opa, etc., are also being explored as possible meningococcal vaccine candidates.

Several OMV vaccines have been developed and tested in clinical trials, and used to stop different epidemics in Norway (Bjune et al., 1991), Brazil (de Moraes et al., 1992), Cuba (Sierra et al., 1991) and New Zealand (Oster et al., 2005). Their efficacy was variable and depended mainly of whether the vaccine strain matched the dominant circulating serosubtype, as the PorA proteins are the major inducer of bactericidal Abs (Borrow et al., 2005).

A broadly protective group B vaccine may be successfully developed as a consequence of the availability of the full genomic sequence of *N. meningitidis* and the discovery of new OMP candidates (Giuliani et al., 2005). Indeed, the reverse vaccinology approach allows identification of the entire repertoire of proteins potentially expressed by the bacteria, and the testing of them as a vaccine candidate without any prior selection based on their *in vitro* expression or their role in virulence and immunogenicity (Rappuoli, 2001).

1.11.4 Hyporesponsiveness to plain polysaccharide meningococcal vaccine

Hyporesponsiveness to the meningococcal polysaccharide capsule has been described following repeated immunisation with meningococcal plain polysaccharide vaccine (Borrow et al., 2000b; Granoff et al., 1998; Granoff and Pollard, 2007; Jokhdar et al., 2004; MacLennan et al., 1999; Richmond et al., 2000). The hypothesis is that plain polysaccharide vaccines may drive established memory B cells to terminal differentiation (as plasma cells) without laying down new memory B cells (Granoff and Pollard, 2007). There would be therefore, fewer Ag-specific memory B cells available for responses after the next dose of vaccine was administered. In contrast, meningococcal conjugate vaccines produce new memory cells and maintain or augment future responses to a challenge dose of vaccine. However, immunological hyporesponsiveness induced by polysaccharide vaccines might be overcome by further vaccination with protein-polysaccharide conjugate vaccines (Borrow et al., 2001b; Richmond et al., 2000).

However, whether the serogroup A meningococcal polysaccharide vaccines induces immunological hyporesponsiveness is controversial (Borrow et al., 2000b; Jokhdar et al., 2004; Kayhty et al., 1984). It has been suggested that this serogroup does not behave as a classical TI Ag. Early studies have reported that immunisation with serogroup A polysaccharide elicited immunological memory that is not typically seen with other polysaccharide Ags (Goldschneider et al., 1973; Kayhty et al., 1980) and was immunogenic even in infants younger than six months of age (Peltola et al., 1977). It has been suggested that this latter finding may have a genetic basis or reflect prior exposure with cross-reacting Ags (Goldschneider et al., 1973).

1.11.5 Serological correlate of protection

The original correlate of protection was established in 1969 using a serum bactericidal Ab assay (SBA) utilising human complement (hSBA). The SBA titre represents a measure of bacterial killing by functional Ab and is expressed as a dilution factor, with titres $\geq 1:4$ predicting protection (Goldschneider et al., 1969a). Later, because of the greater availability and degree of standardization, human complement has been replaced by baby rabbit complement (Borrow et al., 2001a) and a rSBA titre $\geq 1:8$ predicts protection (Borrow et al., 2001a; WHO, 1976) in some laboratories.

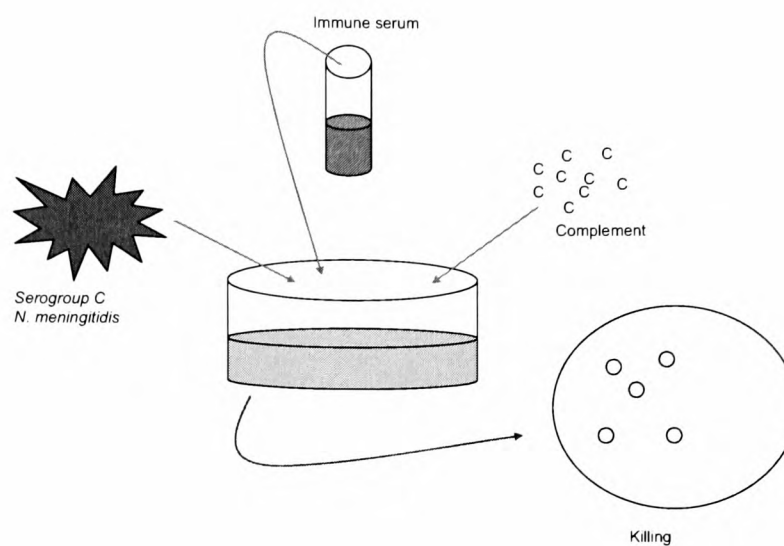


Figure 1.11-2: Serum bactericidal Ab assay; Serial dilutions of human sera containing meningococcal-specific Ab are incubated with appropriate *N. meningitidis* target strains and complement. The specific Abs will bind to the bacteria cell surface through the capsule or cell surface proteins. Then the binding of the C1q subunit of the complement to the surface-bound Ab activates the classical pathway of complement, which finally induces the lysis of the bacteria. The SBA titre for each serum is expressed as the reciprocal serum dilution yielding $\geq 50\%$ killing as compared to the number of target cells present before incubation with serum and complement (Borrow et al., 2005), image kindly given by Dr A.J. Pollard).

The importance of serum bactericidal Abs in protection against group C meningococcal disease was first demonstrated during an epidemic among military recruits in the 1960s. Group C bactericidal Abs were present in baseline sera of about 82% of the recruits. The subjects with detectable bactericidal Abs in serum became carriers of the epidemic strain but did not develop meningococcal disease, while all cases of disease occurred in the 18% of individuals whose baseline sera lacked bactericidal activity (hSBA titres $< 1:4$) (Goldschneider et al., 1969a, b)

In a vaccine efficacy trial, a population-based correlate of protection is used. It looks at the level of Ab acquired by the majority of vaccinated individuals and not acquired by the majority of unvaccinated individuals to establish a cut-off of Ab level to predict protection. The rSBA cut-

off of 1:16 was established in the UK using this approach in a vaccinated population (Borrow et al., 2001a).

The presence of SBA is a reliable marker of protection against meningococcal disease but the absence of SBA does not necessarily imply susceptibility. A mechanism responsible for protective activity in the absence of SBA is the persistence of anticapsular Abs at concentrations that are insufficient to elicit bactericidal activity but that are sufficient to confer protection via opsonophagocytosis (Welsch and Granoff, 2004). The presence of immunological memory is also important, as illustrated both by the response to a polysaccharide challenge and by the maturation of Ab avidity in the months following primary immunisation (Andrews et al., 2003).

1.11.6 Age-related immunity to meningococcal conjugate vaccines

The three MenCVs, first licensed in the UK and now widely used across Europe, Australia, and Canada, are safe and highly immunogenic in all age groups (MacLennan et al., 2000; Richmond et al., 2001a; Richmond et al., 2001b). However, the immunogenicity of this vaccine is still age-dependent, with young children often failing to produce a significant response to the first dose of vaccine and usually requiring two to three doses to develop protective immunity. Studies with MenCV in a three-dose schedule did not show a higher induction of Ab titres with the third dose compared to the second dose (Fairley et al., 1996; Richmond et al., 2001b; Richmond et al., 1999). However, post vaccination Ab titres following one dose of MenCV were significantly lower than after 2 doses of MenCV (Southern et al., 2006), suggesting that two

doses of MenCV in the first year of life induce higher protection than only one dose. In infants, two or three doses of vaccine given during the first six months of life have been shown to induce a bactericidal Ab response of similar magnitude to that induced by the serogroup C plain polysaccharide vaccine and known to be protective in adults (Granoff and Harris, 2004). In older children and adults, a single dose of MenCV is sufficient to elicit protective bactericidal Ab levels in the majority of immunised individuals (Borrow et al., 2001a).

The MenACWY-D prepared by Sanofi Pasteur and licensed in the US and Canada since January 2005 and May 2006 respectively, has been shown to induce protective bactericidal Ab levels against all four serogroups in people older than 2 years of age (Campbell et al., 2002; Rennels et al., 2004). In children, between two and ten years of age, MenACWY-D induced significantly higher and more persistent bactericidal Ab responses than the licensed tetravalent plain polysaccharide vaccine (Pichichero et al., 2005). However, it is modestly immunogenic in infants, although it appears to prime most infants after three doses in infancy (Rennels et al., 2004).

The MenACWY-CRM₁₉₇ developed by Novartis and tested in clinical trials appear to induce seroprotection against all four serogroups in infants, after a three dose schedule given at 2,3, and 4 months or 2, 4 and 6 months of age. The two-dose schedule given at 2 and 4 months of age resulted in lower seroprotection against serogroup A than the three-dose schedule, but the booster dose of MenACWY-CRM₁₉₇ at 12 months of age resulted in seroprotection against all serogroups. However, the MenACWY-CRM₁₉₇ induced lower serum bactericidal activity against serogroup C, in comparison to the monovalent MenCV (Snape et al., 2008b).

1.11.7 Long-term protection after meningococcal conjugate vaccines: Ab persistence versus priming for memory

Long-term protection against disease depends upon Ab persistence and immunological memory. However, studies performed after the introduction of MenCV in the UK suggest that Ab persistence is age-dependent. This vaccine elicits high bactericidal Ab concentrations but, for infants routinely immunised with three doses of vaccine in early life, anti-capsular Ab wanes rapidly after the first year of vaccination to levels that are not protective (MacLennan et al., 2000; Richmond et al., 1999). With increasing age, Ab persistence improves (Table 1.11-2) (Snape and Pollard, 2005; Snape et al., 2008a). Similarly, for the infant schedule, the effectiveness of the vaccine declines after the first year of vaccination (Table 1.11-3) (Trotter et al., 2004).

Timing of vaccination	Time since vaccination	MenC (combined) effectiveness (95% CI) [§]	MenC-CRM ₁₉₇		MenC-TT	
			SBA GMT (95% CI)	% with SBA > 8	SBA GMT (95% CI)	% with SBA > 8
Primary (2, 3, 4 months)	< 1 year	93% (67-99)				
	8 months		16 (12-23)*	75 ¹⁰⁰		
	10 months		13.5 (7.3-25)†	53 ⁵²	21.5 (15.8-29.2)‡	99 ¹⁰⁴
	9-12 months		10.3 (5.2-20.6)†	46 ¹⁰²		
	> 1 year	-81% (-7430 to 71)				
1-3 years (single dose)	4 years		3.2 (1.9-5.5)†	12 ¹⁰²		
	< 1 year	88% (65-96)				
	6 months		51 (30-85)†	75 ⁵⁶	166 (99-281)‡	86 ⁹⁶
			19 (11-32)‡	57 ⁵⁶		
	> 1 year	61% (-327 to 94)				
Adolescence (single dose)	2 years		8.7 (5.8-13.2)†	27 ¹⁰³		
	3 years		2.5 (2.0-3.1)*	25 ⁵⁶		
	< 1 year	96% (89-99)				
	1 year		81.3 (54.5-121.3)*	95 ⁵⁵		
	> 1 year	90% (77-96)				
	3 years			92 ¹⁰⁵		

*Menjugate, Chiron Vaccines. †Meningitec, Wyeth Vaccines. ‡NeisVac-C, Baxter. §SBA in these studies conducted with human complement.

Table 1.11-2: Persistence of SBA geometric mean titre following immunisation with a MenC-CRM197 or MenC-tetanus toxoid conjugate vaccine at various ages (Snape and Pollard, 2005).

However, MenCV has been shown to prime for immunological memory. A primed immune response is of greater magnitude than the primary response and is characterised by the production of higher avidity Abs. Exposure to the unconjugated polysaccharide after priming with MenCV has been shown to elicit an immunological memory response (Borrow et al., 2002; MacDonald et al., 1998; Richmond et al., 2001b; Richmond et al., 1999). Similarly, studies of immune responses to the MenACWY-D and MenACWY-CRM₁₉₇ have shown avidity maturation or at least a 4-fold increase in bactericidal titres following challenge with the plain polysaccharide vaccine, also indicating the induction of immunological memory (Granoff et al., 2005a, b; Snape et al., 2008b).

Following the introduction of routine Hib or MenCV immunisation in infants, it was clear that some individuals with low or undetectable Ab concentrations were still protected against disease and this protection was attributed to herd immunity and immunological memory (Adams et al., 1993; Eskola et al., 1990). However, occasional failures of protection have been reported in primed individuals with low baseline Ab level (MacLennan et al., 2000; Trotter et al., 2004), suggesting that the presence of pre-existing neutralising and/or opsonising Ab was the principal mechanism of defence against invasion by encapsulated bacteria. This could be explained by the fact that it takes a few days to mount a protective Ab response in a primed individual, while encapsulated bacteria can invade the blood and the disease develop within a few hours. In contrast, immunological memory can be sufficient during invasion by microorganisms that require a prolonged incubation period before the disease progresses, such as hepatitis B (4-12 weeks) or hepatitis A (2-6 weeks). Similarly, because of the slow course of rabies, a booster dose of the vaccine can be administered immediately after exposure and can

still elicit a protective Ab response.

1.11.8 Efficacy and effectiveness of meningococcal conjugate vaccines

Efficacy trials were never undertaken for MenCV but effectiveness of the vaccine was monitored post-licensure by an enhanced disease surveillance program (Miller et al., 2001). Since the introduction of MenCV in the UK in 1999, a marked decrease (> 80%) in the incidence of group C meningococcal disease has followed (Snape and Pollard, 2005). Short-term estimates, using the screening method (Farrington, 1993), showed that the effectiveness of the vaccine was 97% for teenagers, 92% for toddlers, and 93% for infants routinely immunised with three doses of vaccine between the second and fourth months of life (Ramsay et al., 2001; Trotter et al., 2004). In the last group, however, protection was of short duration (Table 1.11-3); there was a fall in vaccine effectiveness after routine (2, 3 and 4 months) infant immunisation from 93% (95% CI 67-99) in the first year to -81% (-7430 to 71) at more than 1 year. In contrast, the effectiveness more than 1 year after the single dose regimen employed for 1-2 year olds in the catch-up campaign declined to 61% (-327 to 94%), and effectiveness for all other age groups immunised in the catch-up campaign remained above 80% (Trotter et al., 2004). The confidence intervals for this analysis were wide, as the presence of herd immunity meant that the actual numbers of vaccine failures were relatively small even if direct individual protection was not maintained. This reflects waning bactericidal Ab levels observed in clinical studies (section 1.11.7) and suggests that, although these vaccines prime the infant immune system, immunological memory alone is insufficient to protect this age group. These observations led to an adaptation of the UK immunisation schedule with the addition of a booster dose of MenCV

at 12 months of age (www.advisorybodies.doh.gov.uk/jcvi/childhoodimmunisationoc05.pdf).

Cohort	Age at vaccination	Doses scheduled* by quarter year	Period of observation, by quarter year	Overall		Within 1 year of scheduled vaccination†		More than 1 year after scheduled vaccination†	
				Cases (vaccinated)	Vaccine effectiveness (95% CI)	Cases (vaccinated)	Vaccine effectiveness (95% CI)	Cases (vaccinated)	Vaccine effectiveness (95% CI)
Routine	2-4 months	3	Q1 2000-Q1 2004	28 (21)	66% (6 to 86)	9 (3)	93% (67 to 99)	19 (18)	-81% (-7430 to 71)
Infant catch-up	5-11 months	2	Q3 2000-Q1 2004	13 (5)	85% (46 to 96)	6 (2)	87% (11 to 99)	7 (3)	82% (-8 to 97)
Toddlers catch-up	1-2 years	1	Q3 2000-Q1 2004	25 (10)	83% (60 to 93)	19 (6)	88% (65 to 96)	6 (4)	61% (-327 to 94)
Pre-school catch-up	3-4 years	1	Q3 2000-Q1 2004	37 (2)	98% (91 to 100)	45 (1)	98% (90 to 100)	19 (4)	93% (78 to 98)
Infant school catch-up	4-6 years	1	Q3 2000-Q1 2004	19 (0)	100% (71 to 100)				
Junior school catch-up	7-10 years	1	Q3 2000-Q1 2004	8 (3)	88% (38 to 98)				
Secondary school catch-up	11-16 years	1	Q2 2000-Q1 2004	40 (8)	96% (90 to 98)	45 (4)	96% (89 to 99)	39 (8)	90% (77 to 96)
Sixth form catch-up	17-18 years	1	Q1 2000-Q1 2004	44 (4)	93% (82 to 98)				
Total				214 (53)		124 (16)		90 (37)	

Q=quarter. *Vaccine effectiveness compares children eligible for complete vaccination who had received all scheduled doses versus no doses. Partly vaccinated children were excluded. †For the time change analysis, pre-school, infant, and junior cohorts were combined, as were the secondary school and sixth form cohorts.

Table 1.11-3: Direct effectiveness of MenCV, using the screening method, i.e. through the measure of the proportion of cases in vaccinated children and the estimation of vaccine coverage corresponding to each case (Trotter et al., 2004).

1.11.9 Herd immunity after meningococcal conjugate vaccines

Herd immunity (or community immunity) occurs when the vaccination of a portion of the population (or herd) provides protection to unvaccinated people (John and Samuel, 2000). Ideally, immunisation decreases the spread of the disease, through the prevention of further transmission to others (Fine, 1993). Protein-polysaccharide conjugate vaccines induce herd immunity through reduction of carriage and transmission of *N. meningitidis* (Ramsay et al., 2003a). This may occur through Ab crossing from serum to mucosal surfaces (Zhang et al., 2000). A study by the UK Meningococcal Carriage Group (Maiden and Stuart, 2002), assessing whether the mass immunisation campaign could result in herd immunity, compared meningococcal carriage in students before vaccination (in 1999) with that of students of the same age, one year after mass vaccination. They observed that carriage of serogroup C meningococci was reduced by 66% after the introduction of MenCV, indicating that it induced

sufficient mucosal immunity to inhibit carriage of meningococci expressing the serogroup C polysaccharide (Maiden and Stuart, 2002). Similarly, a herd immunity effect has also been demonstrated by a 67% reduction in serogroup C meningococcal disease for unvaccinated children when comparing 1998/1999 with 2001/2002 (Ramsay et al., 2003a).

1.12 Aims

The aim of this thesis was to assess the cellular basis for the lack of Ab persistence after immunisation with protein-polysaccharide conjugate vaccines in early infancy. Various aspects of the B cell response to immunisation have been investigated to provide insight into the kinetics and magnitude of the primary and secondary B cell response. The main components of the thesis are outlined below:

- 1) An investigation of different methods to assess Ag-specific memory B cells in the laboratory (see section 3) in order to define which method is the most appropriate to study the memory B cell response following immunisation with meningococcal protein-polysaccharide conjugate vaccines in children (section 4 and 5) and rabies vaccine in adults (section 6).
 - i) Limiting dilution assay (LDA) was compared with ELISpot in its ability to detect memory B cell responses before, and 30 days following, a booster dose of MenCV in one-year-old infants primed with MenCV in early infancy (see section 3.2).
 - ii) An ELISpot-based LDA was developed and compared with the standard LDA (ELISA-based LDA) and ELISpot in its ability to detect memory B cell responses to seasonal influenza immunisation in adults (see section 3.3).
- 2) An investigation of MenC-specific B cell responses to a booster dose of MenCV at one year of age after three-dose priming with MenCV in infancy (section 4), in order to assess the persistence of memory B cells 8 months after priming in early infancy and the kinetics of the Ab, plasma cell and memory B cell responses following booster immunisation at one year of

age. Furthermore, the generation of memory B cells and Abs after priming was correlated with the persistence of Ab at one year of age and with the response to a booster dose of MenCV.

3) An investigation of the meningococcal serogroup A, C, W135 and Y memory B cell responses at 5 months of age following a 2-dose primary course of the newly developed quadrivalent meningococcal vaccine conjugated to CRM₁₉₇, which had been shown to be immunogenic in young infants (section 5). The aim of this study was to assess the production of memory B cells after priming with this newly developed meningococcal protein polysaccharide conjugate vaccine and to analyse the kinetics of appearance and disappearance of memory B cells in peripheral blood between 4 and 12 months of age. Furthermore, the influence of maternal Ab present at 2 months of age on the memory B cell response at 5 months of age was assessed.

4) An investigation of a primary B cell response to a novel Ag, the rabies vaccine (section 6), in order to define the key time points to assess B cell responses following primary immunisation, and to assess if the magnitude and kinetics of the B cell responses are different during priming and boosting immunisation. This may improve our understanding of the low induction of memory B cells and low persistence of Abs in children after primary immunisation. 10 healthy volunteers immunologically naïve to the rabies vaccine received a three-doses primary course of the rabies vaccine at one-month intervals, and the kinetics of rabies-specific Ab, plasma cell, and memory B cell responses was assessed following the first and third dose of vaccine. The results were compared to a group of immune individuals, who received a single booster dose of vaccine.

5) An investigation of the influence of baseline MenC-Ab levels on the post-booster Ab increase following booster immunisation with MenA/C or MenCV at one year of age (section 7), in order to extend the findings of the study described in section 4, where a higher increase in Ab post-booster immunisation was observed in the children who had lower Ab levels pre-booster, compared to those with higher pre-booster Ab levels. The serum IgG responses from four studies previously conducted by the Oxford Vaccine Group were pooled and analysed to assess the effect of baseline MenC-specific IgG concentrations on the increase in MenC-IgG concentrations post-booster, using a multilevel modelling technique within groups receiving 1 of 8 different vaccine schedules.

2 Materials and Methods

All the reagents were obtained from Sigma-Aldrich, UK, unless otherwise stated.

2.1 Samples

Venous blood was obtained with fully informed consent either as part of an Oxford Vaccine Group clinical trial or from volunteers for the study of memory B cell response to seasonal influenza immunisation (section 3.3). Nurses or clinical research fellows undertook the process of fully informed consent and venesection for the studies of B cell responses to meningococcal vaccines (section 3.2, 4 and 5) and I undertook these processes for the studies of memory B cell responses to seasonal influenza vaccination (section 3.3) and rabies vaccination (section 6). Blood was collected by needle and syringe into Falcon tubes containing lithium heparin for PBMCs separation and clotted tube for serum separation. The heparin tubes were then transported at room temperature to the laboratory for use within 6 hours. Indeed, cell separation is best undertaken immediately using fresh samples, because recently activated cells may be especially susceptible to apoptosis. The clotted tubes were transported in a cool box and on arrival in the laboratory placed in 2-8°C until separation within 24 hours of sampling. The serum samples were then stored in freezer at ≤ -18 °C (to -80 °C) for further testing.

2.2 Preparation of peripheral blood mononuclear cells (PBMCs)

2.2.1 Reagents

- Lymphoprep: contains sodium Diatrizoate 9.1%(w/v), polysaccharide 5.7%(w/v), density 1.077 g/ml, osmolality 290 mOsm, endotoxins <1.0 EU/mL (Axis-Shield Diagnostics, UK)
- Complete medium: RPMI-1640 Hepes modification (500 ml) + penicillin / streptomycin (50 U/ml, 0.05 g/ml respectively, 5ml) and glutamine (2 mM, 5 ml)
- 10% FCS: complete medium (450 ml) with heat inactivated foetal calf serum (50 ml)
- PBS-EDTA/FCS: A total of 5 PBS tablets and Ethylenediaminetetraacetic acid di-sodium salt dihydrate (EDTA, 0.744 g) were dissolved in distilled water (1L), the pH was then adjusted to 7.2. Following sterilisation by autoclaving, FCS (5 ml) was added to give a final concentration of 0.05%. The solution was stored at 4°C.

2.2.2 Protocol

A maximum volume of 4 ml (before one year of age) and 5 ml (after one year of age) of heparinised blood from children and 20 ml from adults was available for the separation of peripheral blood mononuclear cells (PBMCs). The blood was diluted 1:2 in complete medium. The PBMCs were then separated by density gradient centrifugation over a lymphoprep (Ulmer et al., 1984). The buffy coat layer of PBMCs was transferred into a fresh universal tube, taking care not to transfer any lymphoprep. PBMCs were then washed once in complete medium and three times with PBS-EDTA/FCS.

After the final wash, cells were resuspended into 1 ml of 10% FCS for quantification by adding 50 μ l of cells to 50 μ l trypan blue (0.4%) and 50 μ l PBS. The cells in a 10 μ l volume of this mix were counted using a haemocytometer with a central 5x5 row by column grid and counting 5 squares within this grid. The number of cells obtained was then multiplied by 5 to give the number of cells in 25 squares, then by 3 allowing for the cell dilution, and then by 10^4 to give the number of cells/ml of PBMC suspension. The number of cells/ml was then divided by the required concentration of cells for the assay, which for cell culture and *ex vivo* ELISpot was 2×10^6 , giving the final volume in ml of cell suspension.

2.3 Activation of memory B cells in culture with polyclonal stimulants

2.3.1 Description of the assay

Memory B cells do not secrete Ab spontaneously; they need to be activated in culture with polyclonal stimulants to differentiate into Ab secreting cells (ASCs). A number of mitogens have been used to activate human memory B cells *in vitro*. For example the unmethylated cytosine-phosphorothioate-guanine (CpG) DNA (Bernasconi et al., 2002; Crotty et al., 2004), pokeweed mitogen (PWM) (Crotty et al., 2004), *Staphylococcus aureus* cowan strain (SAC) (Crotty et al., 2004; Nanan et al., 2001), interleukin-2 (IL2) (Bernasconi et al., 2002; Nanan et al., 2001), lipopolysaccharide (LPS) and concanavalin A (ConA) (Crotty et al., 2004). The mechanisms of action are known for some of these mitogens; CpG-ODN are bacterial DNA fragments (or oligodeoxynucleotides), containing unmethylated, cytosine-guanine sequences that can activate B cells via the TLR4 and TLR9 (Bauer et al., 2001; Bernasconi et al., 2002; Takeshita et al., 2001). TLR9 is upregulated on activated and memory B cells and the CpG-TLR9 interaction lowers the activation threshold of the B cells (Krieg, 2002). The most potent ODN sequence for activating human B cells was identified as (5'TCGTCGTTTTGTCGTTTTGTCGTT-3') and was given the name ODN 2006 (Hartmann et al., 2000; Krieg et al., 2000). PWM is a TD mitogen that activates T cells co-cultured with B cells, leading to provision of T cell help to the resting B cells (Heilmann, 1987; Nespoli et al., 1978). SAC induces polyclonal activation of B cells in a T cell independent manner by cross-linking 2 or more BCR but appears to activate only B cells recently exposed to their specific Ag (Falkoff et al., 1982; Suzuki et al., 1988). T cell factors, such as IL2 allows non-cognate T cell

help. IL2 receptor is upregulated in activated B cells (Tvede et al., 1989). LPS acts through TLR4 (Bernasconi et al., 2003). However, the combination of these mitogens acts in synergy to activate B cells *in vitro*, allowing expansion and identification of Ag-specific memory B cells.

IL2 or PWM are necessary for the activation of memory B cells by limiting dilution as these T cell mitogens allow to keep the T cells in excess or not limiting. Indeed, the calculation of the memory B cell frequency by limiting dilution is only possible if only the cells assessed are limiting but not the supplier cells.

The specificity of this assay for memory B cells has been demonstrated previously. Crotty et al. (2004) have shown that the cells detected in this assay possess the surface phenotype of human memory B cells, CD27 (Crotty et al., 2004). It was shown that plasma cells originally present in PBMCs could not survive for more than 2-3 days in culture without specific cytokines or the presence of stromal cells (e.g. fibroblasts) or cell signalling by a variety of cell surface proteins (Cassese et al., 2003; Minges Wols et al., 2002; Pihlgren et al., 2006) and therefore, could not be detected by the post-culture enzyme-linked-immunospot (ELISpot) assay. It was also suggested that naïve B cells are not activated in culture with polyclonal mitogens, because they require BCR triggering in the presence of costimulation via CD40 ligand and the presence of promitotic cytokines such as IL2 and IL15 (Banchereau et al., 1994; Bernasconi et al., 2002; Rajewsky, 1996).

A variety of combinations of the polyclonal mitogens cited above have been compared for their ability to activate memory B cells. Crotty et al. (2004) have identified that PWM/SAC/CpG was the best combination to induce polyclonal proliferation of IgG memory B cells and their

differentiation into ASCs (Crotty et al., 2004) (Figure 2.3-1). Others have found that the culture conditions that induce the greatest production of ASCs after culture of PBMCs are SAC or PWM alone, or combinations of SAC/IL2, SAC/PWM or SAC/PWM/CpG (E. Clutterbuck and D. Kelly, unpublished observations).

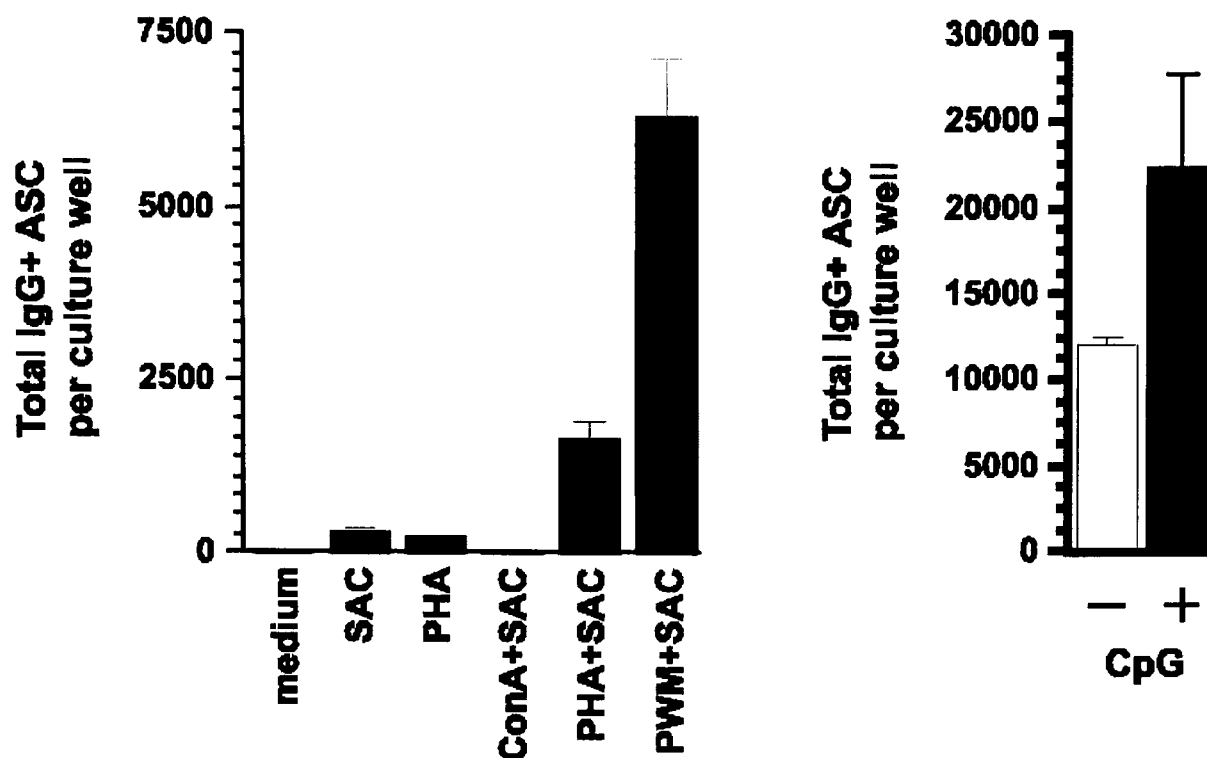


Figure 2.3-1: A variety of mitogens were screened by Crotty et al. (2004) for maximal activity inducing polyclonal proliferation of IgG-memory B cells and their differentiation into ASCs. PBMCs of healthy donors were cultured for 5 days with varying concentration of polyclonal mitogens. After 5 days, the cells were washed and plated onto ELISpot plates coated with anti-human Ig-Abs, and ASCs were detected using an α -IgG γ secondary Ab. The left graph shows a comparison of several mitogens. Medium, medium alone. SAC, 1/10,000. Phytohemagglutinin (PHA), 2.0 μ g/ml. Concanavalin A (ConA), 3 μ g/ml. PWM, 1/100,000. Of the mitogens tested, PWM and SAC provided the best proliferation and differentiation of memory B cells into ASCs. Error bars were calculated as the standard error of mean of three samples. The right graph shows a comparison of ASC production in PWM+SAC supplemented or not with CpG. Addition of CpG resulted in a 1.5 times increase in the total IgG-ASCs observed (Crotty et al., 2004).

Finally, culture duration has also been assessed previously; Crotty et al. (Crotty et al., 2004) and others (E. Clutterbuck and D. Kelly, unpublished observations) have demonstrated that culture of PBMCs for 5-6 days gave the maximal numbers of ASCs.

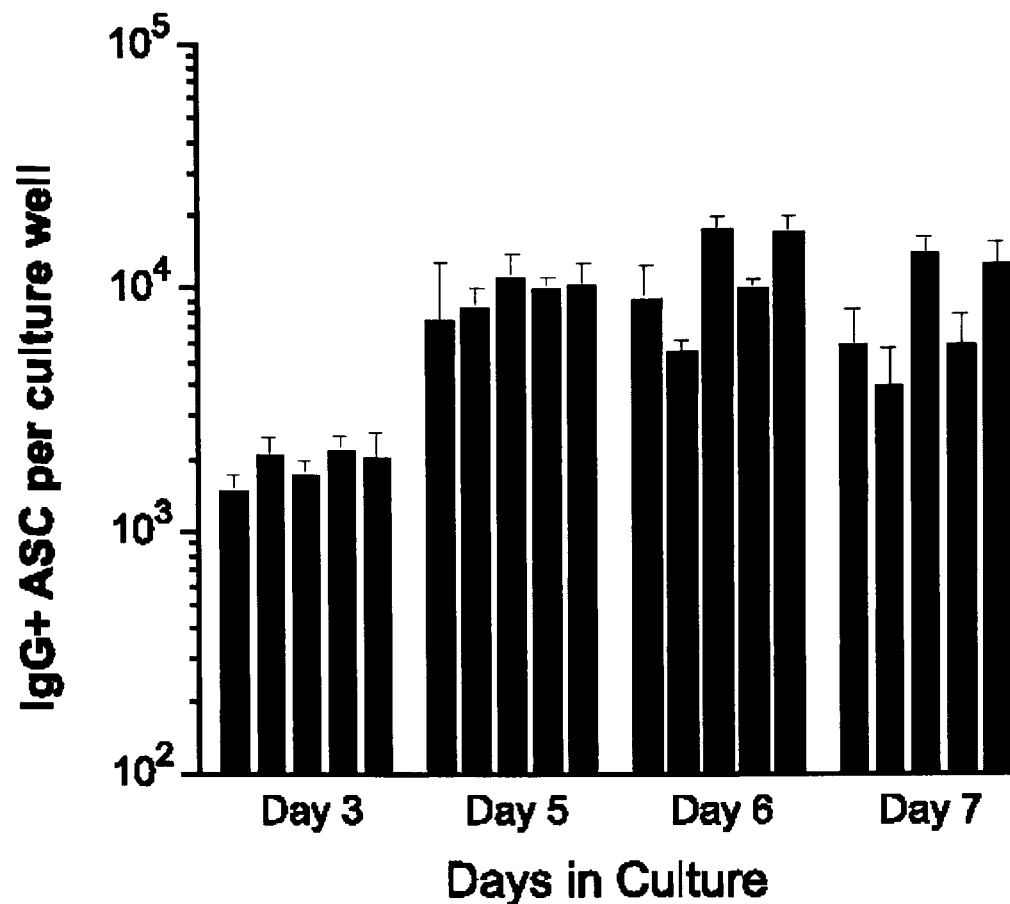


Figure 3-2: The optimal culture duration was screened by Crotty et al. (2004); the kinetics of memory B cell expansion and differentiation into ASCs was assessed by ELISpot after stimulation with PWM and SAC, after 3, 5, 6, and 7 days. Five individuals are shown. For each individual, six replicates wells were stimulated. Error bars show the full growth range among the six replicates. Maximal IgG-ASCs were detected on days 5 and 6 post-culture (Crotty et al., 2004).

2.3.2 Reagents

- 10% FCS (as previously defined)
- PWM (1mg/ml)
- CpG-2006 TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT (200µg/ml) in distilled water (Invitrogen, UK)
- SAC (Calbiochem, UK)
- 96 well, round bottom, cell culture treated, sterile cell culture plates (Fisher, UK)

2.3.3 Protocol

PBMCs prepared from peripheral blood were re-suspended in complete medium with 10% FCS at a final concentration of 2×10^6 PBMCs/ml and added to 96-well round-bottomed culture plates to give 2×10^5 PBMCs/well (100 μ l/well). Stimulated medium, containing SAC (1/5000), PWM (83 ng/ml) and CpG-2006 (2.5 μ g/ml) in 10% FCS was added to the cells to give a total volume of 200 μ l/well. The cells were incubated at 37°C in 5% CO₂ for 5 days for children and 6 days for adults, because for the childrens samples it was observed that 5 days of culture gave a higher number of ASCs than 6 days. After culture, the cells were harvested, washed and re-counted and the memory B cell frequency was expressed in relation to re-counted cultured lymphocytes. This contrasts with the limiting dilution assay, where the frequency of memory B cells was expressed in relation to the seeded PBMCs (see section 2.6).

2.4 Determination of Ag-specific plasma cells and memory B cells by ELISpot

2.4.1 Description of the assay

The ELISpot assay allows the quantification of Ag-specific memory B cells and plasma cells in human blood. The ELISpot assay can be performed either directly from blood (*ex vivo*) to assess Ag-specific plasma cells or after 5 or 6-day polyclonal stimulation of the PBMCs to assess Ag-specific memory B cells. The post culture ELISpot allows the detection of memory B cells that have differentiated into ASCs *in vitro*.

The specificity of the assay to detect Ag-specific plasma cells and memory B cells is evident by the fact that there are no cells detected in naïve subjects before immunisation, but that the specific-cells start to appear around day 7 post immunisation (Clutterbuck et al., 2006).

The negative controls used in this assay are duplicate wells coated with PBS. Ideally, an Ag, to which all humans are naïve would be utilised. For example, keyhole limpet hemocyanin has been used as a control (Crotty et al., 2004). An alternative negative control for this assay would be to put cells from the same naïve subject on each ELISpot assay; however, this is not practical. Similarly, it is not practical to use post-immunisation PBMCs as positive controls. The only positive controls used in this assay were wells coated with IgG to allow the detection of total IgG-ASCs and wells coated with diphtheria toxoid or tetanus toxoid Ags, as most people are immune to these Ags. However, the latter Ags can only be used as a positive control

for the memory B cell ELISpot as Ag- specific plasma cells are rarely found in peripheral blood at a steady state. Possible alternative methods for the development of both positive and negative controls would be to freeze plasma cells from immune or naïve donors, or to use a cell line (from immune or naïve donors) that could be continuously maintained and then differentiated into plasma cells as required.

2.4.2 Reagents

- 10% FCS (as previously defined)
- Purified tetanus toxoid (Statens Seruminstitut, Denmark)
- Purified diphtheria toxoid (Statens Seruminstitut, Denmark)
- Purified CRM₁₉₇ toxoid (Novartis Vaccine, Italy)
- Methylated human serum albumin (mHSA) (NIBSC, UK)
- Serogroup A (98/722), C (98/730), Y (01/426), and W135 (01/428) meningococcal polysaccharide (NIBSC, UK)
- Goat-anti-human immunoglobulin (Caltag laboratories, Burlingame, USA)
- MultiScreenTM-IP 96 well PVDF membrane ELISpot plates (Millipore, UK)
- Alkaline phosphatase conjugate: Goat-anti-human IgG (Calbiochem, UK)
- Substrate solution: alkaline phosphatase substrate kit (a 5-bromo-4-chloro-3-indolyl phosphate in nitro blue tetrazolium dissolved in aqueous dimethylformamide) (Bio-Rad, UK)
- 10xPBS (phosphate buffered saline): NaCl (80 g), KH₂PO₄ (3.14 g), Na₂HPO₄-7H₂O (20.6 g),

KCl (1.6 g) were dissolved into distilled water (1L). The pH was then adjusted to 7.2. Following sterilisation by autoclaving, the solution was stored at room temperature.

- 1xPBS (phosphate buffered saline): 10xPBS (100 ml) was added to distilled water (900 ml), and the pH was corrected to 7.2.

- PBS-Tween: Tween20 0.25% v/v, in 1xPBS (VWR International, UK)

- PBS-EDTA/FCS (as previously defined)

2.4.3 Protocol

2.4.3.1 Preparation of ELISpot plates

ELISpot plates were coated with either serogroup A, C, Y and W135 meningococcal polysaccharide (5 µg/ml) conjugated to mHSA (5 µg/ml), CRM₁₉₇ (10 µg/ml), diphtheria toxoid (10 µg/ml), tetanus toxoid (5 µg/ml), Hib polysaccharide (2 µg/ml), H1N1 subtype of the influenza A virus (2.5 µg/ml), dog kidney cell rabies vaccine (DKCV) (15 µg/ml) or goat-anti-human immunoglobulin (Ig) (10 µg/ml) in sterile PBS (100 µl/well). PBS alone was added to the Ag-blank wells. The ELISpot plates were then stored at 4°C until use (no longer than 2 months). Before adding the cell suspension, pre-coated ELISpot plates were washed (three times with PBS) and blocked with 10% FCS (200 µl/well) for a minimum of 30 minutes.

Subject		1	2	3	4	5	6	7	8	9	10	11	12
	A	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Hib	Hib
	B	Crm	Crm	Diph	Diph	Tet	Tet	Ig 1:10		Ig 1:100		PBS	PBS
	C	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Hib	Hib
	D	Crm	Crm	Diph	Diph	Tet	Tet	Ig 1:10		Ig 1:100		PBS	PBS
	E	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Hib	Hib
	F	Crm	Crm	Diph	Diph	Tet	Tet	Ig 1:10		Ig 1:100		PBS	PBS
	G	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Menc	Hib	Hib
	H	Crm	Crm	Diph	Diph	Tet	Tet	Ig 1:10		Ig 1:100		PBS	PBS

Table 2.44-1: Example of ELISpot plate layout (used for MenC-Infant study, section 4). 200,000 PBMCs or cultured lymphocytes were added to each well except Ig 1:10 and Ig 1:100. MenC = serogroup C meningococcal polysaccharide, Dip = diphtheria toxoid, Tet = tetanus toxoid, Ig 1:10 = total immunoglobulin (IgG or IgA or IgM) with one-tenth dilution of PBMC suspension (20,000 PBMCs/well), Ig 1:100 = total immunoglobulin (IgG or IgA or IgM) with 1/100 dilution of PBMC suspension (2,000 PBMCs/well). The increased number of wells for the MenC relative to other Ags was to increase the sensitivity of the assay for this Ag.

2.4.3.2 Detection of plasma cells

PBMCs (2×10^6 cells/ml) were added to the pre-coated ELISpot plates (100 μ l/well), and incubated overnight at 37°C in 5% CO₂. The PBMCs were removed from the plate before washing four times with PBS-Tween (200 μ l/well), and once with PBS (200 μ l/well), followed by a 4 hour incubation with either IgG-, IgA- or IgM-alkaline phosphatase conjugate, depending on study specifications, at a concentration of 1:5000 (100 μ l/well). Wells were then washed four times in PBS-Tween (200 μ l/well) and three times in dH₂O (200 μ l/well) before adding the substrate solution (50 μ l/well). The plates were developed, allowing the spots to be observed, and reaction was stopped using dH₂O (200 μ l/well). Finally, the plates were washed four times with dH₂O (200 μ l/well) before being dried in a drying oven.

2.4.3.3 Detection of memory B cells

After 5 or 6 days of culture, the cells were re-suspended and washed three times in PBS-EDTA/FCS. The cultured cells were then added to pre-coated ELISpot plates (2×10^5 cells /well) incubated overnight and developed as for the *ex-vivo* ELISpot (see above).

2.4.3.4 Calculation of B cell frequencies

Spots, corresponding to the zone of Abs secreted by individual ASCs, were counted using an ELISpot reader ELR02 (AID) and ELISpot software, version 3.2.3 (Cadama Medical Ltd., Stourbridge, UK). Spot-forming cells were counted and confirmed by visual inspection. Identical settings were used for all plates but different settings were used for the different Ags. The operator was blinded to which sample was being counted.

Ab forming spots were visualised as large, spherical shapes with “fuzzy” granular edges. Spots that did not fit this description were not counted and considered as *in vitro* artefacts.

The number of replicates depended on PBMC yield which varied, so varying numbers of cultures were analysed per donor and donors with the most cells had more replicates of B-cell assays for each Ag tested. The mean number of spots was calculated for each Ag from the replicates. Results were expressed as the number of ASCs per million PBMCs.

Some researchers express frequencies of memory B cells per total PBMCs (Bernasconi et al., 2002; Dorfman et al., 2005), whilst others express the memory B cell frequency as a percentage

of total IgG-ASCs (Crotty et al., 2003a; Nanan et al., 2001; Tangye et al., 2003). It has been suggested that the latter is more accurate, because the number of memory cells observed in the ELISpot assay after 5 days culture does not directly reflect the number of specific memory B cells seeded into a culture well. It was proposed that, despite *in vitro* expansion, the ratio of memory B cells per total IgG-memory B cells does not change. However, it was observed that the total number of IgG-memory B cells varied between individuals and time points. A more direct method of calculating the frequency of memory B cells involves the use of limiting dilution methods, which allows the detection of memory B cell precursors (see section 3). In this thesis, the frequency of memory B cells is expressed per million cultured lymphocytes (for memory B cells) and per million PBMCs (for plasma cells).

2.5 Determination of *N. meningitidis* serogroups A, C, Y and W135 IgG anticapsular Ab concentration by ELISA

2.5.1 Reagents

- Immulon 2-HB flat-bottomed 96-well polystyrene microtitration plates (Thermo Electron Corporation)
- 0.2 µm filters
- mHSA and serogroup A, C, W, Y meningococcal polysaccharide (as previously defined)
- Conjugate: monoclonal Ab– human IgG Fc PAN (1, 2, 3, and 4) HRP conjugated (Stratech Scientific Ltd.)
- 10x PBS (as previously defined)
- 10x Tris-Buffered Saline (TBS): NaCl (320 g), KCl (6.40 g), Trizma Base (3.76 g), Tris HCl (58.24 g), Brij-35 (132 ml) were dissolved into dH₂O (4 L). The pH was then adjusted to 7.2-7.4 using HCl or NaOH. This solution was stored at room temperature. For 1xTBS, 100 ml of 10xTBS was added to 900 ml of dH₂O and then the pH was corrected to 7.2-7.4.
- Substrate buffer solution: Working mixture of Na₂HPO₄-7H₂O (2.57 ml, of a stock solution of 53.61 g/litre) with C₆H₈O₇-7H₂O (2.43 ml, of a stock solution of 21.01 g/litre) in sterile dH₂O (5 ml) with 1 tablet of tetramethylbenzidine and 1 µl of H₂O₂.
- Wash buffer: TBS
- Coating buffer: PBS
- Blocking buffer: 5% NBBS (v/v) in PBS.
- Sample diluent/conjugate buffer: 5% NBBS (v/v), 0.001% Brij 35 (v/v) in PBS

2.5.2 Protocol

Meningococcal serogroup A, C, Y and W135 anticapsular IgG concentrations were determined by ELISA following a previously described method (Gheesling et al., 1994). Immulon 2-HB flat-bottomed 96-well polystyrene microtiter plates were coated with either serogroup A, C, Y or W135 meningococcal polysaccharide (5 µg/ml) conjugated to mHSA (5 µg/ml) in sterile PBS (100 µl/well) and incubated at 4°C overnight. Following blocking, eight two-fold dilutions of the reference serum (starting dilution 1:400 for A and 1:150 for C, Y and W135) (CDC 1999) and test sera (starting dilution 1:25) were made directly in the microtiter plate by well-to-well transfer with a multichannel pipette. The reference serum was assayed in triplicate and test sera were assayed in duplicate. Additionally, an internal quality control (an anti-meningococcal adult immune serum) was diluted (1:200, 1:1000, 1:4000) to yield optical densities (OD) approximately on the high, middle, and low portions of the reference curve. After overnight incubation at 4°C, microtiter plates were developed with a 1: 4000 dilution of the conjugate in sample/diluent buffer for 2.5 h at room temperature, followed by the substrate buffer solution, and the reaction was stopped after 30 min with 2 M H₂SO₄. The OD of each well was then read at 450 nm.

2.6 Determination of antigen-specific memory B cells by limiting dilution assay (LDA)

2.6.1 Activation of memory B cells using a LDA

2.6.1.1 Reagents

- Complete medium (as previously defined)
- 10% FCS (as previously defined)
- 5% FCS: complete medium (475 ml) with heat inactivated foetal calf serum (25 ml)
- PWM, CpG and SAC (as previously defined)
- IL2 (Novartis Vaccines, Italy)
- 96 well, round-bottom, cell culture-treated, sterile cell culture plates (Fisher, UK)

2.6.1.2 Protocol

PBMCs prepared from peripheral blood were re-suspended in complete medium with 5% FCS at a final concentration of 4×10^6 PBMCs/ml. To each well of a 96-well round bottomed culture plate complete medium with 5% FCS was added (100 μ l/well). PBMCs were then added to the first row of the culture plate. Double dilutions of the PBMCs were then performed from the first

row to the last row. Stimulated medium, which contained either CpG-2006 (5µg/ml) and IL2 (1000 UI/ml), or SAC (1/5000), PWM (83 ng/ml) and CpG (2.5 µg/ml) was added to the wells of the first 8 columns. 5% FCS was added to the wells of the last 4 columns (100 µl/well) (Figure 2.6-1). Cells were cultured in a total volume of 200 µl/well and incubated at 37°C in 5% CO₂ for 10 days. At the end of the culture period the plates were subject to centrifugation and the cultured supernatant was harvested and put on to a fresh cell culture plate and frozen for later analysis. For the ELISpot-based LDA (Slifka and Ahmed, 1996) the cells were washed in the plate three times with PBS-EDTA. Following the last wash 10% FCS was added to each well (100 µl/well). The cells were harvested with a manual multichannel pipette and added to a blocked ELISpot plate, pre-coated with the appropriate Ag and the standard ELISpot protocol was followed (as before).

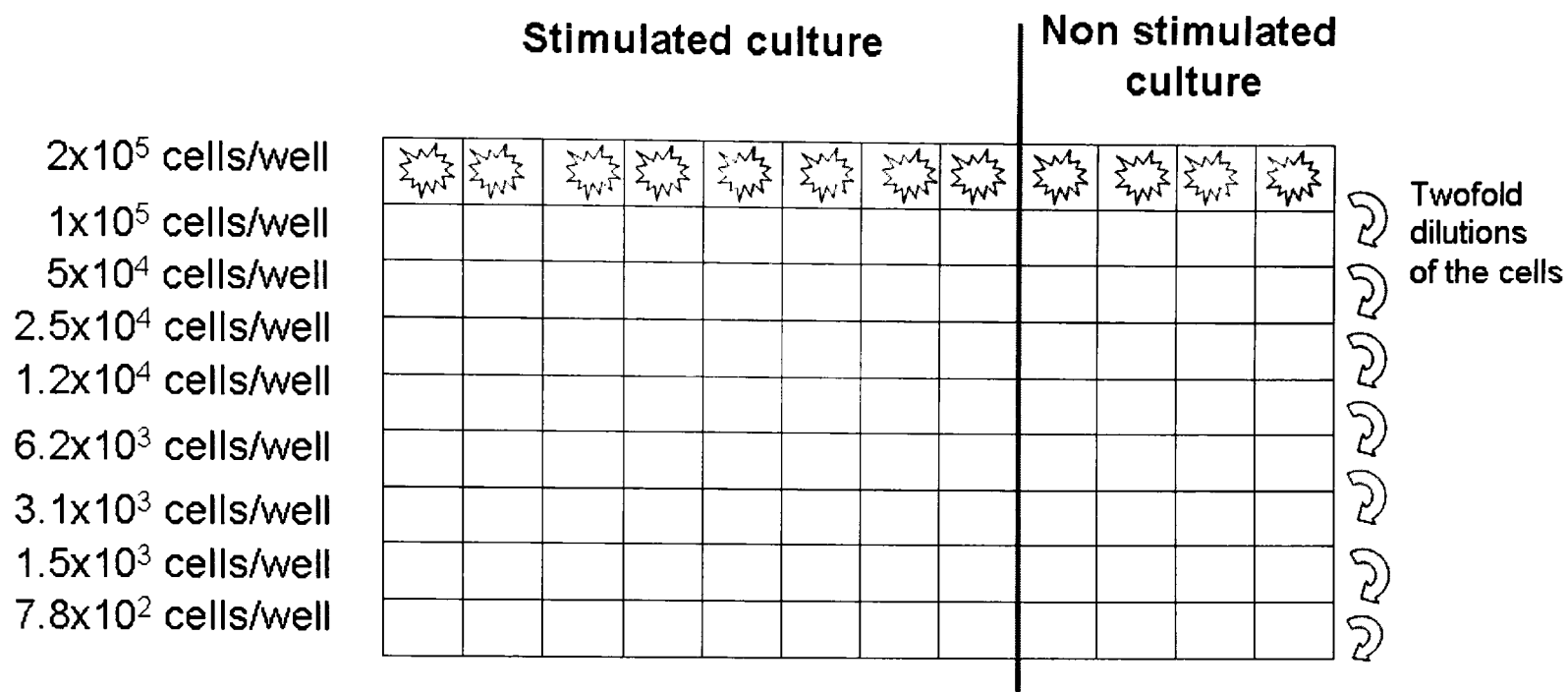


Figure 2.6-1: Plate layout for the activation of memory B cells using a LDA. PBMCs were cultured in graded doses in a total volume of 200 μ l/well in 96-well flat-bottomed plates. PBMCs were serially diluted (twofold dilutions) and stimulated medium containing either CpG/IL2 or SAC/CpG/PWM were added to each well of the first 8 columns. 5% FCS was added to each well of the last 4 columns.

2.6.1.3 Comparison of two different culture media to activate memory B cells for use in a LDA

The detection of memory B cells either by ELISpot or by LDA requires the activation of the cells to induce their differentiation into ASCs. In the first study of “comparison of methods to assess memory B cell frequencies” (section 3.2), SAC/CpG/PWM (medium 1) was used for the ELISpot assay and CpG/IL2 (medium 2) was used for the LDA. Medium 1 had been determined previously as the best culture medium to activate memory B cells for the ELISpot assay (Crotty et al., 2004). Medium 2 was chosen for the LDA because this assay was adapted from a protocol used by Novartis Vaccines (Siena) where CpG/IL2 was used for the activation of memory B cells.

The two media were compared for their ability to activate memory B cells for use in the LDA. The frequency of influenza H1N1-, diphtheria toxoid-, tetanus toxoid- memory B cells was assessed using PBMCs obtained from the same individual using medium 1 (SAC 1:5000, CpG 2.5 µg/ml and PWM 83ng/ml) and medium 2 (IL2 100 UI/ml and CpG 5 µg/ml) following 10 days of culture. The results (Figure 2.66-2) show that medium 1 provides the best culture conditions to activate memory B cells *in vitro* for the LDA.

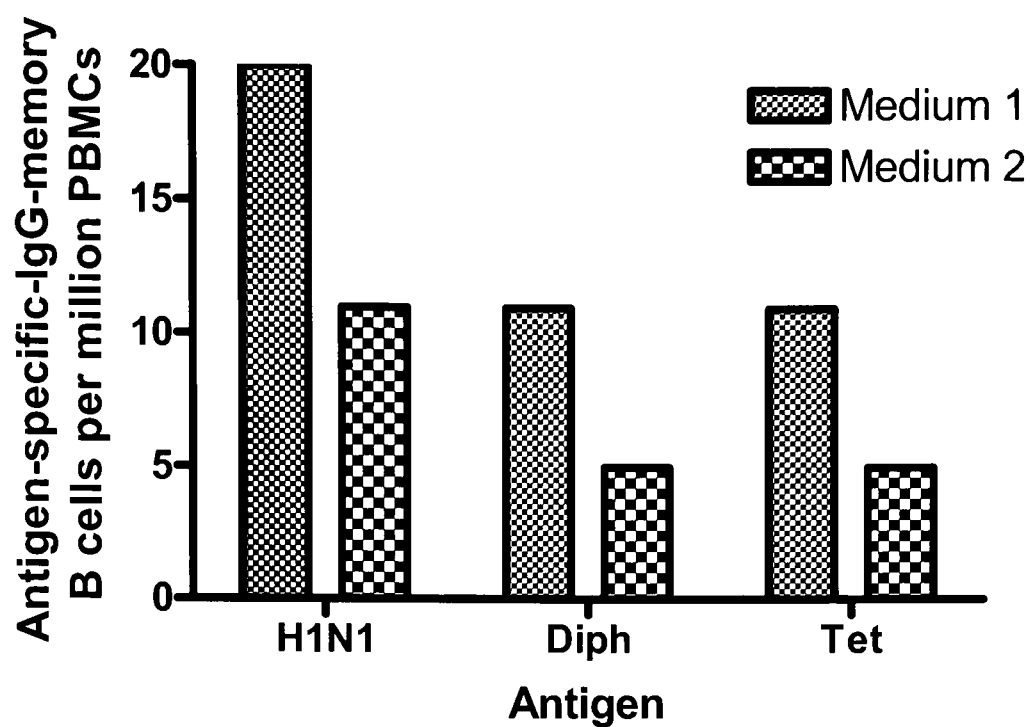


Figure 2.66-2 : Comparison of two different culture media for their ability to activate memory B cells for use in the LDA. The frequency of H1N1-, diphtheria toxoid-, tetanus toxoid-memory B cell frequencies was assessed using PBMCs from the same individual stimulated *in vitro* under limiting dilution with medium 1 (SAC 1:5000, CpG 2.5 $\mu\text{g}/\text{ml}$ and PWM 83ng/ml) or medium 2 (IL2 100 UI/ml and CpG 5 $\mu\text{g}/\text{ml}$) following 10 culture. The results are from one experiment in one individual.

2.6.2 Enumeration of B cells, IgG-B cells and memory B cells in PBMCs by flow cytometry

2.6.2.1 Reagents

- anti-CD19 PerCp (BD Biosciences Ltd., Oxford, UK)
- anti-CD27 PE (BD Biosciences Ltd., Oxford, UK)
- anti-IgG Fitc (BD Biosciences Ltd., Oxford, UK)
- G1G2a FITC/PE (BD Biosciences Ltd., Oxford, UK)
- G2G1 PercP (BD Biosciences Ltd., Oxford, UK)
- BD fluorescent-activated cell sorter (Facs) tubes (BD Biosciences Ltd., Oxford, UK)
- Facs Lysis solution (BD Biosciences Ltd., Oxford, UK)
- Cell Fix solution: 1% paraformaldehyde solution in PBS
- 1x PBS

2.6.2.2 Protocol

PBMCs from each donor were washed as described above (section 2.1) and re-suspended in complete medium with 5% FCS at a concentration of 2×10^6 cells/ml. The cell suspension (50 μ l) was incubated with labelled Abs (5 μ l), anti-CD19 PerCp, anti-CD27 PE, anti-IgG FITC, at room temperature in the absence of light for 15 minutes. The cell suspension (50 μ l) was also

incubated with irrelevant Abs (10 μ l of G1G2a Fitc/PE and 5 μ l of G2G1 PercP) as control. The cells were washed twice, first with Facs lysis solution (500 μ l) and then with PBS (500 μ l) before fixing with 1% paraformaldehyde solution in PBS. Analysis was performed using a three-colour BD FacSCAN (fluorescence-activated cell sorters) and BD CellQuest software version 3.1. Cells were selected on the basis of size and granularity by forward- and side-scatter measurements, and the lymphocyte populations were gated accordingly. A maximum of 3000 events were collected for each PBMC sample (Figure 2.6-3).

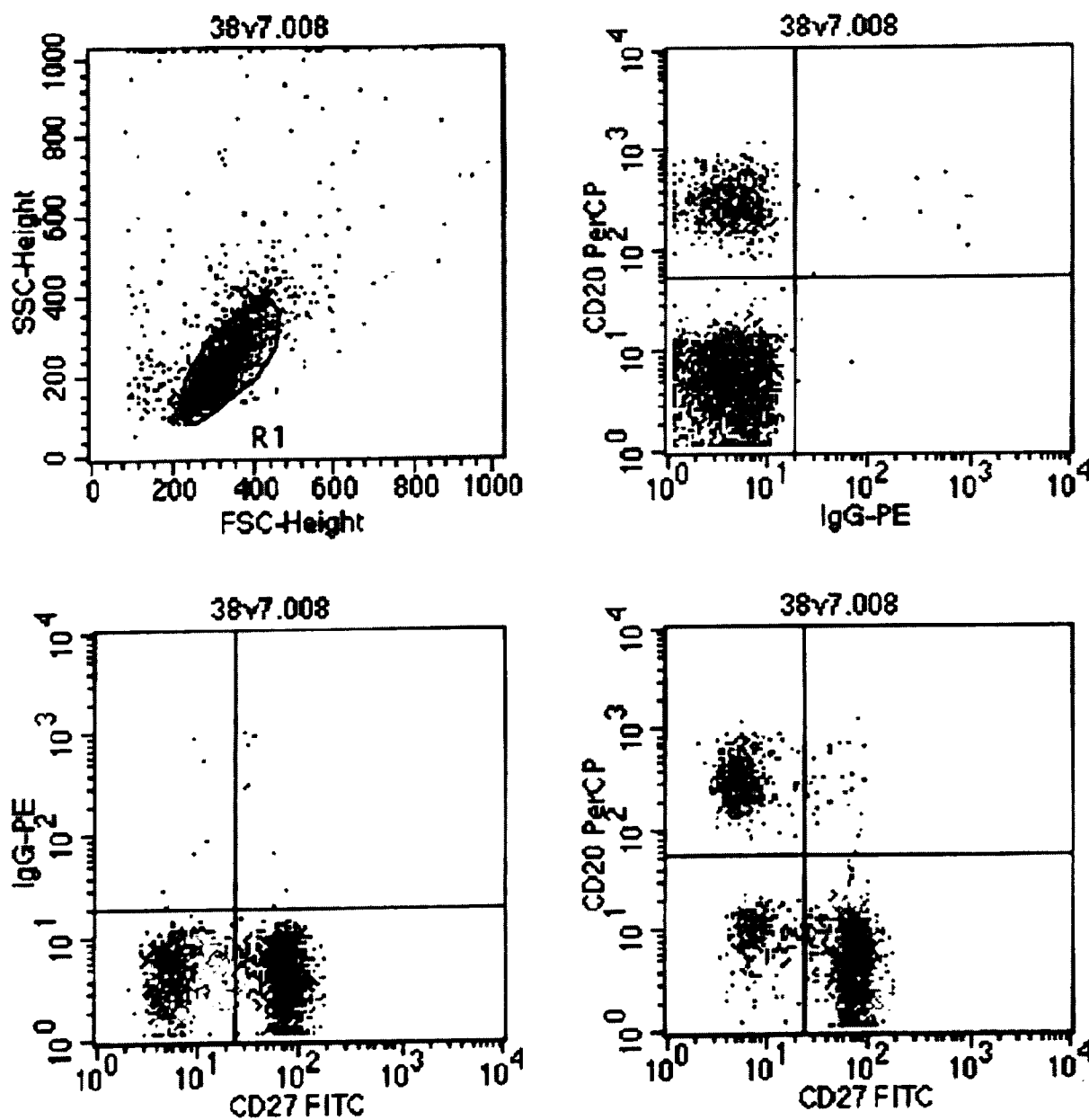


Figure 2.6-3: Enumeration of the B cell subpopulation by flow cytometry. The lymphocytes were gated on the basis of side scatter versus forward scatter (R1). The percentage of cells expressing cell surface marker CD20 only, or CD20 and IgG, or CD20 and CD27, or CD27 and IgG was determined.

2.6.3 Determination of anti-diphtheria toxoid-, CRM₁₉₇-, tetanus toxoid- and H1N1- specific IgG concentration in cultured supernatant by ELISA

2.6.3.1 Reagents

- Microplate Nunc Maxisorp Immuno 96-well flat-bottomed U-shaped plates (Fisher Scientific)
- Purified tetanus toxoid, diphtheria toxoid, CRM₁₉₇ toxoid, H1N1 (as previously defined)
- Anti-human IgG (γ -chain specific) alkaline phosphatase Ab, produced in goat, affinity-isolated Ab
- Substrate solution buffer: alkaline phosphatase yellow liquid substrate system ELISA (5ml) with p-nitrophenyl phosphate (pNPP) tablets (5 tablets)
- Wash buffer: PBS-Tween
- Coating buffer: PBS
- Blocking buffer: 5% dried milk powder (w/v) in PBS
- Sample diluent/conjugate buffer: 0.05% (v/v) Tween, 0.1% dried milk powder (w/v) in PBS

2.6.3.2 Protocol

Microplate Nunc Maxisorp Immuno 96 well flat-bottomed, U-bottom plates were coated with either CRM₁₉₇ (10 μ g/ml), diphtheria toxoid (10 μ g/ml), tetanus toxoid (5 μ g/ml), H1N1 (2.5 μ g/ml) in PBS buffer (100 μ l/well). Plates were sealed and incubated at 4°C overnight. Plates were blocked after discarding the plate contents but without washing (200 μ l/well) and

incubated for 2 hours at room temperature. Plates were washed with PBS-Tween and supernatant (50µl) from defrosted cultured plates was added to the ELISA plates using exactly the same layout as the culture plates. In each plate, a positive control serum was added to the last row and serially diluted from 1:100 to 1: 51200. Complete medium from the cultured plate was added to two wells, which corresponded to the blank wells. Plates were then incubated at room temperature for 1 hour with a 1:4000 dilution of the conjugate in sample diluent/conjugate buffer. Plates were washed and substrate solution buffer was added (100µl/well). Plates were incubated for exactly 30 minutes in the absence of light at room temperature. To stop the reaction 2M NaOH (50µl/well) was added and plates were incubated for precisely 5 minutes in the absence of light. The OD of each plate was read at 405 nm (with 630 nm reference filter) using a Dynex Microplate Reader (ThermoLabs Inc., Basingstoke, UK).

2.6.4 Detection of anti-serogroup C *N. meningitidis* specific IgG concentration in cultured supernatant by ELISA

The concentration of anti-serogroup C *N. meningitidis*-specific IgG in cultured supernatant was determined using the ELISA described in section 2.5. Defrosted culture supernatant (50 µl) was added to each ELISA well following exactly the same layout as the corresponding culture plate. On each ELISA plate, a positive control serum was added in the last row and serially diluted from 1:100 to 1: 51200 (pink in table 2.6-1), and complete medium from the cultured plate was added in two wells, which corresponded to the blank wells (blank in table 2.6-1).

2.6.5 Determination of the frequency of Ag-specific memory B cells

2.6.5.1 Determination of the positives and negatives “wells” for the presence of antigen-specific antibody

Using the OD obtained from ELISA results, the number of wells that were positive and negative for the presence of Ag-specific Ab was determined for each cell dilution of the LDA plate.

The OD obtained from the non-stimulated cultures (green in table 2.6-1) served as a cut-off in order to define the positive and negative culture wells for Ag-specific Abs. Without polyclonal stimulants, memory B cells are not expected to differentiate into plasma cells and, therefore, the Abs detected in non-stimulated culture wells correspond to pre-existing Ag-specific plasma cells. To eliminate the “background” Ab from pre-existing Ag-specific plasma cells, the cut-off for selecting positive culture wells was the mean of all non-stimulated culture wells plus three standard deviations.

cell conc	Nb of +	Nb of -												
2x10 ⁵	7/8	1/8	0.395	0.093	0.465	0.927	1.224	1.68	1.576	0.836	0.095	0.099	0.088	0.084
1x10 ⁵	7/8	1/8	0.372	0.092	0.164	0.392	0.172	0.48	0.182	0.103	0.073	0.073	0.081	0.073
5x10 ⁴	5/8	3/8	0.108	0.088	0.093	0.101	0.128	0.114	0.097	0.22	0.068	0.081	0.073	0.068
25x10 ⁴	0/8	8/8	0.057	0.075	0.076	0.066	0.076	0.076	0.071	0.076	0.075	0.069	0.07	0.068
12.5x10 ⁴	0/8	8/8	0.062	0.074	0.066	0.067	0.067	0.073	0.072	0.073	0.074	0.067	0.069	0.068
6.25x10 ⁴	0/8	8/8	0.059	0.061	0.098	0.061	0.071	0.071	0.067	0.07	0.069	0.068	0.069	0.069
3.125x10 ⁴	0/8	8/8	0.058	0.064	0.07	0.064	0.074	0.069	0.065	0.067	0.07	0.065	0.069	0.069
			3.429	3.154	2.533	2.03	1.486	0.799	0.512	0.31	0.202	0.128	0.068	0.064



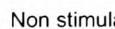



	positive control serum		stimulated cultures (negatives for specific antibodies)		Non stimulated cultures
	non stimulated cultures		stimulated cultures (positives for specific antibodies)		average
	blank				stdev
					cut off

Table 2.66-1: Example of OD obtained by ELISA from the supernatant of a cell culture plate tested for the presence of influenza-specific-IgG. The mean of all non-stimulated culture wells (green) plus three standard deviations, gives the cut-off to define the positive and negative wells. In the example in the table above the cut-off is an OD of 0.0985 (see the small table below right) and all wells ≥ 0.0985 are defined as positive (in yellow) and all wells < 0.0985 as negative (red) for the presence of influenza-specific Ab. For the positive control, an immune serum is serially diluted on the last row of the ELISA plate (pink). For the negative control 5% FCS, which has been used for resuspension of the cells and which was incubated in individual wells on the same plate as the cells, is used (blank wells). The number of negative and positive cultures for each cell dilution is written on the left of the table.

2.6.5.2 Measurement of Ag-specific memory B cell frequencies by LDA using the Reed & Muench method

Reed and Muench developed a method for estimating the 50% endpoints in biological procedures in 1938 (Reed, 1938). The 50% endpoint was defined as the dilution of a drug to test at which half of the test animals reacts or dies. This endpoint is less affected by small chance variations than is any other. Later the Reed & Muench method was adapted to interpret frequency of memory B cells by LDA. Essentially, it looks at the 50% endpoint on the basis of the accumulation of the number of positive and negative culture wells for each cell dilution. The number of accumulated positive culture wells for each cell dilution is calculated by the addition of the number of positive culture wells from the lowest cell dilution to the highest cell dilution. The number of accumulated negative culture wells is calculated by the addition of the number of negative culture wells from the highest cell dilution to the lowest cell dilution. In fact, it is assumed that a positive culture at a given cell dilution would also be positive at a lower cell dilution and a negative culture at a given cell dilution would also be negative at any higher cell dilution. Therefore, for a given cell dilution the cumulative number of positive wells would be the number of positive wells at the given cell dilution, plus those at higher cell dilution, and the cumulative number of negative wells would be the number of negative wells at this cell dilution, plus those at lower cell dilution. The ratio of positive wells for each cell dilution is calculated as follows:

$$\frac{(\text{cumulative positive})}{(\text{cumulative positive} + \text{cumulative negative})}$$

Then, the following formula

$$PD = \frac{(\text{ratio at dilution next above}) - 50 \text{ percent}}{(\text{ratio next above 50 percent}) - (\text{ratio next below 50 percent})}$$

gives the proportional distance (PD) of the endpoint above the dilution giving the next below the 50 percent positive wells. Since the dilutions are increasing on a logarithmic scale, it is necessary to obtain the final reading as follows:

$$\log \text{ of lower dilution} + PD \times \log 2 (\text{dilution factor}) = \text{sum of log (50 percent endpoint)},$$

which makes the endpoint at the dilution of $2^{\text{sum of log (50 percent endpoint)}}$, which is the cell dilution corresponding exactly to 50% of the positive wells. This dilution is assumed to correspond to the dilution at which there is exactly one Ag-specific-memory B cell precursor (MBCp) per well. The frequency of Ag-specific-memory B cells can be expressed in relation to the total number of PBMCs, B cells, memory B cells or IgG-B cells. The percentage of each B cell subpopulation in the PBMCs is assessed before culture by flow cytometry (section 2.6.2).

Input PBMC 1st well	CD20+ (% of PBMC)	CD20+CD27+ (% of PBMC)	CD20+IgG+ (% of PBMC)			
200000	20.87	1.77	0.54			
dilution	positive	negative	cum pos	cum neg	ratio	
1.0000000	8	0	22	0	1.0000	
0.5000000	6	2	14	2	0.8750	
0.2500000	4	4	8	6	0.5714	
0.1250000	2	6	4	12	0.2500	
0.0625000	1	7	2	19	0.0952	
0.0312500	1	7	1	26	0.0370	
0.0156250	0	8	0	34	0.0000	
PD						0.2222
$\log_2(\text{dilabove}) + (\text{PD} \cdot \log_2(0,5))$						-2.2222
dilution corresponding 50% of positive wells						0.2143
N° of PBMC corresponding to 50% of Ag-positive wells						42862
N° of B corresponding to 50% of Ag-positive wells						8945
N° of Ag-ASC precursors/1M B cells						112
N° of CD27+ corresponding to 50% of Ag-positive wells						759
N° of Ag-ASC precursors/1M memory B cells						1318
N° of IgG+ B cells corresponding to 50% of Ag-positive wells						231
N° of Ag-ASC precursors/1M IgG+ B cells						4320

Table 2.66-2: Measurement of Ag-specific memory B cell frequency by the LDA using the Reed & Muench method. At the top of the table, the number of PBMCs/well in the first cell dilution and the percentage of each B cell sub-population in PBMCs measured by flow cytometry are reported. The number of positive and negative culture wells for the presence of Ag-specific Ab is reported for each cell dilution. The cumulative number of positive and negative culture wells for the presence of Ag-specific Ab is shown for each cell dilution. The cell dilution corresponding exactly to 50% of positive wells is also calculated. This cell dilution corresponds to one Ag-specific memory B cell precursor (MBCp) per well. The frequency of Ag-specific-memory B cells can then be expressed in relation to the total number of PBMCs, B cells, memory B cells or IgG-B cells.

2.6.5.3 Measurement of Ag-specific memory B cell frequencies by LDA using the Poisson method

The Poisson distribution is a binomial discrete distribution that supposes that events happen randomly and independently in time at a constant rate. Applied to immunology by Lefkovits and Waldman in 1979 (Lefkovits and Waldmann, 1999), the Poisson statistical analysis states that when a population of cells, c , carrying an undefined number of specific precursor cells is randomly and independently distributed among wells, w , in a 96-well microplate, the number of precursor cells per well, r , should follow the Poisson distribution, as follows: $\frac{c}{w} = \mu$ (the average number of cells/well) and F_r (the fraction of wells with r precursors) = $\frac{\mu^r \cdot e^{-\mu}}{r!}$.

If r is considered to be zero F_r will be the fraction of wells that are devoid of any specific precursor (negative fraction) and the formula becomes $F_0 = \frac{\mu^0 \cdot e^{-\mu}}{0!}$ or $F_0 = e^{-\mu}$, where

F_0 represents the fraction of negative culture wells, e is the base of the logarithm and μ represents the average number of specific precursors per well. Assuming a mean number of 1 Ag-specific MBCp/well ($\mu=1$), the number of wells with 0 cell on average is 0.37 (37%) for F_0 , when substituted in the above formula: $F_0 = e^{-1} = 0.37$.

Adapted to the LDA to measure the frequency of Ag-specific MBCp, the values of negative culture wells for different PBMCs inputs are measured and the cell dilution corresponding to

37% of negative culture wells corresponds to 1 Ag-specific memory B cell per well.

The graphical representation of the Poisson distribution is based on a semi-log plot. The formula $\mu = -\ln F_0$ means that the negative logarithm of the fraction of negative culture wells is linearly proportional to the mean number of precursor cells per culture wells. It follows that, if the negative logarithm of the fraction of negative culture wells ($-\ln F_0$) is plotted on the y axis, and the cell input (μ) at the linear scale on the x axis, it is expected that the experimental points will fit a straight line passing through the origin and that the intercept of the Poisson straight line with the 37% horizontal line will define the position of $\mu = 1$. A “LDA program” has been developed by Lefkovits and Waldman (1999) to perform the calculation procedures of LDA according to the Poisson distribution (Lefkovits and Waldmann, 1999) (Figure 2.6-4).

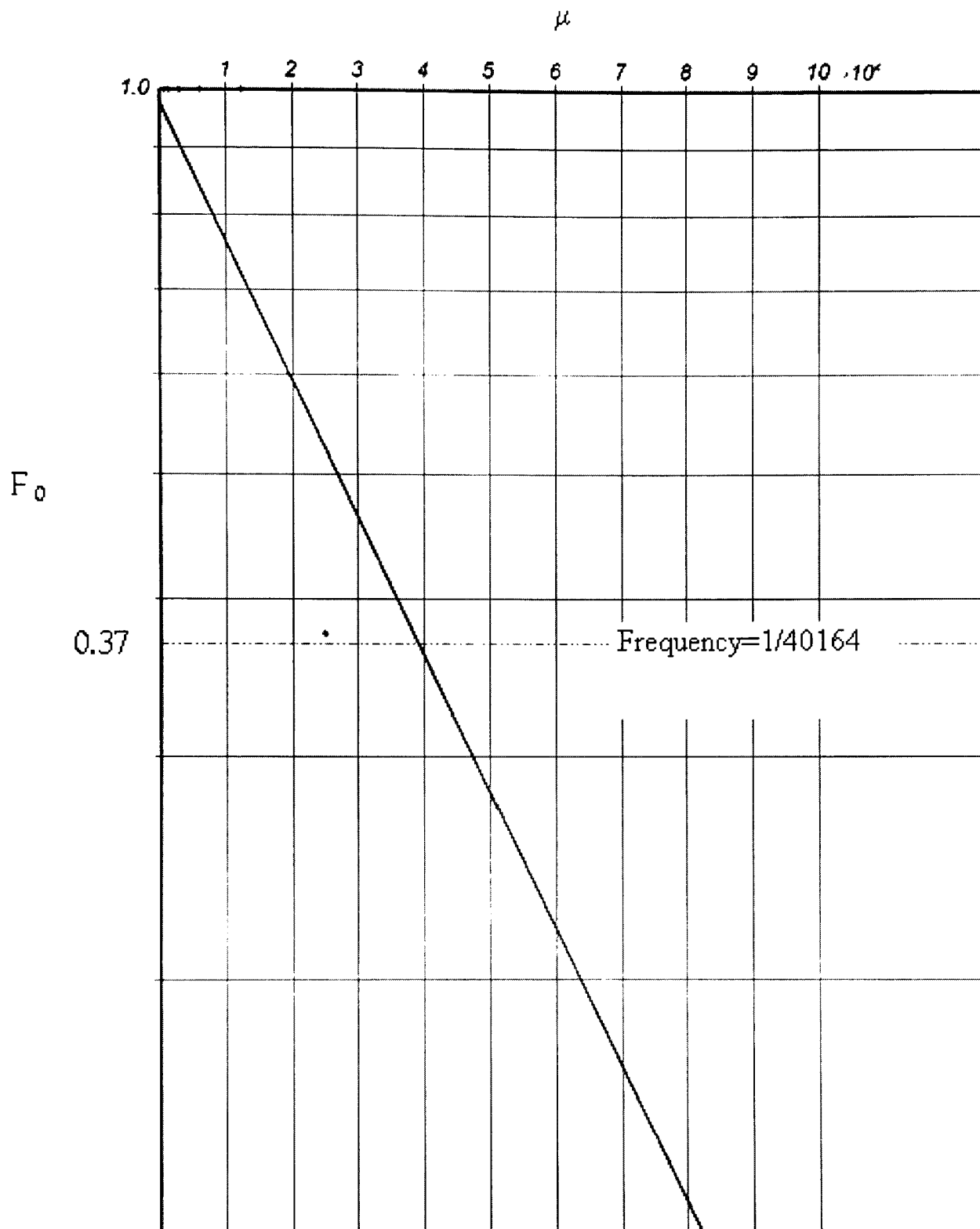


Figure 2.6-4: Graphical representation used for measurement of Ag-specific memory B cell frequencies by LDA using the Poisson distribution. The fraction of negative culture wells is the fraction of wells that are negative for the presence of Ag-specific Ab for each cell dilution. The negative logarithm of the fraction of negative culture wells ($-\ln F_0$) is plotted on the y axis, and the cell input (μ) at the linear scale on the x axis. The intercept of the Poisson straight line with the 37% horizontal line defines the position of $\mu = 1$, i.e. the cell dilution corresponding to exactly one Ag-specific MBCp per well.

2.7 Determination of Ag-specific memory B cells by an ELISpot-based LDA

2.7.1 Description of the assay

The ELISpot-based LDA follows the same basic principles as the standard LDA (ELISA-based LDA), but it directly measures the ASCs derived from the MBCp instead of measuring the accumulated Abs that they produce. The culture duration is 5 days for the ELISpot-based LDA as for the standard ELISpot. In contrast, the ELISA-based LDA requires 10-12 days of *in vitro* stimulation of memory B cells for the same degree of sensitivity (Slifka and Ahmed, 1996). It was shown that after 20-36 hours of culture with polyclonal stimulants, memory B cells proliferate and start to differentiate into ASCs (Tangye et al., 2003). After 5 days, the majority of memory B cells will be terminally differentiated and the newly generated ASCs will start to secrete Ab in culture medium. After 10 days, sufficient Ab might have accumulated in culture medium, however, after this time the resultant ASC might have started to die without specific signals from supporting stromal cells (Crotty et al., 2004; Pihlgren et al., 2006).

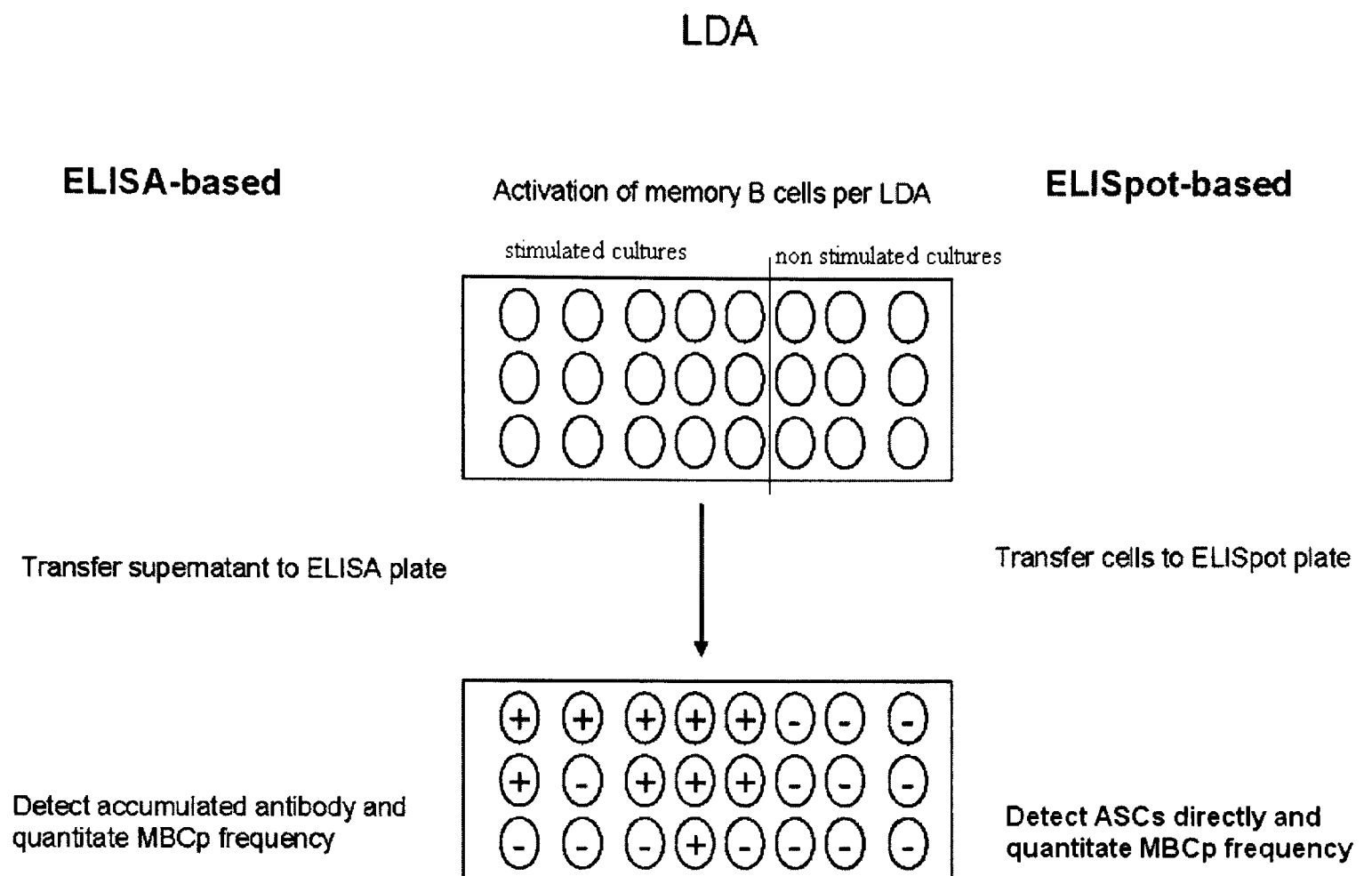


Figure 2.7-1: This diagram illustrates the process of ELISpot based and ELISA based LDA.

The ELISpot- based LDA is similar to ELISA-based LDA except that the quantitation of MBCp is determined at the level of ASCs instead of measuring specific Ab that is secreted by these cells into the culture medium.

2.7.2 Protocol

The memory B cells were cultured under limiting dilution as for the ELISA-based LDA (section 2.5.1). At the end of the culture, the culture plates were subject to centrifugation and the cultured supernatant was removed. The cells were washed in their culture plate three times with

PBS-EDTA/FCS (0.05%). Following the last wash, 10% FCS (100 μ l/well) was added and the cells were harvested with a manual multichannel pipette and added to a blocked ELISpot plate, pre-coated with the appropriate Ag. Then the standard ELISpot protocol was followed (see section 2.3). The cultured supernatant was transferred to a new cell culture plate for direct comparison of the ASCs detected by ELISpot and of the accumulated Abs, that have been produced by these ASCs in culture supernatant, by ELISA (see section 3.3.3.4).

Pre-existing Ag-specific plasma cells were determined in the non-stimulated part of the culture plate because memory B cells are not activated to differentiate into ASC in the absence of polyclonal stimulants. The average number of Ag-specific ASC detected in the non-stimulated cultures (4 last columns) (see Figure 2.6-1) served as a cut-off in order to define the positive and negative culture wells for Ag-specific ASC. This represented a background number of a maximum of one pre-existing ASC/well at the first cell dilution (2×10^5 PBMCs) and there were no Ag-specific ASC detected in the following cell dilutions. Therefore, individual wells with > 1 ASC were scored positive for containing an Ag-specific ASC. The proportion of positive and negative culture wells was determined for each cell dilution. From the proportion of positive and negative culture wells at each cell dilution, Ag-specific memory B cell frequencies were calculated using the Reed & Muench or Poisson methods as for the ELISA-based LDA (see section 2.6.5.2 and 2.6.5.3).

3 Comparison of methods to detect memory B cells in peripheral blood

3.1 Introduction

3.1.1 Why is assessment of memory B cells important?

As discussed earlier in section 1.9, immunity to *N. meningitidis* and other encapsulated bacteria is principally mediated through the binding of IgG to a specific bacterial Ag followed by complement activation or opsonophagocytosis. Protective immunity after immunisation has traditionally been correlated with Ab production. For MenCV, a serum bactericidal Ab titre \geq 1:4 using human complement indicates induction of a protective immune response. However, in young children, circulating Abs drop to non-protective levels after the first year of immunisation (MacLennan et al., 2000; Richmond et al., 1999). There is convincing evidence that protective immunity can persist after immunisation when Abs are no longer detectable, as following the introduction of routine Hib immunisation in infants, it became clear that some individuals with low or undetectable Ab concentrations were still protected against disease (Adams et al., 1993; Eskola et al., 1990). Thus, quantitative parameters other than serum Ab levels may reflect persistent immunity more appropriately.

B cell activation and differentiation into plasma cells and memory B cells is the principal determinant of long-term humoral immunity elicited by immunisation. Animal studies have

shown that plasma cells can persist for several years in bone marrow niches after their activation and continue to secrete Abs. Although the existence of long-lived plasma cells in humans remains controversial, these cells might be responsible for the long-term maintenance of Abs (Manz et al., 2002; Slifka et al., 1998). Memory B cells are programmed to rapidly proliferate and differentiate into plasma cells during a new exposure to an Ag. If this memory B cell response occurs rapidly enough, the vaccinated individual should be protected from the invading pathogen by newly synthesised Abs. Memory B cells may also be responsible for long-term maintenance of Abs through their continuous differentiation into plasma cells (Bernasconi et al., 2002; Slifka et al., 1998).

Previous studies assessing the relationship between memory B cells and Abs at a steady state have reported strong (Bernasconi et al., 2002), moderate (Crotty et al., 2003a; Dorfman et al., 2005) and negative correlations (Leyendeckers et al., 1999; Nanan et al., 2001). Similarly, contradictory results have been reported between the correlations of Ab and memory B cell responses, or Ab and plasma cell responses after immunisation (Bernasconi et al., 2002; Kelly et al., 2006). Therefore, memory B cell and plasma cell responses following immunisation should be assessed as an independent correlate of long-term protection.

3.1.2 Why is it difficult to estimate the number of B cells in the laboratory?

Studies of plasma cells and memory B cells in humans are complicated by the fact that their tissues of residence are not easily accessible; at a steady state, plasma cells reside in bone marrow (Slifka et al., 1995) and most memory B cells are thought to reside in secondary lymphoid organs (Maruyama et al., 2000) or in the bone marrow (Osmond, 1986), although

they may also re-circulate between secondary lymphoid organs through the blood (Nanan et al., 2001).

At rest, Ag-specific plasma cells are present at extremely low frequencies in peripheral blood but their frequencies briefly increase around day 6-7 post-immunisation with varied Ags in adults and children (Bernasconi et al., 2002; Clutterbuck et al., 2006; Kelly et al., 2006). This transient appearance of Ag-specific plasma cells in peripheral blood following immunisation is thought to represent newly generated plasma cells passing through the circulation en route to their long-term residential location (the bone marrow).

Several human studies have detected memory B cells specific for different Ags (diphtheria toxoid, tetanus toxoid, and smallpox) at rest in peripheral blood at a frequency between 0.01 and 1% of the total IgG-memory B cells, several years following immunisation (Crotty et al., 2003a; Nanan et al., 2001). Memory B cells specific for TI-Ags (e.g. serogroup C meningococcal polysaccharide) were observed at a frequency of $\leq 0.01\%$ several months or years after primary immunisation in adults and children (Kelly et al., 2006) (section 4). However, Ag-specific memory B cells increase in frequency in peripheral blood following immunisation and their frequency appears to be stable for one month after challenge with TD-Ags (Clutterbuck et al., 2006; Kelly et al., 2006). These cells might represent a mixture of newly generated memory B cells transiently travelling through the blood to secondary lymphoid organs and pre-existing re-circulating memory B cells. Therefore, the determination of the Ag-specific memory B cell pool might be difficult to extrapolate from the limited measurement of memory B cells in peripheral blood.

The process of cell separation and preparation is complex, because the cells are difficult to handle, can be activated easily and loss of cells may occur during this process. This process is best undertaken immediately using fresh samples, rather than batched for analysis at a later date as is the case for the serum samples. Recovery of frozen cells is easily compromised and recently activated cells may be especially susceptible to apoptosis. A high number of cells is needed to assess B cell frequencies, compared for example to the small amount of serum needed to assess Ab levels by ELISA. All these difficulties are particularly important when assessing immune response following immunisation of children because only a small volume of blood is available for testing and the frequency of specific memory B cells can be very low, especially for polysaccharide Ags.

Furthermore, the use of positive and negative controls is complicated in Ag-specific B cell assays. The detection of Ag-specific plasma cells and memory B cells utilises the property of Ag-specific Ab secretion. Memory B cells do not secrete Ab and should therefore, first be activated to proliferate and differentiate into plasma cells. The best negative or positive controls for Ag-specific B cell assays would be cells of a donor known to be immune or naïve to the Ag tested. However, it is neither practical nor ethical to bleed people repeatedly for the purpose of assay controls. Possible alternative methods for the development of both positive and negative controls would be to freeze plasma cells from immune or naïve donors, or to use a cell line (from immune or naïve donors) that could be continuously maintained and then differentiated into plasma cells as required. However, developments of such methods are complicated by the maintenance of cell viability. By contrast, serum Ab concentration can be measured by ELISA, an established and reproducible method, and the serum of naïve and immune donors can be stored for many years and remain stable.

Finally, a limitation of memory B cell assays is the difficulty in directly estimating the frequency of Ag-specific MBCp; memory B cells are activated to proliferate and differentiate into ASCs by culture with polyclonal stimulants and the resultant ASCs are detected through the secretion of Ag-specific Abs. However, the number of Ag-specific ASCs detected in the ELISpot plate does not exactly reflect the number of Ag-MBCp seeded in culture as the latter cells proliferate before differentiating into ASCs. Some research groups have tried to overcome this problem by expressing the memory B cell frequency as a percentage of total IgG-secreting cells (Crotty et al., 2003a; Nanan et al., 2001; Tangye et al., 2003). However, it has been demonstrated that the total number of IgG-memory B cells varied between individuals and time points (unpublished observations). The frequency of memory B cells can also be expressed per million cultured lymphocytes (Kelly et al., 2006). The use of the limiting dilution technique appears to overcome this problem (see below).

3.1.3 Techniques available to assess Ag-specific B cell frequency in the laboratory

There are currently three different methods to assess Ag-specific B cell frequencies in humans; the ELISpot, the LDA, and flow cytometric analysis. The last method, to be Ag-specific, requires a method of labelling the Ag. This has been shown to be possible for protein Ag (Odendahl et al., 2005), but appears to be more complicated for polysaccharide Ags, which bind non specifically to various cell types. Therefore, only the ELISpot and the LDA assays will be discussed in this chapter. The first step of the two methods is the same: separation of the

PBMCs from whole blood.

In the ELISpot assay, cells are first cultured and then transferred to an ELISpot plate, and Ag-specific ASCs are detected by an immunoenzymatic method. A spot develops in the well where an ASC has secreted one or several Abs that are specific for the coated Ag. It is assumed that each spot corresponds to one ASC. The number of spots in each well is counted with an ELISpot reader and also checked by eye. ELISpot allows the assessment of plasma cells (ex vivo ELISpot) and memory B cells (ELISpot after culture) (see section 2.3). To assess memory B cell frequencies, the memory cells are cultured with mitogens to drive the memory B cells to differentiate into ASCs (see section 2.2). At the end of the culture period, the cultured lymphocytes are washed, re-counted and re-suspended in a known concentration before being transferred to the ELISpot plate. The memory B cell frequency is expressed as a proportion of cultured lymphocytes.

In the LDA, memory B cells are cultured *in vitro* and the concentration of the Abs secreted into the supernatant by the memory B cells, which have differentiated into ASCs, is measured (see section 2.5). The cultured supernatant containing the secreted Abs is harvested after 10 days of culture and frozen for later quantification of the Ag-specific Abs by ELISA. In the LDA, cells are titrated before culture using the maximum number of replicates for each cell dilution that is possible for the number of PBMCs available. Then for each cell dilution, the proportion of Ab positive and negative cultured wells is determined and these proportions are used to deduce the frequency of Ag-specific memory B cells. The definition of a positive or negative well is critical in the interpretation of the assay because the proportions of positive and negative culture wells are used to calculate the B cell frequency. It is possible to have a parallel plate with non-

stimulated medium with exactly the same cell titrations as the stimulated plate. This control plate containing non-stimulated PBMCs is used to set a cut-off for positivity, since no memory B cells should differentiate into ASC, and therefore the highest OD of this plate represents the background (or Abs secreted by plasma cells originally present in PBMCs before culture). Several different statistical methods can be used to deduce the number of MBCp from the proportion of positive and negative culture wells at each cell dilution. The two methods most frequently used are the “Poisson method” developed by Lefkovits and Waldman in 1979 (Lefkovits and Waldmann, 1999) and the “Reed & Muench method” developed by Reed and Muench in 1938 (Reed, 1938) (see section 2.5.5). Basically, the Poisson method defines that the cell dilution corresponding to 37% of negative wells corresponds to one precursor cell per well. The Reed & Muench method defines that the cell dilution corresponding exactly to 50% of positive wells corresponds to one precursor cell per well.

Using the LDA, memory B cells can only be assessed at rest because Ag-specific memory B cells cannot be detected as long as there are Ag-specific plasma cells in circulation. If there are pre-existing plasma cells in the culture medium, the signal given by the non-stimulated cultures, which serve as the cut-off for defining the positivity of a culture well for the presence of Ag-specific Ab, will be too high. However, with ELISpot it is never certain whether some pre-existing plasma cells survive in the stimulated medium with the support of other PBMCs. Therefore, memory B cells detected 1-2 weeks following immunisation with ELISpot might include these plasma cells.

A modified LDA of Ag-specific memory B cells has been developed by Slifka et al. (1996) for an ELISpot assay (Slifka and Ahmed, 1996). In this assay, the cells are cultured by limiting

dilution as for the standard LDA (ELISA-based LDA) but after culture, instead of harvesting the supernatant from the cells, the cells themselves are harvested and transferred to an ELISpot plate. In contrast to the ELISA-based LDA, which measures the accumulated specific Abs produced by ASCs, the ELISpot-based LDA identifies the ASCs directly. To define if a culture well is positive for the presence of Ag-specific ASCs, several non-stimulated culture wells are run for each cell dilution. This allows the determination of a cut-off of positivity for each cell dilution. The frequency of MBCp is calculated with the same statistical methods as for the standard ELISA-based LDA. This method has several advantages: it requires less days of culture (5 days) in comparison to the ELISA-based LDA, which requires at least 10 days of culture before enough specific Ab accumulate in the culture medium. Furthermore, the ELISpot-based LDA might be more sensitive than the ELISA-based LDA as it directly detects the Ag-specific ASCs.

By performing an ELISpot-based LDA in parallel with the ELISA-based LDA, it would be possible to link the number of Ag-specific ASCs detected by ELISpot in each well with the level of Ag-specific Abs secreted in the culture medium by the same cells detected by ELISA. This has been achieved in the second study, “The influenza adult study” (section 3.3).

3.1.4 Structure of this chapter

Two studies were carried out to compare LDA and ELISpot assays. The first study, discussed in section 3.2, assessed MenC-specific memory B cells in 33 children before and 30 days following immunisation with MenCV, using ELISpot and the ELISA-based LDA. For the ELISA-based LDA the two statistical methods described in sections 2.5.5.2 and 2.5.5.3 were

compared for their ability to estimate MenC-specific memory B cell frequencies. Then the results obtained by the ELISA-based LDA were compared to the ELISpot assay.

A second study discussed in section 3.3 was carried out assessing influenza-specific memory B cell frequencies in healthy adults following immunisation with seasonal influenza vaccine. In this study, ELISpot-based LDA was compared to the ELISA-based LDA. The two LDA assays were then compared to the standard ELISpot assay.

3.2 Memory B cell responses to MenCV in one-year-old children

3.2.1 Introduction

The introduction of MenCV in the UK in 1999 led to a marked decline in the number of cases of serogroup C meningococcal diseases. The vaccine is highly immunogenic in all age groups, inducing high bactericidal Ab levels; however, in young infants these Abs decline rapidly, despite the persistence of immunological memory. In infants given a primary course of MenCV at 2, 3 and 4 months of age, the vaccine effectiveness was shown to be poor more than one year after priming (Trotter et al., 2004). It is unclear why MenCV elicits only short-lived Abs in young infants.

In humans, memory B cells can be detected in peripheral blood many years following immunisation (Crotty et al., 2003a; Nanan et al., 2001) and are thought to be the principal determinant of long-term maintenance of Abs and to mediate secondary immune responses following re-challenge with the Ag (Ahmed and Gray, 1996). The persistence of MenC-specific memory B cells after primary immunisation with MenCV in infancy has not yet been determined. Such information could help to increase our understanding of long-term persistence of Abs in young infants. However, the measurement of memory B cells in young infants can be challenging due to the smaller volume of blood available and because Ag-specific memory B cell frequencies are lower in infants. In this study, two different methods were compared for their ability to assess MenC-specific memory B cells in the peripheral blood of one-year-old infants, who had been primed with three doses of MenCV.

3.2.2 Methods

3.2.2.1 Subjects and vaccines

Thirty-three children were recruited at 12 months of age to a phase IV, open-label study to investigate the B cell memory immune responses to a booster dose of MenCV (Menjugate[®], Novartis Vaccines and Diagnostics GmbH&Co., Marburg, Germany) after three-dose priming with MenCV in infancy (study M14P5E1; OxREC number CO6/Q1604/N41; Eudract number 2006-000732-28) (see as well section 4). After informed consent by the parents, participants received 0.5 ml MenCV dose. The 0.5 ml dose contained 10 µg of the polysaccharide and between 12.5 and 25 µg of the conjugate CRM₁₉₇ (mutant protein derived from diphtheria toxoid) and was adsorbed on aluminium phosphate. Five mls of venous blood was obtained from each child before and 30 days after the booster immunisation. The PBMCs were isolated immediately from the fresh blood and cultured for 6 days for ELISpot and 10 days for LDA. Each experiment was performed only once.

3.2.2.2 Measurement of MenC-, CRM₁₉₇- and diphtheria toxoid-memory B cell frequencies by ELISpot

MenC, CRM₁₉₇- and diphtheria toxoid- specific memory B cells were assessed by ELISpot following the methods described in 2.2-2.4). CRM₁₉₇- and diphtheria toxoid- memory B cells were also assessed as the serogroup C meningococcal polysaccharide is conjugated to the mutant peptide related to diphtheria toxoid, CRM₁₉₇, in the MenCV.

3.2.2.3 Measurement of MenC-, CRM₁₉₇ and diphtheria toxoid-memory B cell frequencies by limiting dilution

After separation, PBMCs were re-suspended at a final concentration of 4×10^6 PBMCs/ml and cultured for 10 days with CpG/IL2 (CpG 5 μ g/ml, IL2 1000IU/ml) under limiting dilution conditions following the methods described in section 2.6. At the end of the culture, the cultured supernatant was analysed by ELISA for the presence of MenC-, CRM₁₉₇- and diphtheria toxoid- specific Abs following the methods described in sections 2.6.3 and 2.6.4. Using the OD obtained in each well of the ELISA plate, the number of wells that were positive and negative for the presence of Ag-specific Abs for each cell dilution of the LDA plate was determined. The frequency of MenC-, CRM₁₉₇- and diphtheria toxoid- specific MBCp was determined by LDA using two statistical methods (Poisson and Reed & Muench) described in section 2.6.5.

3.2.2.4 Statistical analysis

Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. Ag-specific memory B cell frequencies were summarised using medians with 5th – 95th percentile. Within group comparisons of B-cell numbers at varying time points were made using the Wilcoxon signed ranks test for paired data. The correlation between the different methods was compared with the Spearman's rank correlation coefficient, using untransformed B cell numbers.

3.2.3 Results

3.2.3.1 Recruitment

All 33 children completed the study. However, because of insufficient sample volumes or assay failures, not all data points were available for each time point (see number of participants for each data point in Table 3.2-1).

3.2.3.2 Memory B cell responses

Before immunisation with MenCV at one year of age, MenC-memory B cells were not detectable in peripheral blood with the two methods; ELISpot and (ELISA-based) LDA. However, ELISpot was capable of detecting CRM₁₉₇- and diphtheria toxoid- memory B cells in circulation, whereas LDA could not (table 3.2.1). Following immunisation, MenC-, CRM₁₉₇- and diphtheria toxoid- memory B cells could be detected in peripheral blood by ELISpot and LDA (table 3.2.1).

Ag	Time post vaccination (days)	LDA using the Reed & Muench method	LDA using the Poisson method	ELISpot
MenC-memory B cells Median (5 th -95 th Percentile) [Participants]	Day 0	0 (0-0) [0]	0 (0-0.8) [21]	0 (0-5) [24]
	Day 30	7 (0-35) [20]	3.3 (0-13) [20]	20 (0-115) [23]
CRM ₁₉₇ -memory B cells Median (5 th -95 th Percentile) [Participants]	Day 0	0 (0-10) [13]	0 (0-7.1) [13]	5 (0-20) [22]
	Day 30	6 (0-21.5) [20]	2.6 (0-11) [20]	35 (5-90) [21]
Diphtheria toxoid memory B cells Median (5 th -95 th Percentile) [Participants]	Day 0	0 (0-8) [14]	0 (0-4) [14]	12.5 (5-45) [22]
	Day 30	3.5 (0-15.5) [20]	3.5 (0-13.8) [19]	72.5 (10-220) [22]

Table 3.2-1: MenC-, CRM₁₉₇- and diphtheria toxoid- memory B cell responses (in ASC per million PBMCs) measured by LDA using the Reed & Muench or Poisson statistical methods, and by ELISpot pre- and post-immunisation (30 days post-immunisation) with MenCV at one year of age after three dose priming with MenCV in infancy.

Results showed a significant increase in MenC-specific memory B cell frequencies, between pre- and post-vaccination (30 days post-vaccination) by ELISpot and LDA. However, for the other Ags assessed, there was a significant increase in memory B cell frequencies only by ELISpot (Figure 3.2-1).

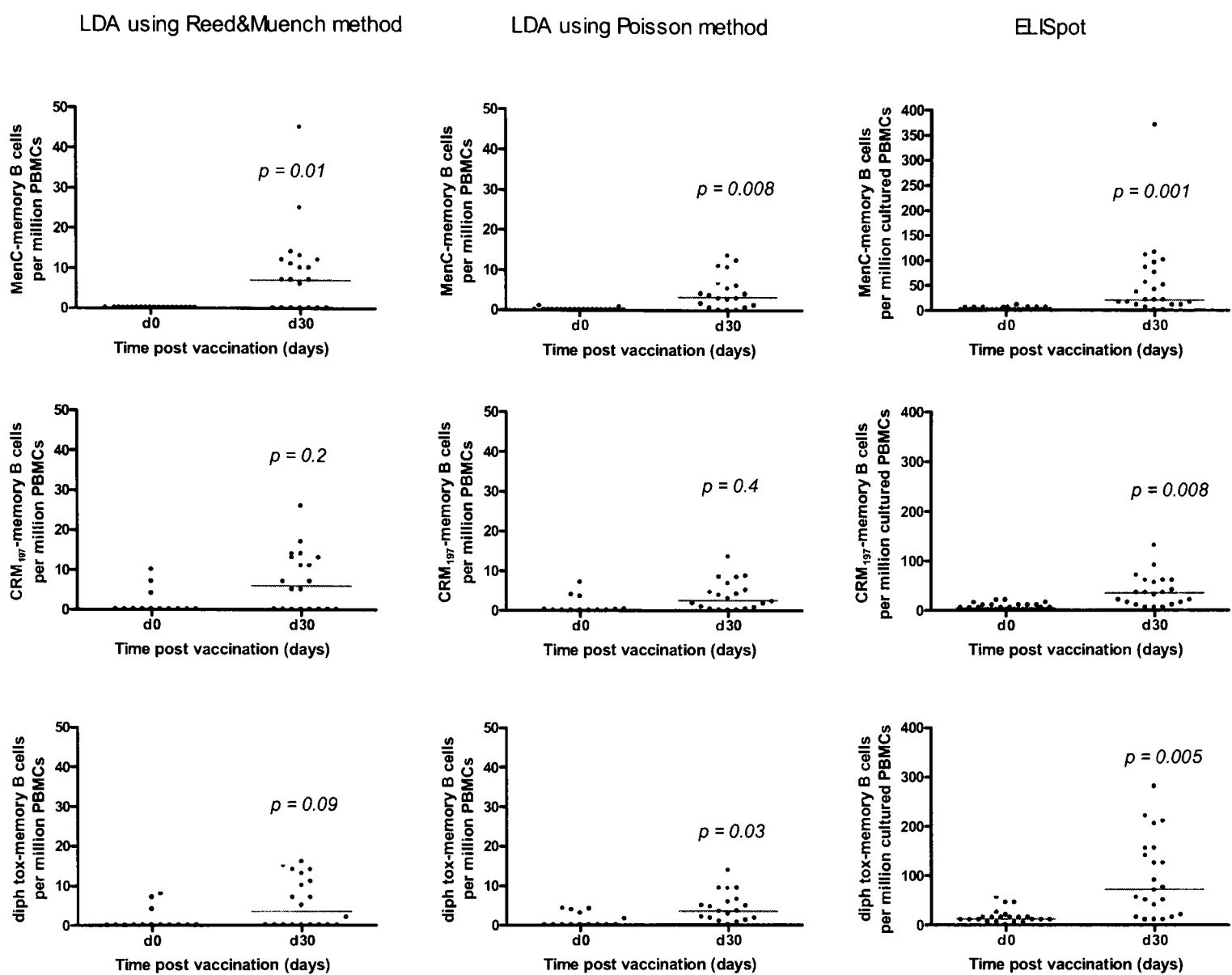


Figure 3.2-1: MenC- (top row), CRM197- (middle row) and diphtheria toxoid- (lower row) specific memory B cells were assessed pre- and 30 days post-immunisation by LDA using the Reed & Muench and Poisson statistical methods, and by ELISpot. The horizontal bars represent the median number of specific memory B cells at each time point. The Wilcoxon signed ranks test for paired data was used to compare pre-and post-vaccination median values (see p values on each graph).

3.2.3.3 Sensitivity of the two assays to detect memory B cell responses

In order to obtain a better comparison of the different techniques in terms of their respective sensitivity to detect Ag-specific memory B cells before and after vaccination, the different values of Ag-specific memory B cell frequencies were plotted in one single graph (Figure 3.2-2). The results show that the ELISpot technique is more sensitive than the LDA technique.

Before immunisation, and when the Ag-specific memory B cell frequencies are low, there is often a signal by ELISpot but no signal from LDA. In general, the frequencies of Ag-specific-memory B cells obtained by ELISpot are higher than the ones obtained by LDA.

- LDA using the Reed & Muench method
- ▲ LDA using the Poisson method
- ▼ ELISpot

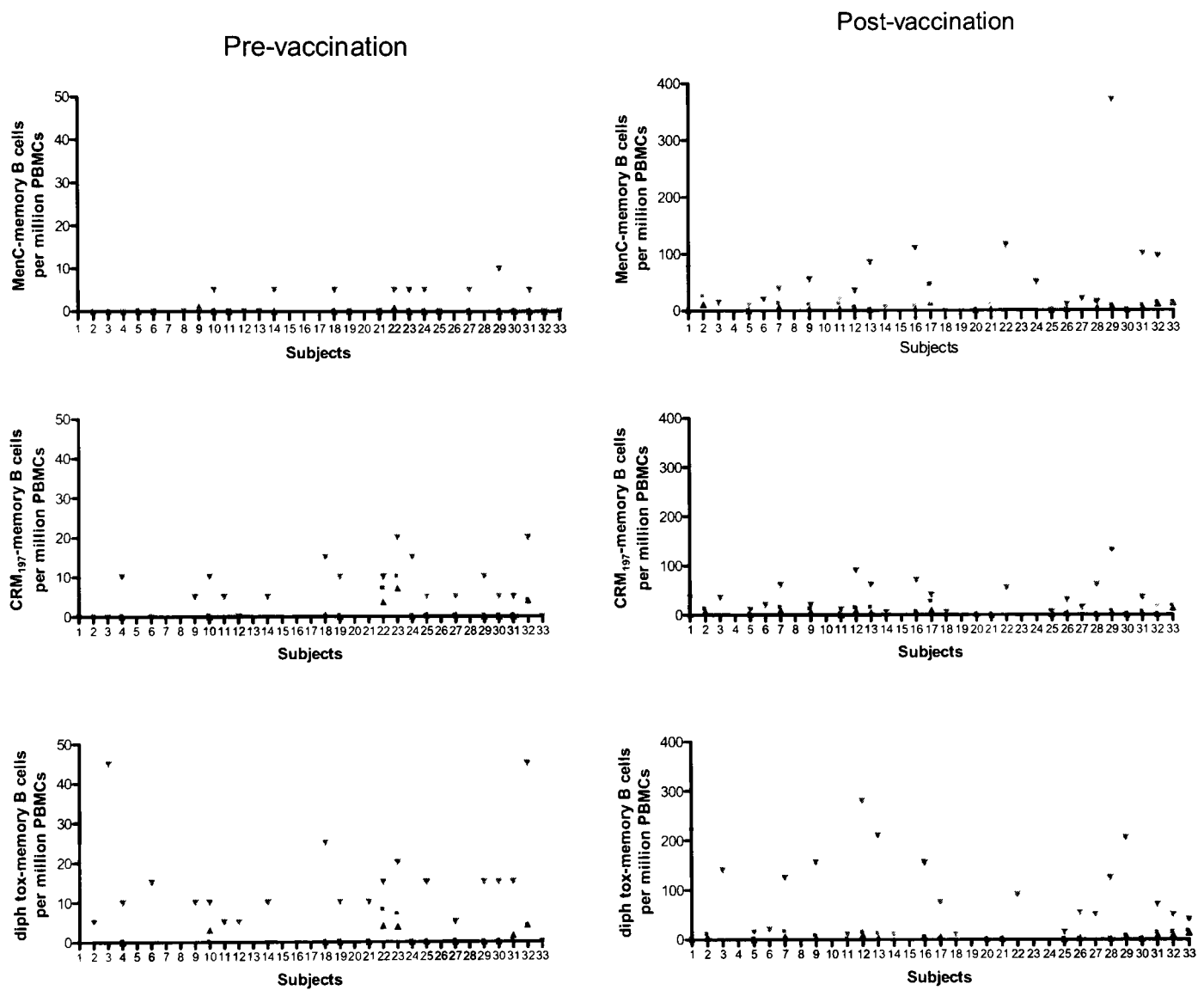


Figure 3.2-2: The LDA using the Reed & Muench and Poisson statistical methods and the ELISpot were compared in terms of their sensitivity to detect memory B cells specific for MenC, CRM₁₉₇ and diphtheria toxoid before and after immunisation.

3.2.3.4 Correlations

Spearman Rank correlations were calculated between each method, before and after immunisation and for each Ag. Results showed significant correlations between the two statistical methods; Reed & Muench and Poisson, used to interpret the frequency of Ag-specific memory B cells by LDA, before immunisation (CRM₁₉₇ $r = 0.77$, $p < 0.002$, $n = 13$; diphtheria toxoid $r = 0.73$, $p = 0.003$, $n = 14$) and after immunisation (Table 3.2-2).

Before immunisation, it was not possible to assess MenC-specific memory B cell frequency by LDA using the Reed & Muench statistical method, because the MenC-specific memory B cell frequency was too low and this method requires that at least the top cell dilution has 50% of its cultures positive (see section 2.6.5.2). Therefore, comparison between LDA-Reed & Muench and other methods could not be used for MenC, before immunisation.

There was no correlation between ELISpot and LDA methods, before (data not shown) and after immunisation (Table 3.2-2), for all three Ags assessed.

The LDA using the Reed & Muench statistical method allows expression of the frequency of Ag-specific-memory B cells in relation to total PBMCs, B cells, IgG-B cells and memory B cells. The percentage of each B cell subpopulation in the PBMCs is assessed before culture by flow cytometry (according to the methods described in section 2.6.2). With the ELISpot method, frequency of memory B cells can be expressed in relation to PBMCs or as a percentage of total IgG-ASCs. Using the different possibilities to express memory B cell frequencies by

LDA and ELISpot, there was found to be no correlation between the two techniques, except for diphtheria toxoid-memory B cells. There was a correlation between the ELISpot method and the LDA using the Reed & Muench statistical method, when memory B cell frequencies, measured by ELISpot were expressed as a percentage of total IgG-ASCs, and those measured by LDA were expressed in relation to B cells ($r = 0.66$, $p = 0.01$, $n = 14$), memory B cells ($r = 0.56$, $p = 0.03$, $n = 14$) and IgG-B cells ($r = 0.65$, $p = 0.01$, $n = 14$).

Ag	Methods	LDA using the Reed & Muench method			LDA using the Poisson method		
		r	p	n	r	p	n
MenC	LDA using the Reed & Muench method						
	LDA using the Poisson method	0.85	<0.0001	20			
	ELISpot	0.02	0.9	16	0.3	0.3	16
CRM ₁₉₇	LDA using the Reed & Muench method						
	LDA using the Poisson method	0.85	<0.0001	20			
	ELISpot	0.4	0.1	15	0.6	0.02	15
Diphtheria Toxoid	LDA using the Reed & Muench method						
	LDA using the Poisson method	0.8	<0.0001	19			
	ELISpot	0.3	0.2	16	0.2	0.6	15

Table 3.2-2: The memory B cell frequencies obtained with the LDA using the Reed & Muench and Poisson statistical methods, and the ELISpot were correlated for each Ag. Only post-vaccination results are reported in this table. Spearman's rank correlation coefficient (r) with the p-values and the number of participants tested for each association (n).

3.2.3.5 Percentage of B cell sub-populations in PBMCs

As part of the LDA using the Reed & Muench statistical method, the percentage of each B cell sub-population in relation to PBMCs had to be determined by flow cytometry. The pre- and post-immunisation mean percentage of each B cell sub-population in all 33 children was assessed and reported graphically in Figure 3.2-3. Before immunisation the percentages of each B cell sub-population in relation to PBMCs were CD20 27.8% (95%CI 23.2-32.4), CD20IgG 0.8% (0.5-1), CD20CD27 2.3% (1.7-2.9), CD27IgG 0.4% (0.2-0.6). After immunisation, the percentage of each sub-population had not statistically changed. The percentages of each B cell sub-population in relation to PBMCs were: CD20 26.8% (95% CI 22.8-30.7), CD20IgG 0.9% (0.7-1.1), CD20CD27 2.4% (1.9-3), CD27IgG 0.5% (0.3-0.6).

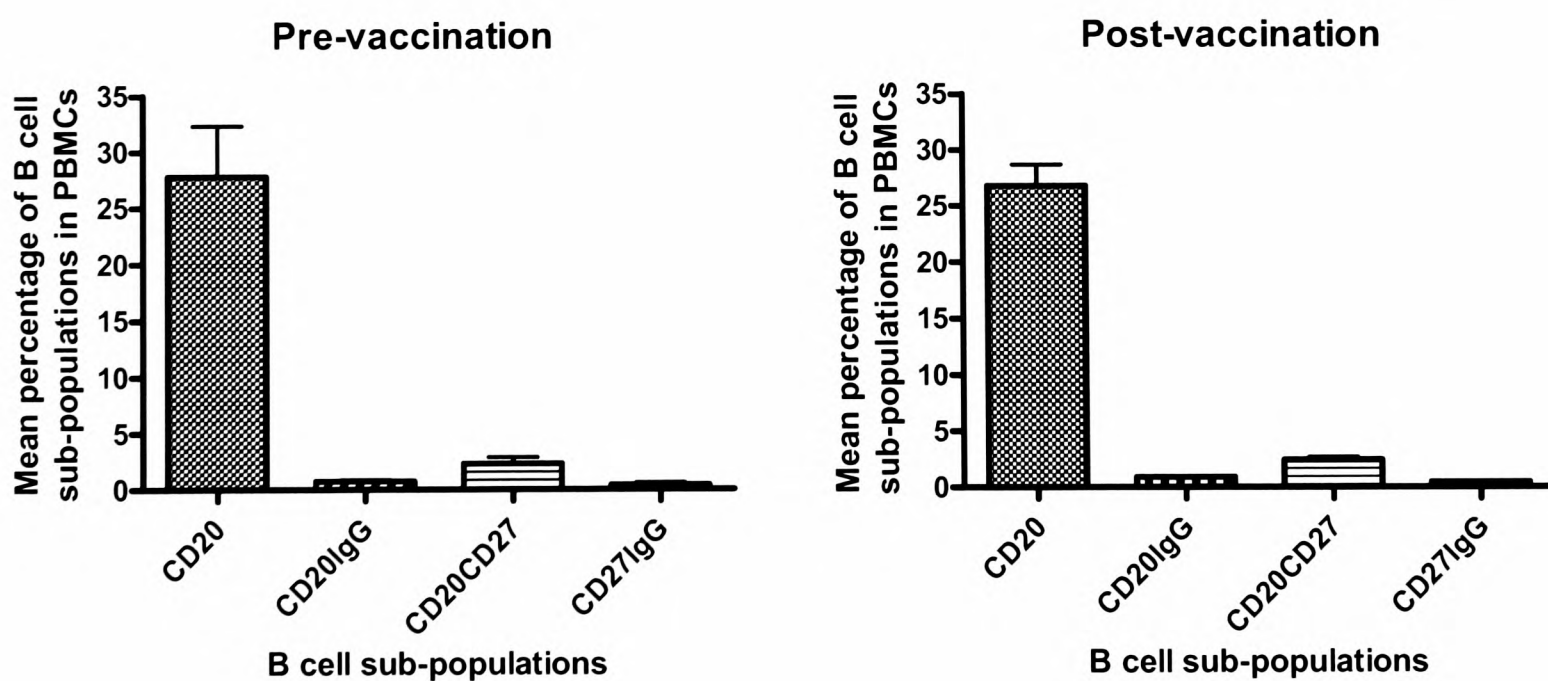


Figure 3.2-3: The percentage of the B cell sub-populations in PBMCs of 33 one-year-old children was determined before and 30 days following a booster dose of MenCV using the flow cytometry method described in section 2.6.2. The data represents the mean and standard error of the mean.

Before immunisation, there were between 0.2 and 0.6% IgG-memory B cells per PBMCs. Assuming an average of 0.4% of IgG-memory B cells per PBMCs, from the 200,000 PBMCs per well in the first cell dilution of LDA culture plates there should be around 800 IgG-memory B cells per well. The frequency of Ag specific memory B cells is between 0.01-1% of total IgG-memory B cells in adults (Crotty et al., 2003a; Nanan et al., 2001). Assuming that the frequency of polysaccharide specific memory B cells in children is among the lowest of cell frequencies, it can be extrapolated that there was less than one MenC-specific memory B cell per well in the first cell dilution of LDA culture plates before immunisation. This explains why the frequency of MenC-memory B cells measured by LDA was often equal to zero. However, one MenC-memory B cell per well can proliferate and differentiate into ASC allowing the MenC-ASC to be detected by ELISpot. However, the MenC-Abs secreted by the resultant ASCs and accumulated in culture medium may still not be sufficient to be detected by ELISA.

3.2.4 Discussion

The development of assays capable to detect memory B cell responses after infant immunisation is important for the evaluation of long-term protection offered by new vaccines. In this study the LDA using two statistical methods (Reed & Muench and Poisson) and the ELISpot assays were compared for their ability to detect MenC-, CRM₁₉₇-, and diphtheria toxoid- memory B cell responses, in a clinical trial where 33 children primed with MenCV in early infancy were challenged with a booster dose of MenCV at one year of age.

The results obtained with the two different statistical methods, Reed & Muench and Poisson for the LDA, correlated well for the three Ags assessed pre- and post-immunisation, however, the

Poisson method appears to be more sensitive than the Reed & Muench method. This is because the only requirement of the Poisson method is that at least two cell dilutions have responding culture wells for the presence of Ag-specific Abs, even if these cell dilutions have a very low proportion of positive culture wells. In contrast, the Reed & Muench method requires that at least the top cell dilution has 50% of positive culture wells. Therefore, the Reed & Muench method appears to be “more statistically accurate” but less sensitive than the Poisson method.

The LDA and ELISpot methods did not correlate. There are several possible explanations. First, the culture medium used for the activation of memory B cells was not the same for LDA and ELISpot. LDA used CpG/IL2 and ELISpot used CpG/SAC/PWM. Second, the culture duration was different; in LDA the activation of the cells lasted 10 days, but in ELISpot it lasted only 5 days. Third, the two methods are completely different in the way they assess Ag-specific memory B cell frequencies; LDA uses Ab accumulation in supernatant during the 10 days of culture and the memory B cell frequency is estimated with a statistical method from the proportion of positive and negative cultures for each cell dilution. In contrast, the ELISpot directly measures ASCs that have differentiated from MBCp during culture. Finally, an important difference between LDA and ELISpot is that, after culture, cells are re-counted and re-suspended before being added to an ELISpot plate and therefore, with the ELISpot the memory B cell frequency is expressed in relation to the cultured PBMCs. In contrast, using the LDA the frequency of memory B cells is expressed in relation to the number of PBMCs seeded into each culture well.

The ELISpot method appeared to be more sensitive than the LDA method. This might be because in the ELISpot ASCs differentiated from memory B cells are directly assessed but in

the LDA ASCs are indirectly assessed, as the Abs secreted by ASCs in supernatant during the culture are assessed by ELISA. The ELISA is thought to be very sensitive and able to detect very low concentrations of Abs in serum. However, it is possible that the Abs secreted into the supernatant by Ag-specific memory B cells are sometimes at frequencies too low to be detected by ELISA. The first cell dilution used in LDA was similar to the cell dilution used in the ELISpot (except that the cell dilution for ELISpot was re-adjusted after culture). This frequency corresponds to one MenC-specific ASC per well. The number of Abs secreted by each ASC is unknown, however, if there is only one Ag-specific ASC per well at the top cell dilution of culture LDA plates, the concentration of Abs in the supernatant might not be sufficient to be detected by ELISA.

3.2.5 Conclusions and further work

These results suggest that the ELISpot method is better for assessment of low memory B cell frequencies, such as polysaccharide specific-memory B cells in infant samples. This is explained by the fact that ELISpot directly assesses ASCs that have differentiated from cultured memory B cells, whereas LDA assesses Abs produced by these ASCs in cultured supernatant. However, culture of memory B cells induces proliferation of these cells, before their differentiation into ASCs. Therefore, the frequency of Ag-specific memory B cells obtained by ELISpot does not reflect exactly the frequency of Ag-specific memory B cells present in the peripheral blood. In contrast, LDA can directly estimate the frequency of the original Ag-specific memory B cells. The higher sensitivity of ELISpot is, therefore, also attributed to the proliferation of memory B cells that occurs during culture. An ELISpot-based LDA would allow the combination of the high sensitivity of the ELISpot with the direct estimation of

memory B cell precursors. In the next study, three different methods are compared; the ELISA-based LDA, the ELISpot-based LDA and the standard ELISpot.

3.3 Memory B cell responses to seasonal influenza-vaccine in adults

3.3.1 Introduction

A study comparing the ELISA-based LDA with ELISpot for their ability to detect memory B cell responses pre- and post-immunisation with MenCV in infants has shown that the ELISA-based LDA was not suitable for the assessment of low memory B cell frequencies in small blood volumes. However, the ELISA-based LDA has several advantages over ELISpot. It is possible to freeze the supernatant for later analysis or for second testing; there is a control for the pre-existing plasma cells (by culturing some of the cells in parallel without polyclonal stimulants); the frequency of MBCp can be directly assessed; and there is not the subjectivity of the ELISpot for manual counting of spots.

In this study, the two methods LDA and ELISpot were compared for their ability to detect high memory B cell frequencies in adults. The memory B cells specific to the subtype H1N1 of the influenza A virus were assessed by three methods following seasonal influenza-immunisation in healthy adults. The three methods utilised were the ELISA-based LDA, the ELISpot-based LDA and the standard ELISpot. The ELISpot-based LDA follows the same basic principles as the ELISA-based LDA, but it provides more rapid quantitation of MBCp frequencies because it directly identifies the ASCs derived from the MBCp, instead of measuring the accumulated Abs that they have produced (Slifka and Ahmed, 1996).

3.3.2 Materials and Methods

3.3.2.1 Subjects and Vaccines

Between October and December 2006, 18 adult volunteers were recruited to assess the frequency of memory B cells specific for the H1N1 subtype of the influenza A virus, following seasonal influenza-immunisation. The influenza vaccine used in the winter season 2006/2007 contained genetic variants of both the H1N1 and H3N2 subtypes of the influenza A virus (Health Protection Agency, 2008b). After informed consent by the participants, 20 ml of venous blood was obtained between days 17 and 62 post-immunisation.

3.3.2.2 Measurement of influenza-specific memory B cell frequencies by the standard ELISpot, the ELISA-based LDA and the ELISpot-based LDA

PBMCs were separated from the 20 ml of fresh blood for the different memory B cell assays according to the method described in section 2.2. The ELISpot assay for the detection of H1N1-specific-memory B cells was undertaken according to the methods described in section 2.3 and 2.4. The ELISA-based and ELISpot-based LDA were undertaken following the methods described in sections 2.6 and 2.7. The same culture medium as for the standard ELISpot was used for the activation of the memory B cells for LDA, as the comparison of both culture media SAC/CpG/PWM versus CpG/IL2 had demonstrated that SAC/CpG/PWM was also appropriate for the activation of memory B cells for the LDA (see section 2.6.1.3). Two LDA-culture plates per subject were prepared; for the ELISpot-based LDA the plate was incubated for 5 days and, for the ELISA-based LDA, the plate was incubated for 10 days. At the end of the incubation

period the cultured supernatant was harvested and transferred to an ELISA plate for determination of H1N1-specific-Abs in culture medium by ELISA (see section 2.6.3), and the remaining cells were added to a blocked ELISpot plate, pre-coated with H1N1, for determination of H1N1-specific memory B cells by ELISpot (according to the methods described in section 2.4).

Only the statistical method of Reed & Muench was used to determine the frequency of H1N1-MBCp by the ELISA-based LDA (see section 2.6.5). The frequencies of H1N1-MBCp were assessed using the ELISpot-based LDA according to the methods outlined in section 2.7; pre-existing H1N1-specific plasma cells were determined in the non-stimulated part of the culture plate (4 last columns) (see Figure 2.6-1), and the average number of cells detected served as a cut-off in order to define the positive and negative culture wells for H1N1-specific ASC in the stimulated part of the plate. This represented a background number of a maximum of one pre-existing ASC/well at the first cell dilution (2×10^5 PBMCs) and in general there were no cells in the higher dilutions. Therefore, individual wells with > 1 ASC were scored as positive for containing an H1N1-specific ASC. The proportion of positive and negative culture wells was determined for each cell dilution, and from these proportions, Ag-specific MBCp frequencies were calculated using the Reed & Muench method as for the ELISA-based LDA (see section 2.6.5.2). The B cell sub-populations were not assessed in this study and therefore, the frequency of H1N1-specific MBCp using the LDA (Reed & Muench) was only expressed in relation to million PBMCs.

3.3.2.3 Statistical analysis

Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. Ag-specific memory B cell frequencies were summarised using medians with 5th – 95th percentile. The correlation between the different methods was compared with the Spearman's rank correlation coefficient, using the untransformed B cell numbers.

3.3.3 Results

3.3.3.1 Recruitment

Eighteen healthy adults agreed to give a sample of blood following seasonal influenza-immunisation. Samples from all 18 of these adults were available for the ELISpot-based LDA and for the standard ELISpot. One individual had not enough PBMCs to perform the three assays; therefore, the ELISA-based LDA was not carried out for this volunteer and only 17 samples were available for the ELISA-based LDA.

3.3.3.2 Sensitivity of the three assays to detect memory B cell responses

Memory B cells specific to the subtype H1N1 of the influenza A virus were assessed by ELISpot, the ELISA-based LDA, and the ELISpot-based LDA between days 17 and 62 following seasonal influenza-immunisation. Using the standard ELISpot assay, all adults had

detectable H1N1 specific-memory B cells in peripheral blood, but using the two LDA methods all individuals except two had detectable H1N1-memory B cells in peripheral blood.

In order to better compare the different techniques in terms of their respective sensitivity to detect H1N1 specific-memory B cells following seasonal influenza-immunisation, the different values of memory B cell frequencies were plotted in one single graph (Figure 3.3-1). The results show the ELISpot assay to be more sensitive than the two LDA assays; in two individuals there were no H1N1 specific-memory B cells detected using the two LDA methods but, the ELISpot was able to detect memory B cells in these volunteers. In general, the frequencies of H1N1-memory B cells obtained by ELISpot were higher than the ones obtained by LDA (Figure 3.3-1 and Table 3.3-1).

- ELISpot-based LDA
- ◆ ELISA-based LDA
- ELISpot

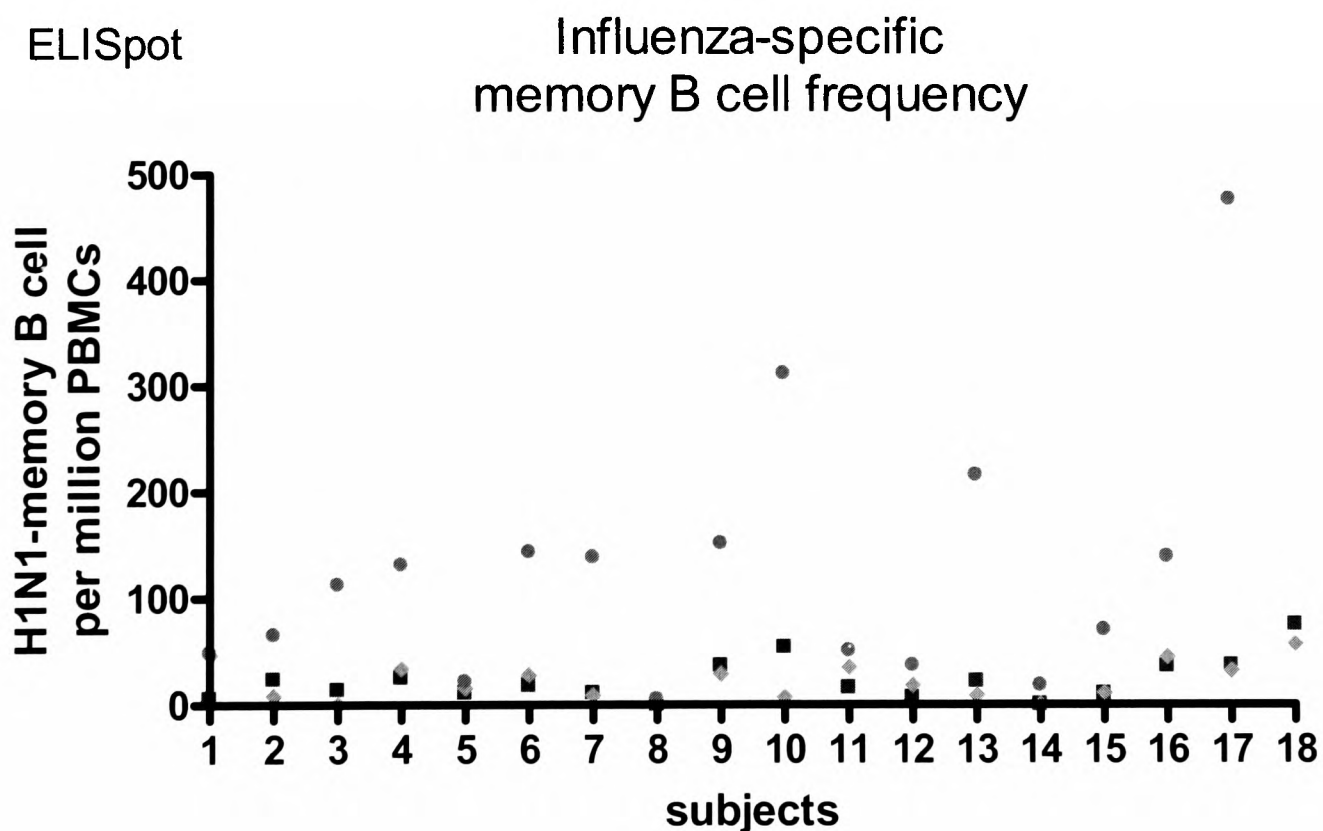


Figure 3.3-1: Frequency of H1N1-specific memory B cells assessed in 18 volunteers by an ELISpot-based LDA, the ELISA-based LDA and the ELISpot.

H1N1 specific-memory B cells per million PBMCs	ELISA-based LDA	ELISpot-based LDA	Standard ELISpot
Median (with 5 th – 95 th percentile) [participants]	17 (0-75) [18]	18 (0-57) [17]	96.5 (5-476.3) [18]

Table 3.3-1: Number of H1N1-specific memory B cells per million PBMCs measured by the ELISpot-based LDA, the ELISA-based LDA and the standard ELISpot following seasonal influenza immunisation.

3.3.3.3 Correlations

Spearman's rank correlation coefficients were calculated to compare each technique. There was a significant correlation between the ELISpot-based LDA and the standard ELISpot ($r = 0.76$, $p = 0.0002$). However, the ELISA-based LDA did not correlate with the ELISpot-based LDA nor with the standard ELISpot (see table 3.3-2).

	ELISpot-based LDA			ELISpot		
	r	p	n	r	p	n
ELISA-based LDA	0.29	0.26	17	0.03	0.9	17
ELISpot-based LDA				0.76	0.0002	18

Table 3.3-2: Correlations between the different methods. Spearman's rank correlation coefficient (r) with the p-values (p) and the number of volunteers tested for each association (n).

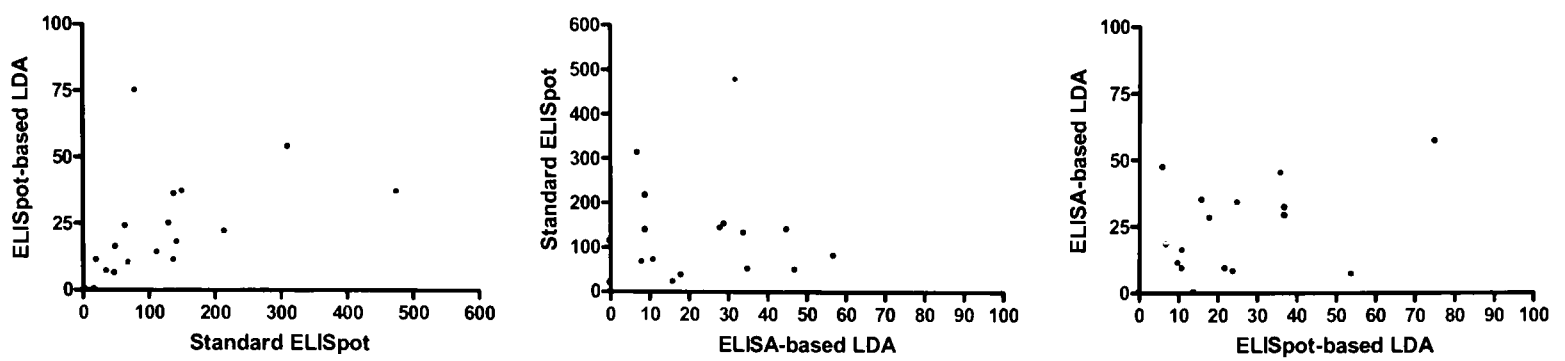


Figure 3.3-2: Correlations between the three different methods (ELISA-based LDA, ELISpot based LDA and standard ELISpot), using the Spearman’s correlation method (see Table 3.3-2 for the r and p values of each correlation).

3.3.3.4 Comparison of the number of B cells detected in the ELISpot plates and the concentration of Abs detected in the supernatant by LDA

After culture of PBMCs by limiting dilution, the supernatant was transferred into an ELISA plate and tested for the presence of H1N1-specific Abs in the cultured supernatant, and the cells were transferred into an ELISpot plate for direct detection of H1N1-specific ASCs. Direct comparison of the cells detected by the ELISpot, and of the Abs secreted by these cells in culture medium and detected by the ELISA, showed no correlation. The results show that the ELISpot wells with a lot of cells are not always the same wells as those with high OD readings in the ELISA plate and vice versa (see Table 3.3-3).

	Non stimulated culture								Stimulated culture			
	1	2	3	4	5	6	7	8	9	10	11	12
No of H1N1-specific ASCs detected by ELISpot from PBMCs cultured under limiting dilution	33	30	75	25	15	5	9	10	0	0	0	0
	32	45	54	48	9	68	53	43	0	0	0	0
	28	25	23	37	65	51	38	45	0	0	0	0
	16	5	1	5	4	2	4	3	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	1	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
OD corresponding to H1N1-IgG concentrations detected by ELISA of cultured supernatant of the same PBMCs	0.331	0.506	1.184	0.254	0.395	0.274	0.241	0.248	0.238	0.247	0.238	0.206
	0.111	0.108	0.121	0.115	0.133	0.126	0.129	0.103	0.129	0.134	0.159	0.123
	0.089	0.087	0.086	0.086	0.091	0.086	0.084	0.083	0.106	0.107	0.105	0.1
	0.074	0.079	0.074	0.076	0.077	0.079	0.08	0.07	0.081	0.086	0.087	0.085
	0.071	0.069	0.072	0.07	0.07	0.07	0.072	0.068	0.075	0.078	0.072	0.074
	0.071	0.077	0.072	0.074	0.076	0.082	0.084	0.062	0.061	0.072	0.077	0.063
	0.068	0.07	0.067	0.068	0.07	0.069	0.07	0.068	0.069	0.067	0.071	0.071
No of H1N1-specific ASCs detected by ELISpot from PBMCs cultured under limiting dilution	14	23	66	21	23	25	3	72	1	1	2	1
	12	19	45	17	25	27	5	12	0	0	0	0
	6	2	1	1	5	15	2	8	0	0	0	0
	0	0	19	0	0	0	0	5	0	0	0	0
	3	0	2	0	0	4	0	1	0	0	0	0
	0	0	0	0	0	0	0	11	0	0	0	0
	0	1	0	0	0	0	0	0	0	0	0	
OD corresponding to H1N1-IgG concentrations detected by ELISA of culture supernatant of the same PBMCs	0.631	0.997	0.867	1.235	0.838	1.082	0.292	1.957	0.133	0.107	0.107	0.134
	0.458	0.613	2.409	0.978	1.41	2.143	0.785	0.951	0.108	0.086	0.081	0.098
	0.39	0.113	0.123	1.268	0.219	0.22	0.22	0.123	0.082	0.081	0.081	0.085
	0.091	0.09	0.48	0.18	0.139	0.105	0.187	0.244	0.08	0.072	0.068	0.08
	0.094	0.083	0.086	0.083	0.083	0.086	0.073	0.078	0.072	0.071	0.068	0.072
	0.069	0.068	0.067	0.069	0.07	0.071	0.071	0.099	0.079	0.064	0.06	0.078
	0.068	0.069	0.068	0.068	0.069	0.067	0.07	0.068	0.069	0.069	0.069	0.073

Table 3.3-3: Direct comparison of the ELISpot-based LDA and ELISA-based LDA. Twofold dilutions of PBMCs were seeded into 96-well plates (see section 2.6.1) and stimulated in vitro with SAC/CpG/PWM for 5 (top) and 10 days (bottom) before being tested for H1N1-specific ASCs by ELISpot and H1N1-specific Abs by ELISA. After culture, the culture medium was transferred into an ELISA plate and the cells into an ELISpot plate, with exactly the same layout as the culture plate. The results show no correlation between the ELISpot wells with many cells and the ELISA wells with high OD and vice versa.

3.3.4 Discussion

The present study is the first to compare the standard ELISpot, an ELISA-based LDA and an ELISpot-based LDA as detection methods of influenza-specific memory B cells in the peripheral blood of adult volunteers. The limiting dilution methods have the advantage over the standard ELISpot to estimate the frequencies of MBCp. Indeed, the polyclonal stimulation of the memory B cells has the convenience of expanding cells of low frequency; however, without the use of limiting dilution, it is not possible to relate the final number of ASC to the frequency of MBCp as polyclonal stimulation is associated with proliferation of memory B cells prior to differentiation into ASC. The ELISA-based LDA has the advantage over the ELISpot assays in being more practical and reproducible as the cultured supernatant can be frozen for later analysis.

The results of this study confirm previous findings that the standard ELISpot is the most sensitive method for detecting Ag-specific memory B cells in peripheral blood and that the ELISA-based LDA does not correlate with the standard ELISpot (see section 3.2). However, in the study there was a correlation between the ELISpot-based LDA and the standard ELISpot but not between the two LDA methods. The direct comparison of the ELISpot and ELISA wells described in this section confirmed the non-correlation between the ELISA-based and ELISpot-based LDA. This could have several explanations; the ELISA-based LDA detects Abs that have accumulated in the supernatant during culture, whereas the ELISpot-based LDA measures the ASCs differentiated from MBCp at one time point. During the culture, some cells might secrete Abs and then die and the different cells might secrete different amounts of Abs. It is also

possible that the ELISpot-based LDA detects pre-plasma cells or memory B cells that have just differentiated into plasma cells but have not had time to secrete Ab (plasmablasts). Finally, some cells might be lost during the process of washing the cells in the culture plates before transferring them to the ELISpot plate. However, in this study of memory B cell responses to seasonal influenza-immunisation, the three different methods found that all participants had a high frequency of influenza-specific memory B cells.

3.3.5 Conclusions

In conclusion, while the standard ELISpot appears to be the more appropriate method to detect low memory B cell frequency in samples obtained from children, the ELISA-based and ELISpot-based LDA can yield comparable results and could be used in future studies evaluating induction of memory B cells after immunisation in adults.

4 B cell responses to a booster dose of MenCV at one year of age

4.1 Introduction

N. meningitidis is one of the principal causes of bacterial meningitis and septicaemia in children, with a peak incidence at the age of 6-24 months. In response to an increase in the number of cases of meningococcal disease caused by serogroup C meningococci during the 1990s, MenCV was introduced into the UK routine infant immunisation schedule in 1999 and was offered to all individuals younger than 19 years in a catch-up campaign (Miller et al., 2001).

MenCV induces bactericidal capsular polysaccharide-specific Abs, the presence of which correlate with protection against the disease (Borrow et al., 2001a). Several different factors are thought to contribute to long-term protection after immunisation with conjugate vaccines: herd immunity, persistence of anticapsular Abs in serum and immunological memory (Maiden and Stuart, 2002; Ramsay et al., 2003a; Trotter et al., 2004). However, the only validated correlate of protection is the presence of serum bactericidal activity, measured in an *in vitro* assay using either rabbit (rSBA) or human (hSBA) complement, with a titre \geq 1:8 or 1:4 respectively (Andrews et al., 2003; Borrow et al., 2001a).

The introduction of MenCV in the UK in 1999 led to a marked decline in the number of cases of serogroup C meningococcal disease. However, a study of the direct effectiveness of the vaccine reported that, although the vaccine was highly effective during the first year after immunisation in all age groups, effectiveness could not be demonstrated beyond the first year

after immunisation amongst those receiving a 3 dose infant schedule (Trotter et al., 2004). Other studies have reported a rapid drop in bactericidal Ab titre by one year of age after infant immunisation despite the persistence of immunological memory, which appears to correspond with this decline in effectiveness (MacLennan et al., 2000; Snape et al., 2005).

In support of the view that functional Ab is necessary for long-term protection, studies of Hib and MenC vaccine failures have demonstrated that some children developed Hib or meningococcal infections despite the apparent presence of immunological memory (Auckland et al., 2006; McVernon et al., 2003). Immunological memory in these studies was inferred by the presence of much higher Ab levels during convalescence in the vaccine failures than unimmunised controls.

Most studies that have examined the kinetics of the Ab response in primed individuals have observed an increase in functional Ab by day 5 after immunisation (Bernasconi et al., 2002; Snape et al., 2006). However, a recent study of the Ab response to serogroup C polysaccharide vaccine among children previously primed with only one dose of MenCV at 12-15 months of age, reported an increase in bactericidal Ab levels within 2 to 4 days of re-immunisation (Tsai et al., 2006). These observations imply that immunological memory by itself is too slow to provide protection when a primed individual acquires an invasive strain of these encapsulated bacteria; protection afforded by the secondary rise in Ab following exposure to the organism develops only after several days, whereas the meningococcus invades the blood and disease develops within hours. As a result of these observations, the current immunisation strategy in the UK depends on maintenance of protective Ab levels in the blood through use of a booster dose of both Hib and MenC vaccine in the second year of life

(www.advisorybodies.doh.gov.uk/jcvi/childhoodimmunisationoc05.pdf).

B cells produce Ab and are therefore the principal determinant of long-term protection against encapsulated bacteria after immunisation; indeed it has been shown that memory B cells persist for several years after Ag exposure (Crotty et al., 2003a). It is not clear whether memory B cells reside in secondary lymphoid organs (Maruyama et al., 2000), in the bone marrow (Osmond, 1986), or whether they continuously re-circulate between secondary lymphoid organs through the blood (Nanan et al., 2001). It is presumed that these memory B cells maintain protective Ab levels in serum by continuously differentiating into plasma cells. This phenomenon may be Ag-independent and may occur in response to polyclonal stimuli (derived from microbes or activated T cells) (Bernasconi et al., 2002; Schitteck and Rajewsky, 1990; Sprent and Tough, 1994). Alternatively, generation of plasma cells could be Ag-dependent (via cross-reactive Ags, re-exposure to the Ag or persisting Ag) (Ahmed and Gray, 1996; Ochsenbein et al., 2000). Finally, murine studies have shown that a subgroup of plasma cells generated after immunisation are long-lived and persist in bone marrow and contribute to sustaining long-term Ab production (Manz et al., 1997; Slifka et al., 1998; Slifka et al., 1995).

Understanding the generation and maintenance of Abs by immunisation is of central importance to enhancing long-term protection against polysaccharide-encapsulated bacteria in the first few years of life. In this study, the persistence of MenC-specific B cells and Abs at one year of age after priming with MenCV in early infancy is assessed, and the B cell and Ab responses to a booster dose of MenCV given at one year of age are described. Further, the relationship between the post-primary generation of memory B cells and Abs, and humoral immunity around the 12-month booster dose of vaccine is reported.

4.2 Methods

4.2.1 Subjects and vaccines

A cohort of 72 children of at least 12 months of age who had participated in a previous study assessing their B cell response to the C polysaccharide component during routine infant immunisation with MenCV at 2, 3 and 4 months of age (study M14P5, Eudract number 2004-004962-33) was approached and invited to participate in this “follow-on” study. A total of 33 of those invited agreed to participate. After informed consent by the parents, participants received a dose of 0.5 ml of MenCV (Menjugate[®], Novartis Vaccines and Diagnostics GmbH&Co., Marburg, Germany), by intramuscular injection into the antero-lateral thigh. The 0.5 ml dose contained 10 µg of the polysaccharide and between 12.5 and 25 µg of the conjugate CRM₁₉₇ and was adsorbed on aluminium phosphate. Further, to boost immunity against Hib as per the routine UK schedule (www.advisorybodies.doh.gov.uk/jcvi/childhoodimmunisationoc05.pdf), a dose of Hib conjugate vaccine (Hiberix) was administered concurrently in the opposite anterolateral thigh. The 0.5 ml dose contains 10 µg of the polysaccharide of Hib and 30 µg of the carrier protein tetanus toxoid. A 6 ml blood sample was obtained from each child prior to immunisation and at day 30 after immunisation. The 33 children were also split into 5 subgroups for an additional blood sample obtained on either day 2, 4, 6, 8 or 9 after immunisation, this allowing investigation of the kinetics of the Ab, plasma cell and memory B cell responses after immunisation. The protocol was approved by the Oxfordshire Research Ethics Committees (approval number CO6/Q1604/N41; Eudract number 2006-000732-28).

4.2.2 Anti-*N. meningitidis* serogroup C bactericidal activity as measured by rSBA

A serogroup C *N. meningitidis* bactericidal assay was performed using baby rabbit complement according to standard protocols (Maslanka et al., 1997). Briefly, twofold dilutions of heat-inactivated sera were incubated with the C11 strain of group C *N. meningitidis* and freshly thawed rabbit complement (PelFreez, Brown Deer, WI). The last dilution producing a $\geq 50\%$ reduction in colonies (killing) compared to control wells, containing complement and bacteria, was taken as the end-point rSBA titre.

4.2.3 Anti-*N. meningitidis* serogroup C IgG concentration as measured by ELISA

The serogroup C *N. meningitidis* polysaccharide-specific IgG concentration was determined by ELISA following a previously described method (Gheesling et al., 1994) (see section 2.5).

4.2.4 Anti-diphtheria toxoid IgG concentration as measured by luminex multiplex assay

The diphtheria toxoid-IgG concentration was determined in the laboratories of the Vaccine Evaluation Unit, Health Protection Agency in Manchester, using a Luminex multiplex assay as previously described (Pickering et al., 2002). The results were read on a Bio-plex reader (Bio-Rad, Hemel Hemstead, UK) and the data acquired by use of a computer software package (Bio-plex Manager, Bio-Rad, Hemel Hemstead, UK).

4.2.5 Separation of PBMCs and B-cell ELISpot

A maximum volume of 5 ml of heparinised blood was available for the separation of PBMCs, which were separated according to the methods described in section 2.2. ELISpot methods for the detection of plasma and memory B cells specific for MenC, diphtheria toxoid and CRM₁₉₇ were undertaken according to the methods described in section 2.3 and 2.4.

4.2.6 Statistical analysis

For the purposes of analysis, ELISpot assays in which fewer than 4 spots were detected were treated as though no ASCs were detected. The number of replicate wells used depended on the total number of PBMCs available, which varied between individuals and at the different time points. For MenC specific B cell assays where at least 4 wells of 2×10^5 PBMCs were used the minimum sensitivity of the assay was 5 cells per million PBMCs. For diphtheria toxoid- and CRM₁₉₇- specific B cell assay where at least 2 wells of 2×10^5 PBMCs were used the minimum sensitivity was 10 cells per million PBMCs. Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. rSBA titer and ELISA IgG concentrations were summarised using geometric mean concentration (GMC) and geometric mean titre (GMT) with corresponding 95% confidence intervals (CI). For B-cell numbers medians with 5th – 95th percentile were calculated. Within group comparisons of B-cell numbers and Ab concentrations at varying time points were made using the Wilcoxon signed ranks test for paired data with the p-values adjusted for multiple comparisons using the false discovery rate method (Benjamini et al.,

2001). The correlation between the different variables was compared using the Spearman's rank correlation coefficient, using the log transformed rSBA titer and ELISA IgG concentration but untransformed B cell numbers, with the p-values adjusted for multiple comparisons using the false discovery rate method (Benjamini et al., 2001). Further, we compared children's ability to produce MenC-specific memory B cells at 5 months of age with the persisting protective Ab titer at one year of age, using the Fisher's exact test. Because of the small sample size of this study no multivariate analysis was run.

4.3 Results

4.3.1 Recruitment

Between May and August 2006, all 33 children completed the study. However, because of insufficient sample volumes or assay failures, not all data points were available for each time point (see number of participants for each result of Table 4.3-1).

4.3.2 Kinetics of anti-serogroup C *N. meningitidis* bactericidal activity after immunisation

At one year of age, 8 months after three doses of MenCV at 2, 3 and 4 months of age, the pre-booster rSBA GMT had declined significantly from 1526 to 9 ($p = 0.0007$) and only 9/22 (40.9%) children had an rSBA titre equal or above the putative protective threshold of 1:8 (Table 4.3-1 and Figure 4.3-1). By day two after booster immunisation at one year 1/5 children had an rSBA $\geq 1:8$, by day four 5/6 had an rSBA titre $\geq 1:8$ and from day six onwards, all children had protective levels of bactericidal Ab (rSBA GMT $\geq 1:8$). The rSBA GMTs at day 30 post-booster were significantly higher than the rSBA GMTs pre-booster ($p = 0.001$) with an average of 654 fold rise (95% CI: 257-1664) (Table 4.3-1). The post-booster rSBA GMTs were also significantly higher than the rSBA GMT one month post-primary immunisation ($p = 0.0003$).

Visit	Day [Age]	MenC-rSBA GMT (95% CI) [participants]	MenC-IgG GMC (95% CI) [participants]	Diph-IgG GMC (95% CI) [participants]	MenC-plasma cells median (5 th - 95 th percentile) [participants]	Diph-plasma cells median (5 th - 95 th percentile) [participants]	CRM ₁₉₇ - plasma cells median (5 th - 95 th percentile) [participants]	MenC-memory B cells median (5 th - 95 th percentile) [participants]	Diph-memory B cells median (5 th - 95 th percentile) [participants]	CRM ₁₉₇ -memory B cells median (5 th - 95 th percentile) [participants]
Previous study	[5 months]	1526 (1151, 2023) [n=33]	19.8 (16, 24.5) [n=33]	1.4 (1.1-1.8) [n=32]	NA	NA	NA	11 (0, 107) [n=22]	10 (0, 82.5) [n=20]	NA
Visit 1	0 [12 months]	9 (4, 22) [n=22]	1.1 (0.7, 1.7) [n=22]	0.2 (0.1-0.2) [n=22]	0 (0, 0) [n=23]	0 (0, 0) [n=23]	0 (0, 0) [n=23]	3.5 (0, 9.5) [n=23]	11.5 (0, 52.5) [n=23]	7.8 (0, 17.5) [n=22]
	2	5 (0, 46) [n=5]	1 (0.3, 3) [n=4]	0.1 (0.1-0.4) [n=4]	0 (0, 0) [n=5]	0 (0, 0) [n=4]	0 (0, 0) [n=4]	0 (0, 11.5) [n=4]	0 (0, 14) [n=3]	0 (0, 11.5) [n=3]
	4	128 (18, 901) [n=5]	1.2 (0.3, 4.4) [n=6]	0.1 (0.1-0.3) [n=6]	7 (0, 136) [n=7]	8.5 (0, 37.5) [n=7]	4 (0, 15) [n=7]	2 (0, 6.5) [n=6]	0 (0, 12.5) [n=4]	0 (0, 5) [n=5]
	6	4705 (2290, 9667) [n=5]	16.3 (10.3, 25.7) [n=5]	0.7 (0.2-2.3) [n=4]	112.5 (23, 234) [n=6]	121.5 (21.5, 170) [n=6]	43.8 (11.5, 100) [n=6]	8 (0, 47.5) [n=6]	14 (0, 22.5) [n=6]	10 (0, 17.5) [n=5]
Visit 2	8	18390 (4482, 75459) [n=6]	52.9 (23, 121.6) [n=6]	3.2 (1.2-8) [n=6]	19.5 (5.5, 160) [n=7]	27.5 (0, 66.5) [n=7]	15 (0, 26.5) [n=7]	40 (1, 658.5) [n=4]	81.5 (7.5, 392.5) [n=4]	27.5 (0, 160) [n=4]
	9	13004 (4813, 35131) [n=6]	46.2 (25.9, 82.7) [n=6]	3.3 (2-5.5) [n=5]	11 (7.5, 344) [n=6]	42.5 (0, 156.5) [n=5]	21.5 (0, 55) [n=5]	23 (0, 422.5) [n=5]	86.5 (20, 177.5) [n=4]	40.8 (12.5, 56.5) [n=4]
Visit 3	30 [12 months]	6087 (4179, 8865) [n=28]	27.9 (20.3, 38.4) [n=28]	1.8 (1.3-2.5) [n=28]	0 (0, 5) [n=26]	0 (0, 22.5) [n=22]	0 (0, 12.5) [n=22]	16.5 (0, 111) [n=28]	52.5 (0, 220) [n=25]	32.5 (0, 95) [n=23]

Table 4.3-1: Ab and B cell responses to MenCV at various days following challenge with MenCV at one year of age and at 5 months of age after primary immunisation. MenC-rSBA GMT, MenC- and diphtheria toxoid- IgG GMCs (in µg/ mL and IU/mL), MenC-, diphtheria toxoid- and CRM₁₉₇- plasma cells (in ASCs per million PBMCs), MenC-, diphtheria toxoid- and CRM₁₉₇- memory B cells (in ASCs per million cultured lymphocytes).

4.3.3 Kinetics of anti-*N. meningitidis* serogroup C IgG concentration after immunisation

At one year of age, before the booster dose of MenCV a MenC-specific IgG concentration ≥ 2 $\mu\text{g/ml}$ was seen in 6/22 children, all of whom had a protective rSBA titer ($\geq 1:8$). The pre-booster concentrations of MenC-specific IgG and diphtheria toxoid-specific IgG were significantly lower than the GMC after three-dose primary immunisation ($p = 0.0008$ for MenC and diphtheria toxoid).

Following challenge with MenCV, levels of Abs specific for MenC and diphtheria toxoid increased rapidly. At 30 days after immunisation at one year of age the IgG concentrations were significantly higher than before the booster dose at 12 months of age ($p = 0.001$ for MenC and diphtheria toxoid) with an average fold increase of 26.3 (95% CI: 16.2-42.5) for MenC-IgG and 10.7 (95% CI: 7.2-15.9) for diphtheria toxoid-IgG. Further, 30 days post-MenCV at one year of age, the MenC-IgG concentration was significantly higher than after primary immunisation at 5 months of age ($p = 0.02$). However, the concentration of diphtheria toxoid-IgG was not significantly higher ($p = 0.06$) (Table 4.3-1 and Figure 4.3-1).

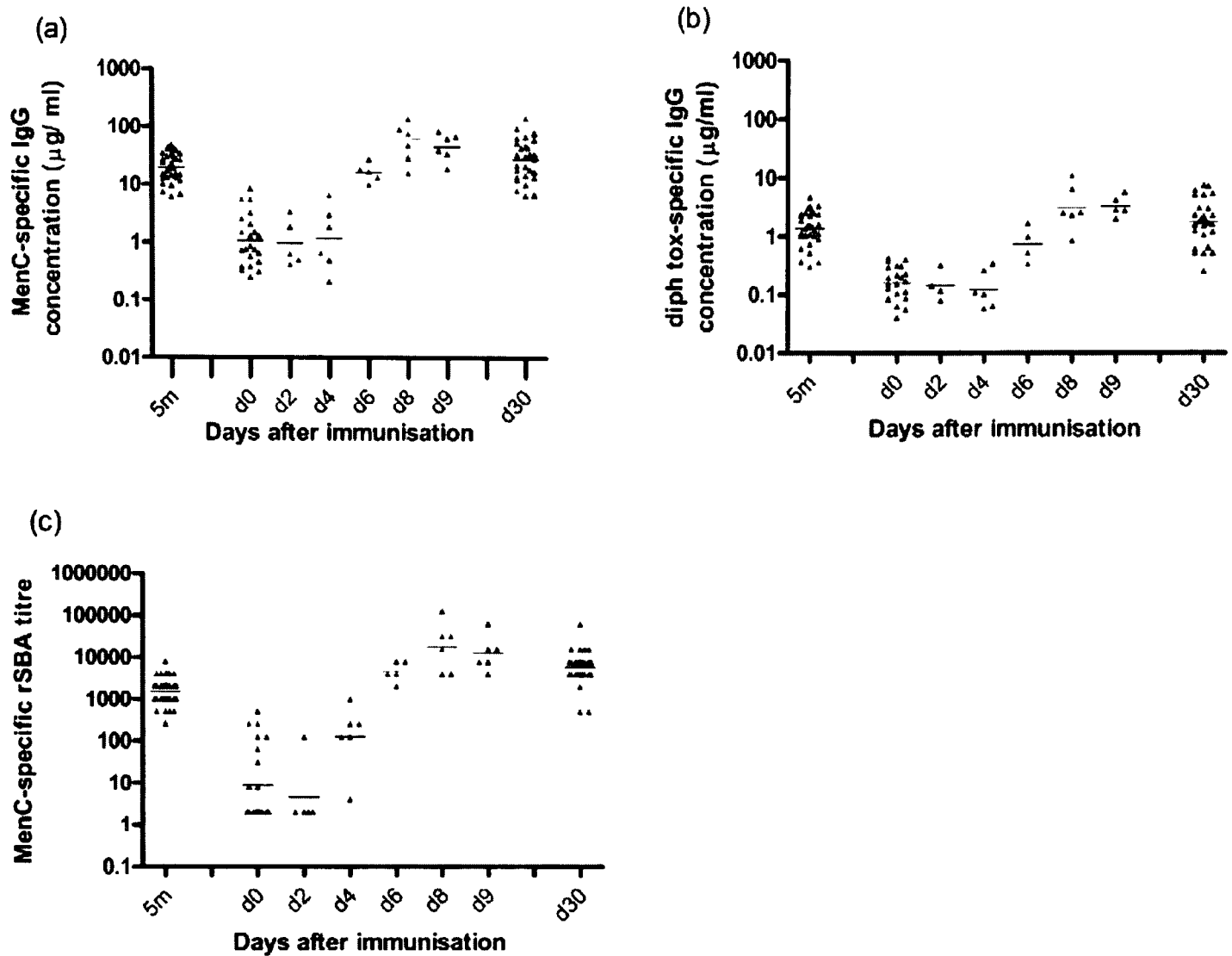


Figure 4.3-1: MenC specific- IgG (a) and bactericidal Ab (c), and diphtheria toxoid specific- IgG (b) responses to immunisation with MenCV at one year of age. (a) Using an ELISA assay, MenC-specific IgG response was measured at 5 months of age after primary immunisation and at various days following immunisation with MenCV at one year of age. The horizontal bars represent the GMC at each time point. (b) Using a Luminex multiplex assay, diphtheria toxoid-specific IgG response was measured at 5 months of age after primary immunisation and at various days following immunisation with MenCV at one year of age. The horizontal bars represent the GMC at each time point. (c) Serum bactericidal activity using the rabbit complement (rSBA) was measured at 5 months of age after primary immunisation and at various days following immunisation with MenCV at one year of age. The horizontal bars on the graph represent the GMT at each time point.

4.3.4 Antibody decline after primary immunisation (from 5 months to 12 months of age) and Ab rise after booster dose of MenCV at one year of age (12 months to 13 months)

After primary immunisation, the decline in MenC-specific Ab (measured by rSBA titer and MenC-specific IgG concentration) was similar for all children (Figure 4.3-2) from 5 months (1 month after priming with 3 dose of MenCV) to 12 months of age. Following the booster dose of MenCV at one year of age, there was a tendency that the children who had lower Ab levels (measured by rSBA titer and IgG concentration) at 12 months of age had higher increase in Ab (Figure 4.3-2). Therefore, the rise in Ab was lower in the children who had higher Ab levels pre-booster, although the post-booster levels in these children were higher (Figure 4.3-2). Further, the children with a rSBA titer at one year of age $< 1:8$ had an average fold increase of 2353 (n = 10), while those with a rSBA $\geq 1:8$ had an average fold increase of 159 (n = 7). The children with an IgG concentration at one year of age $< 2 \mu\text{g/ml}$ had an average of 34.4 fold increase (n = 13), compared with an average fold increase of 10.9 (n = 4) in those with an IgG concentration $\geq 2 \mu\text{g/ml}$.

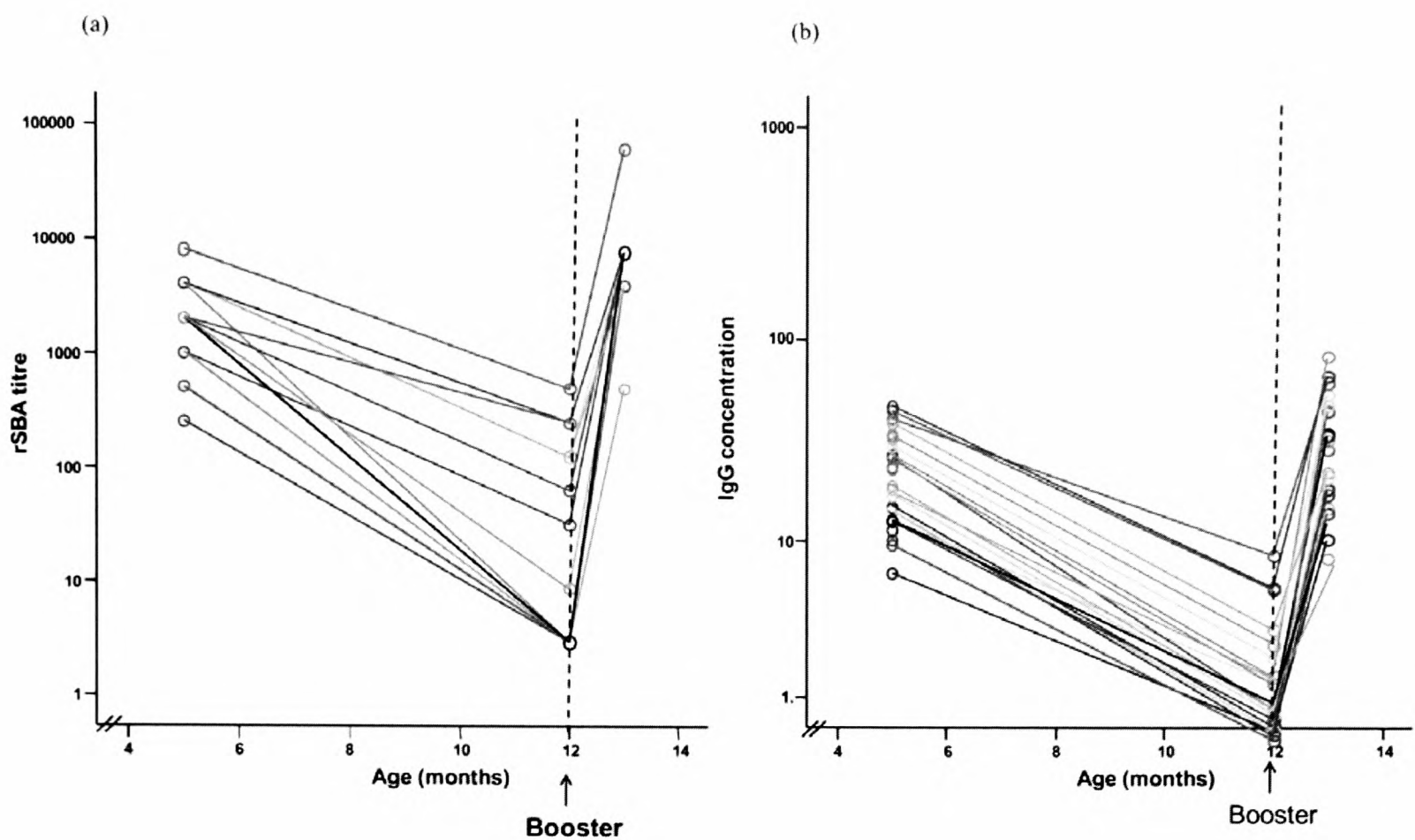


Figure 4.3-2: Ab decline after primary immunisation (from 5 months to 12 months of age) and Ab rise after a booster dose of MenCV at one year of age (from 12 months to 13 months). Results are shown for (a) MenC- rSBA titer and (b) MenC-IgG concentration. Each line colour represents a different child.

4.3.5 Plasma cell frequency measured by *ex vivo* B cell ELISpot

Prior to the booster dose of MenCV at one year of age there were no plasma cells specific for MenC, diphtheria toxoid, and CRM₁₉₇ detected in peripheral blood. The first appearance of MenC-, diphtheria toxoid-, and CRM₁₉₇- specific plasma cells above baseline was seen at day 4 with a peak at day 6 and a rapid decline by day 8-9 (Figure 4.3-3) (Table 4.3-1). There were almost no detectable specific plasma cells at day 30 after vaccination. The median frequency of CRM₁₉₇-specific plasma cells was half the frequency of diphtheria toxoid-specific plasma cells. In comparison to the response to primary immunisation, the peak frequency of plasma cells

was higher after the booster dose of MenCV at one year of age (median of 112.5 MenC-plasma cells per million PBMCs and 121.5 diphtheria toxoid-plasma cells per million PBMCs at day 6 post-one year booster dose, compared to 50 MenC-specific plasma cells and 83 diphtheria toxoid-specific plasma cells per million PBMCs at day 4 post third dose of MenCV at 4 months of age) (data not shown).

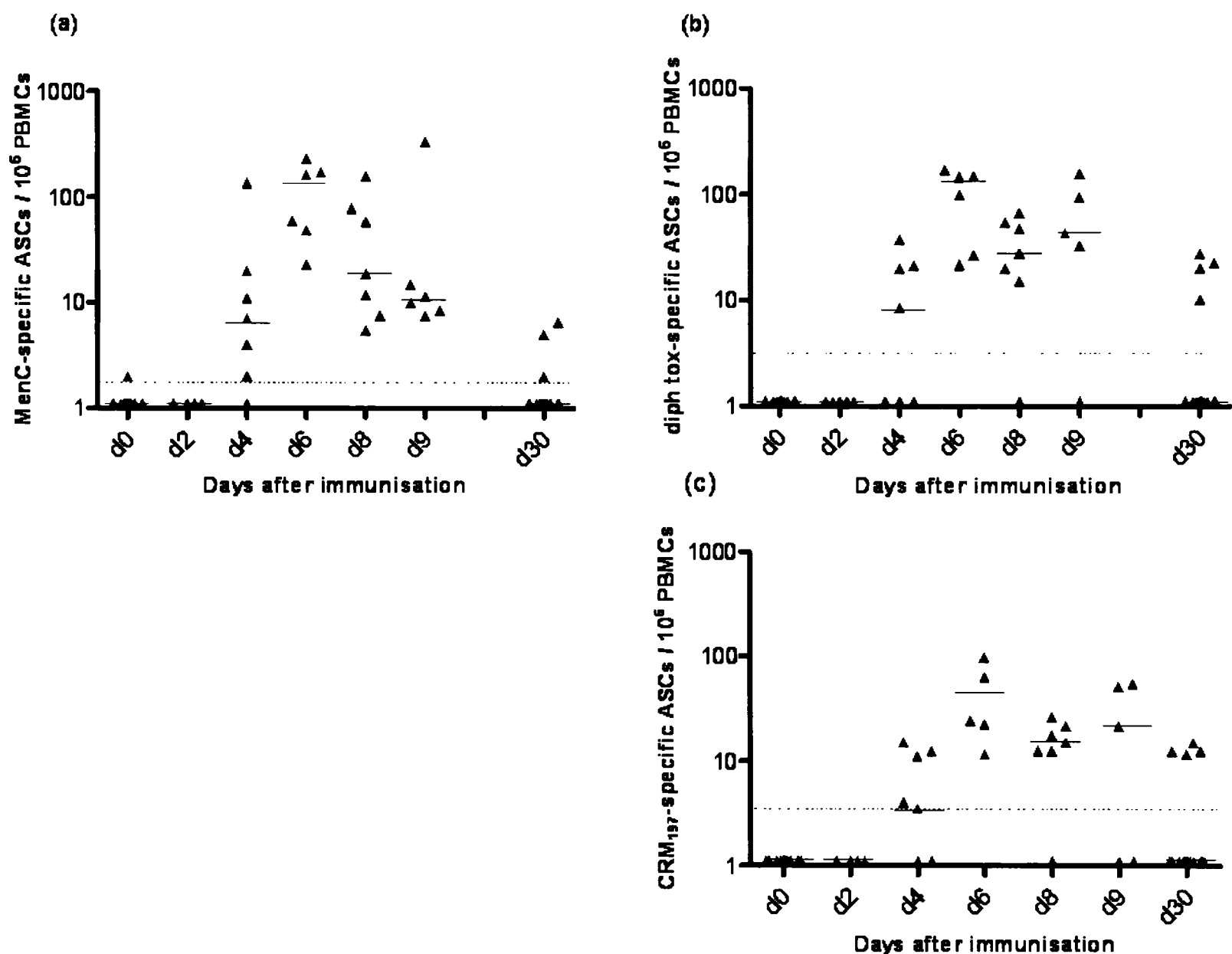


Figure 4.3-3: MenC-, diphtheria toxoid- and CRM₁₉₇- specific plasma cell responses to immunisation with MenCV at one year of age. The children were immunised at day 0 and the frequency of (a) MenC, (b) diphtheria toxoid and (c) CRM₁₉₇- specific-ASCs was measured at various days after immunisation. The horizontal bars represent the median number of specific ASCs at each time point. The minimum sensitivity of the assay is plotted as a broken line on the graph for each figure. In some assays there were fewer PBMCs available for analysis and the minimum sensitivity was twofold higher (not shown on graphs). The zero values have been given the arbitrary value of 1 for illustrative purposes.

4.3.6 Memory B cell frequency by culture B cell ELISpot

Prior to the booster dose of MenCV at one year of age 7/23 (30%) children have been found to have ≥ 5 MenC-memory B cells per million cultured lymphocytes. The median frequency of MenC-memory B cells for the 23 children was 3.5 MenC-memory B cells per million cultured lymphocytes. By contrast, at 5 months of age after primary immunisation 8/22 (36%) children had < 5 memory B cells per million cultured lymphocytes. The median frequency of MenC-memory B cells for the 22 children was 11 MenC-memory B cells per million cultured lymphocytes (Table 4.3-1). The frequency of MenC-memory B cells prior to the one year vaccine dose was significantly lower than the memory B cell frequency reached after primary immunisation at 5 months of age ($p = 0.01$) (Table 4.3-1). However, the frequency of memory B cells specific for diphtheria toxoid had not significantly declined at one year of age compared to the frequency at 5 months (median difference 0, range (-9, 11), $p = 0.6$) (Table 4.3-1).

Following the booster dose of MenCV at one year of age, the first response above baseline was seen by day 8 for MenC-, diphtheria toxoid-, and CRM₁₉₇- specific memory B cells (Figure 4.3-4). At 30 days after immunisation, memory B cells specific for MenC, diphtheria toxoid, and CRM₁₉₇ were still detectable in blood at a significantly higher frequency than before the booster ($p = 0.002$ for MenC, $p = 0.02$ for diphtheria toxoid, and $p = 0.008$ for CRM₁₉₇) These frequencies were also significantly higher than those measured 1 month after primary immunisation for diphtheria toxoid ($p = 0.009$) but not for MenC ($p = 0.2$).

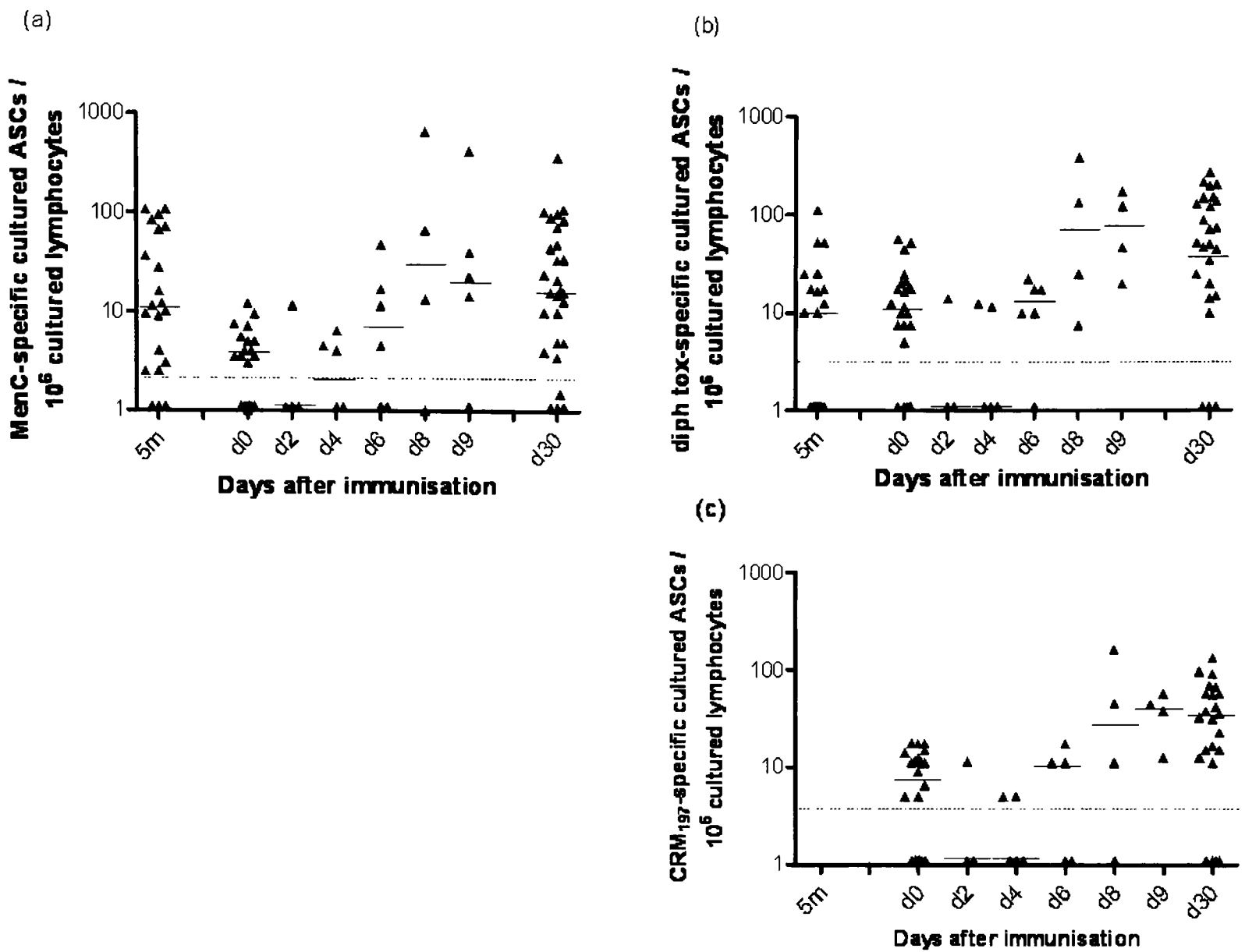


Figure 4.3-4: MenC-, diphtheria toxoid- and CRM₁₉₇- specific memory B cells at 5 months and after immunisation with MenCV at one year of age. Using an ELISpot assay after 6 days culture with SAC/CpG/PWM, the (a) MenC-, (b) diphtheria toxoid- and (c) CRM₁₉₇- specific memory B cell response was measured at various days following immunisation with MenCV at one year of age (and at 5 months of age after primary immunisation for MenC- and diphtheria toxoid-specific memory B cells). The horizontal bars on the graph represent the median number of specific ASCs at each time point. The minimum sensitivity of the assay is plotted as a broken line on the graph for each figure. In some assays there were fewer PBMCs available for analysis and the minimum sensitivity was twofold higher (not shown on graphs). The zero values have been given the arbitrary value of 1 for illustrative purposes.

4.3.7 Correlation between the primary immune response and both persistence of immunity at one year of age and the response to a booster dose of MenCV

At 5 months of age there was a strong evidence of association between the MenC-specific memory B cells and Ab (Table 4.3-2) but not between the diphtheria toxoid-specific memory B cells and Ab ($r = 0.3$, $p = 0.2$).

The level of MenC-specific Ab and memory B cells produced after priming correlated with the persistence of Ab and memory B cells at one year of age, and with the post-booster Ab and memory B cell levels (see Table 4.3-2 and Figure 4.3-5). Similarly, diphtheria toxoid memory B cells and Ab measured at 5 months of age correlated with the persistence of diphtheria toxoid-Ab at one year of age ($r = 0.8$, $p = 0.003$ and $r = 0.7$, $p = 0.002$). However, only the diphtheria toxoid-Ab measured at 5 months of age, and not the memory B cells, correlated with the post-booster Ab levels at 13 months of age ($r = 0.6$, $p = 0.003$).

	Memory B cells at 5 months			IgG at 5 months			rSBA at 5 months		
	r	p	n	r	p	n	r	p	n
IgG at 5 months	0.74	0.001	21						
rSBA at 5 months	0.71	0.001	22	0.84	<0.0001	31			
IgG at 12 months	0.69	0.01	14	0.85	<0.0001	20	0.72	0.001	22
rSBA at 12 months	0.65	0.02	14	0.74	0.001	20	0.67	0.002	22
Memory B cells at 12 months	0.65	0.02	15	0.65	0.003	21	0.67	0.002	23
IgG at 13 months	0.52	0.04	18	0.67	0.001	20	0.55	0.005	28
rSBA at 13 months	0.56	0.03	18	0.52	0.01	26	0.61	0.002	28
Memory B cells at 13 months	0.66	0.003	21	0.61	0.003	26	0.46	0.02	28

Table 4.3-2: Correlation of MenC-memory B cells and Ab at 5 months of age with the Ab and memory B cells measured at 5 months, 12 months and 13 months of age. Spearman's rank correlation coefficient (r) with the p-values adjusted for multiple comparisons (p) and the number of subject tested for each association (n).

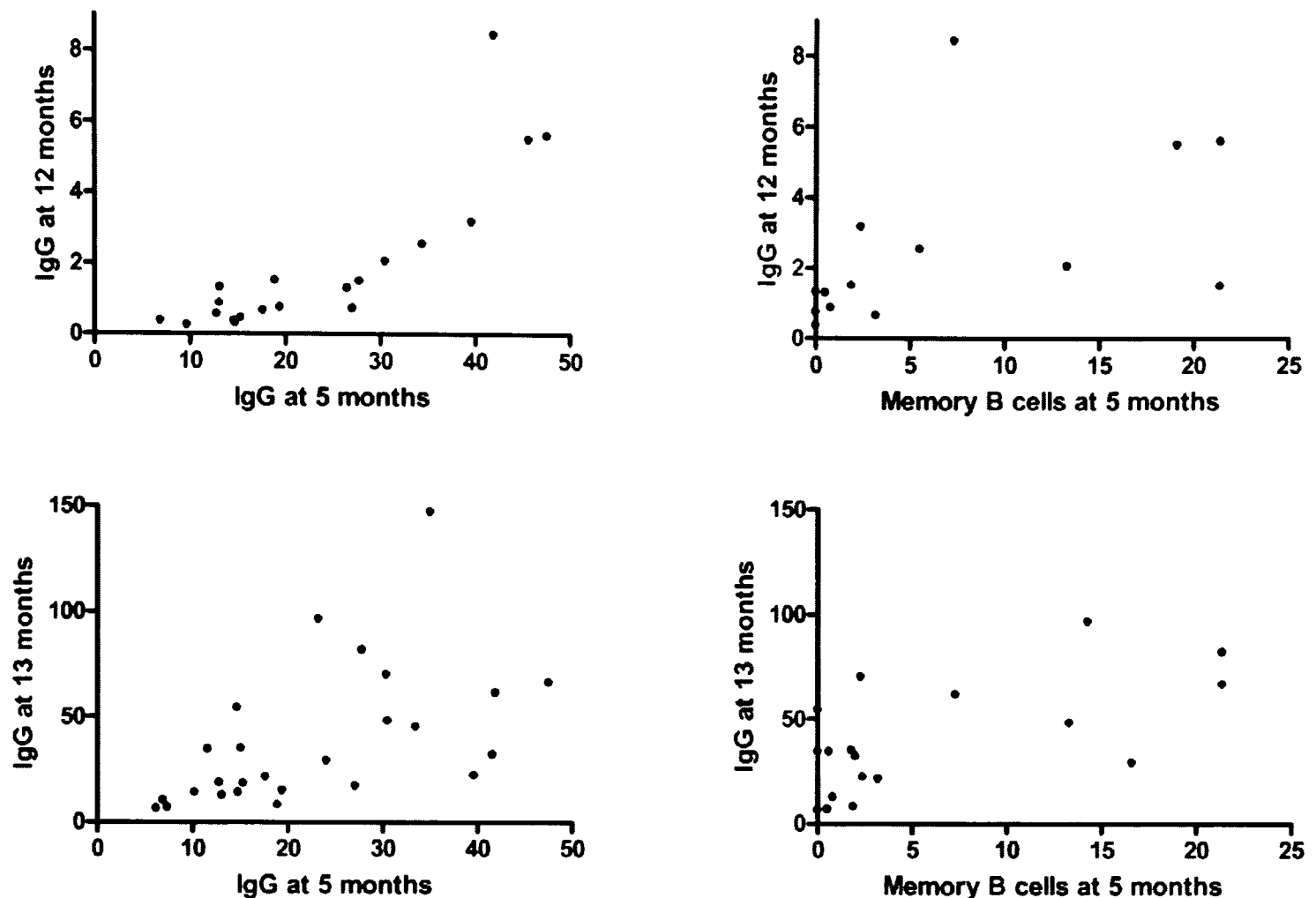


Figure 4.3-5: Correlations between MenC-memory B cells and Ab at 5 months of age with the persisting Ab at one year of age and post-booster Ab levels, using the Spearman’s correlation method (see Table 4.3-2 for the r and p-values of each correlation).

4.3.8 Correlation between memory B cells at 5 months and the rSBA titre at one year of age using the Fisher exact test

The presence or absence of detectable MenC specific memory B cells at 5 months of age (i.e. ≥ 5 MenC-memory B cells per million cultured lymphocytes) was found to have some evidence of correlation with the maintenance of a “protective” rSBA titer ($\geq 1:8$) to 1 year of age ($p = 0.03$) (Table 4.3-3).

No. memory B cells at 5 months	rSBA at 1 year	
	< 8	≥ 8
< 5	5	0
≥ 5	3	6

Table 4.3-3: Correlation between MenC-memory B cells at 5 months of age and rSBA titre at one year of age. The presence or absence of detectable MenC-specific memory B cells at 5 months of age (i.e. ≥ 5 MenC-memory B cells per million cultured lymphocytes) were correlated with the maintenance of a “protective” rSBA titer ($\geq 1:8$) to 1 year of age using the Fisher’s exact test with 1 *df*, ($p = 0.03$).

4.3.9 Correlation between carrier protein-specific and polysaccharide capsule-specific immune response

There was a correlation between diphtheria toxoid-specific and MenC-specific memory B cell frequencies after immunisation ($r = 0.74$, $p < 0.001$, $n = 25$) but not before ($r = 0.29$, $p = 0.19$, $n = 23$). Furthermore, there was a correlation between diphtheria toxoid-specific and MenC-specific Ab concentration before ($r = 0.47$, $p = 0.03$, $n = 22$) and after immunisation ($r = 0.58$, $p = 0.001$, $n = 28$). However, there was no correlation between the diphtheria toxoid-baseline Ab concentration and the post-booster MenC- memory B cell and Ab levels (data not shown).

4.3.10 Correlation between CRM₁₉₇ and diphtheria toxoid B cell frequencies

There was a strong correlation between the diphtheria toxoid- and CRM₁₉₇- memory B cell response after immunisation ($r = 0.89$, $p < 0.001$, $n = 23$) and a moderate correlation before

immunisation ($r = 0.48$, $p = 0.03$, $n = 22$). In general, the frequency of diphtheria toxoid-specific B cells was 1.5-3 times greater than that of CRM₁₉₇-specific B cells, depending of the day after immunisation.

4.4 Discussion

In this study we observed for the first time that the ability of children to produce MenC-specific memory B cells and Ab at 5 months of age during primary immunisation allows both determination of the strength of the immune response to priming and also prediction of the persistence of functional Ab at one year of age and the intensity of the humoral immune response to a booster dose of MenCV at one year of age.

At one year of age, less than 50% of the study children had maintained protective bactericidal Ab levels. These observations support the previous evidence that protective Ab levels do not persist well in children vaccinated with MenCV under 2 years of age (Rennels et al., 2001; Snape et al., 2005; Tsai et al., 2006). By contrast, immunisation of older children and adults results in more persistent protective Ab for some years (Kelly et al., 2006; Snape et al., 2006; Trotter et al., 2004).

Previous studies demonstrated that immunological memory was maintained despite the waning of MenC-specific Abs (Andrews et al., 2003; Borrow et al., 2002; MacLennan et al., 2000). In this study, following secondary immunisation with MenCV at one year of age, MenC-specific Abs increased rapidly and by day 6 all children had achieved a protective rSBA titer. This Ab response to a booster dose of MenCV at one year of age after 3 dose priming in infancy is comparable to the one observed by Tsai et al. (2006) in children primed with a single dose of MenCV at 12-15 months of age and who were challenged with serogroup C polysaccharide at 24 months of age (in this study all children had mounted protective bactericidal Ab levels by day 5) (Tsai et al., 2006).

However, the maintenance of functional Ab rather than the immunological memory appears to be the key determinant of long-term protection against invasive diseases caused by encapsulated bacteria. It is not clear how long-term production of Ab is maintained. It has been suggested that long-lived plasma cells surviving in bone marrow niches (Moser et al., 2006; Odendahl et al., 2005) continue to secrete Ab after the exposure with the Ag. Slifka et al. (1998) have demonstrated that these cells could survive for longer than one year in mice (Slifka et al., 1998). The presence of long-lived plasma cells has not been demonstrated in humans. Alternatively, it was suggested that plasma cells would have to be continuously replaced by the turn over of memory B cells in humans. This phenomenon may be Ag-independent and occur by polyclonal stimulation of B cells (Bernasconi et al., 2002) by microbial products (which stimulate B cells via TLRs), or through bystander T cell help (cytokines secreted by T cells activated by another Ag) (Lanzavecchia, 1983). Others have postulated that this event is Ag-dependent and occurs through cross-reactive Ag or persisting Ag (Ochsenbein et al., 2000). It has been shown that although a GC persists for only 3 weeks following immunisation, memory B cells continue to proliferate and to differentiate into plasma cells in the lymphoid tissues for several months after stimulation in response to the Ag persisting on the follicular DC networks (Holder et al., 1993). Although these mechanisms are unclear in humans, studies in mice indicate that long-lived plasma cells survive in bone marrow niches independently of memory B cells and of the Ag (Manz et al., 1998).

In the present study, there was a strong correlation between the MenC-specific memory B cells and Abs at 5 months of age and also between these two parameters and the persistence of Ab at one year of age. These results suggest that priming with MenCV in early infancy may generate GC of various efficiency in different children. Thus, some children produce a higher number of

memory B cells and plasma cells and are therefore able to produce higher Ab levels after priming. In contrast, the children who generate less efficient GC will have a smaller B cell pool and lower Ab response at 5 months of age (1 month after primary immunisation). The immune response is determined by a combination of genetic and environmental factors but genetic factors predominantly determine the Ab response to vaccines given in early life (Marchant et al., 2006; Newport et al., 2004), while environmental factors influence Ab persistence and avidity maturation (Marchant et al., 2006).

In this study, the decline of the Ab concentrations from 5 months (1 month after primary immunisation) to 12 months was similar in all children. Thus, it is not clear whether B cells have a direct role in sustaining Ab production after priming or whether the level of persisting Ab at one year of age only depends on the biological half life of Ab and would, therefore, mostly be determined by the level of Ab reached after primary immunisation. However, it was observed that the anti MenC IgG GMC was 19.7 $\mu\text{g/mL}$ at 5 months of age (i.e. post primary immunisation), whereas at one year it was 1.1 $\mu\text{g/mL}$. An explanation based solely on Ab half-life (3 weeks (Vieira and Rajewsky, 1988)) is insufficient to account for these high levels. This suggests the existence of other mechanisms determining the long-term persistence of Ab.

Persisting Ab at one year of age correlated more strongly with the level of Ab at 5 months than with the number of memory B cells present at 5 months. However, when children were separated into those who had detectable and undetectable memory B cells at priming, we found that all those with undetectable memory B cells after priming did not maintain protective Ab levels at one year of age. Conversely, 2/3 of the children with detectable memory B cells at 5 months had maintained protective rSBA titer at one year. All children produced high levels of Ab at 5 months

of age (IgG between 6.2 and 47.6 µg/ml and rSBA titer between 512 and 8192); therefore, it is more difficult to define a cut-off of Ab level after priming to predict long-term protection. Therefore, even if the role of memory B cells in the long-term maintenance of protective Ab levels is unclear, it appears that the measure of memory B cells, rather than the Ab at 5 months of age can better discriminate the children who will sustain protective levels of Ab.

The role of B cells generated after priming in the secondary immune response has not been studied previously. In this study, we observed that the children who achieved the highest Ab level following priming also attain the highest Ab level following the booster dose of MenCV. However, there was a difference in the rise of the Ab response: the children with the lower pre-booster Ab level at 12 months of age had the higher increase in Ab compared to the children with higher level at baseline. Similar results have been observed following other vaccines (Danilova et al., 2005a; Danilova et al., 2005b). These findings suggest that there might be a mechanism of feedback above a certain Ab level and Ag-specific B cells might not increase above a certain threshold. Similarly, it has been shown that a third dose of MenCV did not induce a higher Ab titer compared to the second dose (Richmond et al., 1999). It is not clear how this feedback mechanism operates; it could result from competition between the pre-existing Ab and the membrane-bound BCR for the Ag, impairing the activation of B cells (Nanan et al., 2001).

We used the *ex vivo* ELISpot assay to detect cells that spontaneously secrete Ab after PBMCs separation. Clutterbuck et al. (2006) have shown that these cells have the phenotype of plasma cells (CD20⁺CD38⁺CD27⁺ or CD20⁻CD38⁺CD27⁺) (Clutterbuck et al., 2006). In this study, following immunisation with MenCV at one year of age, the first appearance of plasma cells specific for MenC, diphtheria toxoid, and CRM₁₉₇ above baseline was seen at day 4 with a peak at

day 6, followed by a rapid decline in the frequency to day 8-9. These plasma cell kinetics are similar to those observed in previous studies with a variety of vaccine Ags in primed adults: MenC polysaccharide vaccine and MenCV (Kelly et al., 2006); varied serotypes of the heptavalent pneumococcal conjugate vaccine and diphtheria toxoid (Clutterbuck et al., 2006) and tetanus toxoid (Bernasconi et al., 2002), suggesting that the kinetics of the secondary plasma cell response is common to all Ags (TD and TI) and all age groups.

Given the rapidity of the plasma cell response following immunisation in previously primed individuals, these cells are likely to represent plasma cells derived from pre-existing memory B cells. It was shown in humans that memory B cells respond to Ag re-challenge 20-30 hours earlier than naïve B cells, because of their lower activation requirements (Tangye et al., 2003) and their homing to unique Ag-draining sites (e.g. mucosal epithelium) (Liu et al., 1995). Additionally, it was observed in mice that most memory B cells give rise to extra-GC plasma cells upon re-challenge with the Ag (Kroese et al., 1991; Liu et al., 1991). It is also possible that, some of these plasma cells are long-lived plasma cells, translocated from their bone marrow niches by an unknown signal after re-exposure with the Ag or that the transient appearance of the plasma cells in peripheral blood represents their passage from their site of production to their long-term residence in bone marrow (Kelly et al., 2006).

The fate of the plasma cells produced in this day 6/7 burst is unknown. The rise in MenC-Ab was also observed from day 4-6 with a peak by day 8, suggesting that the rapid increase in Ab levels following immunisation is produced by these newly formed plasma cells.

A previous larger study found a weak correlation between the increase in Ab level and the peak plasma cell response following immunisation with polysaccharide conjugate vaccines (Kelly et al., 2006). As mentioned earlier, this could be explained by negative feedback in Ab production among those with pre-existing raised Ab levels. Further, in this study there was a moderate correlation between the memory B cells at 5 months and the post-booster Ab levels. These findings suggest that the rise in Ab from day 0 to day 30 post-booster may have various different origins (e.g. persisting memory B cells and long-life plasma cells, newly formed plasma cells and memory B cells) and depend on several factors (e.g. T helper cell, Ag presenting cells, serum titer of specific Ab).

Polyclonal stimulation with SAC, CpG-DNA and PWM for 6 days induces polyclonal proliferation of IgG⁺ memory B cells and their differentiation into ASCs (Crotty et al., 2004). Further, naïve B cells are not activated in the absence of specific Ag (they require BCR triggering by anti-Ig) (Bernasconi et al., 2002) and pre-existing plasma cells do not survive more than few days without appropriate stromal cells support (Cassese et al., 2003). It is therefore likely that the cells detected in this study after culture are derived from B cells that have a memory phenotype.

It has been suggested that after their production memory B cells continuously re-circulate through the blood and secondary lymphoid organs (Nanan et al., 2001; Salmi and Jalkanen, 1997) and it was therefore postulated that the memory B cells found in the circulation were representative for the entire B-cell pool (Nanan et al., 2001). Previous studies with diphtheria toxoid-, tetanus toxoid-, and smallpox- specific memory B cells have shown their persistence for a prolonged period after immunisation, with a frequency in human peripheral blood of 0.01-1% of total IgG-memory B cells (Crotty et al., 2003a; Nanan et al., 2001). Similarly, in this study we found that

0.02% of total IgG-memory B cells were MenC-specific (median frequency), 0.12% were diphtheria toxoid-specific (median frequency), and 0.05% were CRM₁₉₇-specific (median frequency), by one year of age (8 months after the 3 dose priming schedule). The frequency of persisting MenC-memory B cells was significantly lower than that reached one month after primary immunisation. However, the frequency of diphtheria toxoid-memory B cells was maintained. The factors influencing the maintenance of memory B cells in the circulation after vaccine exposure could be cellular competition (making space for new memory B cells) in secondary lymphoid organs (Crotty et al., 2003a), or the persistence of the Ag in FDC (MacLennan, 1994).

We found in this study that following immunisation at one year of age, MenC-, diphtheria toxoid-, and CRM₁₉₇- specific memory B cells were detected by the end of the first week after immunisation and were still raised above baseline levels at one month (for MenC: 0.26% of total IgG-memory B cells; for diphtheria toxoid 0.67% of total IgG-memory B cells, and for CRM₁₉₇ 0.21% of total IgG-memory B cells). The frequency of MenC-specific memory B cells is one of the lowest values described, though most previous studies have involved adult volunteers. Further, following the booster dose of MenCV at one year of age the MenC-memory B cell frequency was not significantly higher than the frequency reached after primary immunisation (at 5 months of age). This contrasts to diphtheria toxoid-memory B cells, which had reached a higher frequency after the booster dose of MenCV than after primary immunisation. These findings suggest that memory B cells specific for protein Ags better persist in peripheral blood than polysaccharide-specific memory B cells in children. It is possible that polysaccharide specific-memory B cells reside in secondary lymphoid organs because of the retention of polysaccharide Ags as immune complexes in follicular DCs. Additionally, different factors might control the pool size of mature

B cells specific for protein Ags and for polysaccharide Ags. It is possible that re-challenge with the polysaccharide only induces the exit of the MenC-memory B cells from their niches and their maturation rather than their proliferation. However, there was a strong correlation between carrier-specific and MenC-specific memory B cell responses after immunisation but not before immunisation. In addition, there was a correlation between diphtheria toxoid- and MenC- specific Ab before and after immunisation. This suggests that although at a steady state, these cells might not reside in the same place. In general, the children, who have a good immune response to the carrier protein also have a good immunity for the capsule polysaccharide, emphasising the important role of T cell help for good B cell priming and long-term protection. Alternatively, this might simply indicate that there are genetic determinants of the strength of the immune responses irrespective of the stimulus.

There was a small decline in the polysaccharide- and carrier- specific memory B cells detectable in peripheral blood in the first days following immunisation (between day 0 and 6 in figure 4.3-4). This could be explained by the fact that following intramuscular immunisation, the vaccine Ag is transported into the closest secondary lymphoid organs by DCs to be presented to B cells and T cells. This induces a transitory retention of the circulating B cells in those lymphoid organs, and therefore, a temporary decline in peripheral blood memory B cells. However, the memory B cell kinetics we have observed are comparable to those described in previous studies using a variety of vaccine Ags in primed adults (Crotty et al., 2003a; Nanan et al., 2001) and suggest that although MenC-specific memory B cells were only detectable at a low frequency in most children at one year of age, these cells probably persist after priming in secondary lymphoid organs (mostly in mucosal surfaces) (Liu et al., 1995; Maruyama et al., 2000), ready to rapidly proliferate and differentiate upon re-challenge.

Finally, there was a correlation between the diphtheria toxoid- and CRM₁₉₇- memory B cell responses before and after immunisation, and the frequency of diphtheria toxoid-specific B cells was 1.5-3 times greater than the frequency of CRM₁₉₇-specific B cells, depending of the day after immunisation. This could be explained by a smaller pool of T cell clones being stimulated by CRM₁₉₇ compared to diphtheria toxoid, either because the infants had been primed with diphtheria toxoid vaccine, in addition to the MenC-CRM₁₉₇ vaccine at 2, 3 and 4 months of age. Secondly, the cross-reacting material CRM₁₉₇ contains a mutation in the catalytic domain of the toxin molecule, which make it more sensitive to proteolytic degradation and therefore, less immunogenic in comparison to the wild type toxoid. However, it has been reported that treatment with formaldehyde did not affect the Ab response but altered the T cell response to CRM₁₉₇, possibly through the alteration or loss of T cell epitopes (Gupta et al., 1997; McNeela et al., 2000; Porro et al., 1980).

In summary, this study found that the infants who generate efficient GCs (identified by high Ab and memory B cells level) at 5 months of age (1 month after primary immunisation), best maintained protective Ab levels at one year of age. However, one third of the infants in this study produced a very low number of memory B cells after the initial immunisation (< 5 memory B cells per million cultured lymphocytes) and did not maintain protective Ab levels by one year of age. An understanding of the factors that determine the production of adequate GC during priming in early infancy could lead to an improvement in the short and longer-term protection induced by glycoconjugate vaccines that are so important for the protection of young children against serious diseases caused by polysaccharide encapsulated bacteria.

5 B cell responses to a primary course of MenACWY-CRM₁₉₇ at 2 and 4 months of age

5.1 Introduction

In children, *N. meningitidis* is one of the leading causes of meningitis and septicaemia, with the highest incidence occurring between the age of 6 and 24 months, although an additional peak is observed in adolescents. Despite appropriate treatment, the overall mortality rate is 10% (Thorburn et al., 2001; Goldacre et al., 2003; Jensen et al., 2003; de Greeff et al., 2008;; Jacobsson et al., 2008)., and up to 10% of the survivors suffer from life-long sequelae (Oostenbrink et al., 2002; Buysse et al., 2008a; Buysse et al., 2008b). Five serogroups are responsible for the vast majority of meningococcal diseases worldwide: serogroups A, B, C, W135 and Y (Peltola, 1983). A substantial increase in incidence and reported outbreaks due to serogroup C meningococci in the early 1990s led to immunisation campaigns with MenCV in the UK and many other countries (Snape and Pollard, 2005). MenCV was shown to be very effective and its introduction in the UK resulted in a rapid reduction in serogroup C meningococcal disease (Miller et al., 2001), with a decline in serogroup C carriage (Maiden and Stuart, 2002). However, this vaccine cannot offer protection against other serogroups of meningococci.

Tetravalent meningococcal A, C, Y and W135 (MenACWY) conjugate vaccines are currently under development. A MenACWY-diphtheria toxoid vaccine has been developed by Sanofi

Pasteur. However, in contrast to the MenCV, this vaccine was poorly immunogenic in young infants, despite its ability to induce immunological memory (Rennels et al., 2004). This vaccine was licensed for the 2-55 year olds in Canada and for children over 11 years of age in the US in 2005/2006. Therefore, no tetravalent MenACWY conjugate vaccines are currently licensed for use in children younger than 2 years, despite the highest rates of invasive meningococcal diseases occurring in this age group.

A novel tetravalent MenACWY conjugate vaccine, using CRM₁₉₇ as the carrier protein, has been developed by Novartis vaccines and tested in clinical trials in children and adults. The MenACWY-CRM₁₉₇ appears to be immunogenic in young infants as following a 2- or 3- dose primary schedule, protective serum bactericidal activity was induced against the four serogroups in more than 80% of children (Snape et al., 2008b). However, similar to MenCV, following priming in early infancy, a decline in protective Ab was observed less than one year after immunisation. This is despite the induction of immunologic memory (Snape et al., 2008b). However, it has been noted that long-term protection against invasive meningococcal disease relies on the persistence of bactericidal Abs in serum.

In this study, in order to increase our understanding of the induction of long-term protection after immunisation with protein-polysaccharide conjugate vaccines in infants, the memory B cell response specific to meningococcal serogroups A, C, Y and W135 was determined one month after a two-dose primary course of MenACWY-CRM₁₉₇ vaccine at 2 and 4 months of age. Furthermore, the kinetics of the decline in serogroup A, C, Y and W135- specific memory B cells from 4 months to 12 months of age was evaluated and the influence of maternal Ab on the memory B cell response at 5 months of age was assessed.

5.2 Materials and Methods

5.2.1 Study population

216 healthy infants were recruited to a phase II, single centre, open-label, randomised study assessing the serogroups A, C, Y and W135 specific B cell response to a primary and booster course of MenACWY-CRM₁₉₇ vaccine (Novartis Vaccines and Diagnostics Srl). Potential participants were recruited by information letter outlining the planned study, sent via child health computer departments (responsible for the mailout of appointments for routine immunisation) to the parents of all 3-week-old infants in the Thames Valley region. Interested parents were then screened for eligibility criteria. Exclusion criteria included previous meningococcal disease or household contact with meningococcal disease, known hypersensitivity reactions to any vaccines contained within the routine immunisation schedule, severe acute or chronic disease, immune dysfunction, receipt of blood products, bleeding or seizure disorders, and recent receipt of antibiotics or corticosteroids. Written informed consent was obtained from the mothers of all enrolled infants. Ethical approval was obtained from the Oxfordshire Research Ethics Committees (approval number B07/Q1605/41; Eudract number 2006-003476-35). The trial is registered with clinicaltrials.gov (identifier NCT 00488683).

5.2.2 Immunisation and sampling protocol

One dose of 0.5 ml of the MenACWY-CRM₁₉₇ vaccine was administered by intramuscular injection into the right antero-lateral thigh at 2 and 4 months of age. The 0.5 ml dose consisted of *N. meningitidis* serogroup A, C, W135, and Y capsular saccharides (10 µg of serogroup A; 5 µg each of serogroups C, Y, and W135) individually conjugated to CRM₁₉₇ (between 12.5-33 µg for serogroup A, 6.5-12.5 for serogroup C, 3.3-10 µg for serogroups W135 and Y), without adjuvant.

At the same time, as per the routine UK immunisation schedule, concomitant vaccines were administered into the left thigh; the combined diphtheria toxoid, tetanus toxoid, acellular pertussis, Hib, and inactivated polio vaccine (PediaceL, Aventis-Pasteur MSD Ltd) at 2, 3 and 4 months of age, and the 7-valent pneumococcal conjugate vaccine (Prevenar, Wyeth Vaccines) at 2 and 4 months of age.

Following priming with MenACWY-CRM₁₉₇ at 2 and 4 months of age, blood samples were collected in all children at 5 months of age and in a subset of children at various days (0, 7, 14, 49, 90 or 120) following the last dose of vaccine at 4 months, thus allowing investigation of the B cell response after priming. Furthermore, at the time of enrolment into the study a blood sample from all mothers and from a subset of infants was taken, to assess whether the memory B cell response at 5 months of age was influenced by the presence of maternal Ab at 2 months of age.

5.2.3 *N. meningitidis* serogroups A, C, Y and W135-specific IgG concentration as measured by ELISA

Blood samples were separated by centrifugation within 24 hours, and the serum obtained was maintained below -18°C until analysis. The concentration of serogroups A, C, Y and W135 anticapsular IgG concentration was determined by ELISA following a previously described method (Gheesling et al., 1994) (see section 2.5).

5.2.4 Separation of PBMCs and B-cell ELISpot

A maximum volume of 4 ml of heparinised blood was available for the separation of PBMCs, which were separated according to the methods described in section 2.2. The frequencies of meningococcal serogroup A, C, Y and W135 anticapsular- and CRM₁₉₇- specific plasma and memory B cells in peripheral blood were assessed by ELISpot according to previously described methods (see sections 2.2, 2.3, 2.4), except that PBMCs were cultured for 5 days instead of 6 days.

5.2.5 Statistical analysis

For the purpose of analysis, ELISpot assays in which fewer than 4 spots were detected were treated as though no ASCs were detected. Normally, eight replicate wells were used for each meningococcal serogroup and CRM₁₉₇, and so the maximal sensitivity of the assay was 2.5 cells

per million PBMCs. However, when the total number of PBMCs available was limited, at least 8 replicate wells were used for serogroup C and 4 replicate wells for CRM₁₉₇, and the rest of the cells were used to assess the other serogroups (with the following order of priority: W, Y and A). Furthermore, the memory ELISpot was prioritised over the *ex vivo* ELISpot. Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. ELISA IgG concentrations were summarised using GMC with corresponding 95% CI. For B cell numbers means were calculated because there were too many zero values to calculate the medians. Within-group comparisons of B cell numbers at varying time points were made using the Wilcoxon signed-rank test for paired data. The correlation between the different variables was compared using the Spearman's rank correlation coefficient, using the log transformed ELISA IgG concentration but untransformed B cell numbers.

5.3 Results

5.3.1 Recruitment

Between June 2007 and June 2008, all 216 children completed the first phase of the study and received 2 doses of the MenACWY-CRM₁₉₇ vaccine at 2 and 4 months of age. However, because of insufficient sample volumes or assay failure, not all data points were available for each time point (see number of participants for each result in the tables below the graphs). The median age of infants at the time of first immunisation was 8.6 weeks (range 8-11.3 weeks) and at the time of the second immunisation 17.4 weeks (range 16-21.4).

5.3.2 Memory B cell response at 5 months of age

At 5 months of age, one month after 2 doses priming with MenACWY-CRM₁₉₇ vaccine, the proportion of children who had detectable memory B cells (i.e. ≥ 2.5 memory B cells per million cultured lymphocytes) were 18/117 (15.4%) for MenA, 34/170 (20%) for MenC, 19/147 (12.9%) for MenW135, and 16/123 (13%) for MenY. In contrast, 85/168 (50.6%) children had detectable CRM₁₉₇-memory B cells (see table 5.3-1). In the children with detectable memory B cells at 5 months of age, the median frequencies of serogroup-specific memory B cells per million cultured lymphocytes were 3.8 for MenA, 4.2 for MenC, 3.8 for MenW135, 4.2 for MenY, and 7.5 for CRM₁₉₇ (see table 5.3-1). However, the median frequency of memory B cells specific for all

serogroups and CRM₁₉₇ was 0. Furthermore, there was no significant increase in the memory B cell frequency above baseline (p values > 0.05 for the comparison of serogroup specific memory B cell frequencies between 4 months and 5 months of age), although only 3-6 children had their memory B cells assessed at 4 months of age.

5.3.3 Kinetics of appearance of memory and plasma B cells following 2 dose priming with MenACWY-CRM₁₉₇ vaccine at 2 and 4 months of age

Following priming with MenACWY-CRM₁₉₇ vaccine, memory B cells specific for the four serogroups of meningococci and CRM₁₉₇ appeared in peripheral blood from day 7 onwards. Although the peak frequency appeared to be at day 7 for most Ag-specific memory B cells, there was no major variation in the mean frequency of memory B cells or in the percentage of children who had memory B cells detectable in peripheral blood (≥ 2.5 memory B cells per million cultured lymphocytes) between day 0 and 120 post immunisation (see figure 5.3-1 and table 5.3-1).

The plasma cell response to immunisation with MenACWY-CRM₁₉₇ vaccine was only assessed in a very small number of children (1-3 children per time-point) because of insufficient samples volumes. No increase in plasma cells was detected following immunisation, except a small increase in CRM₁₉₇-specific plasma cells by day 7 after the last dose of vaccine at 4 months of age in the two children tested at that time-point (data not shown).

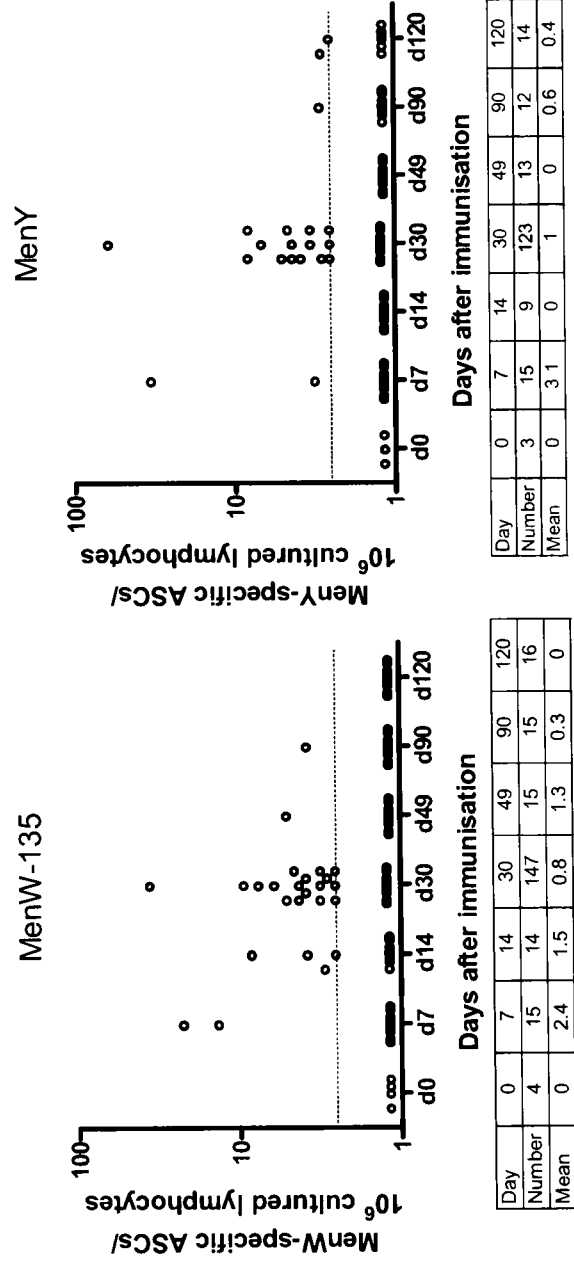
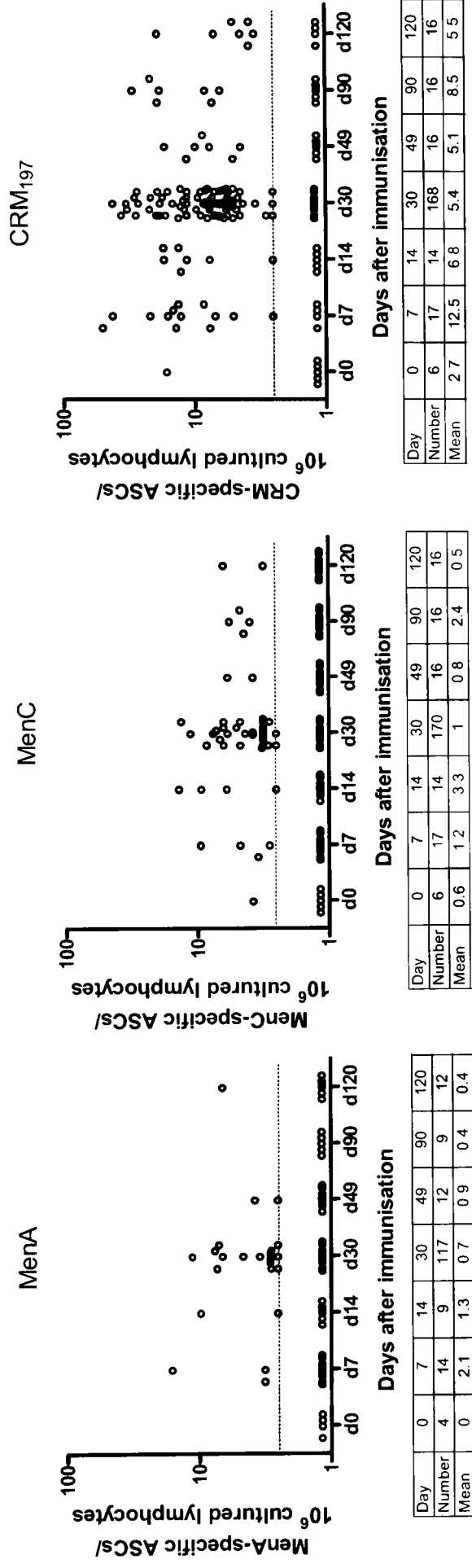


Figure 5.3-1: Using an ELISpot assay after 5 days culture with SAC/CpG/PWM, the memory B cell response specific to serogroups A, C, Y, W135 and CRM₁₉₇ was measured at various days following a primary course of immunisation with MenACWY-CRM₁₉₇ at 2 and 4 months of age. The minimum sensitivity of the assay is plotted as a broken line on the graphs for each figure. In some assays there were fewer PBMCs available for analysis and the minimum sensitivity was slightly higher. The zero values have been given the arbitrary value of 1 for illustrative purposes. The tables below each graph indicate the number of children tested for each time point and the mean frequency of Ag-specific memory B cells per million cultured lymphocytes.

Day [Age]	No children with ≥ 2.5 MenA-memory B cells/million (in %) [median for them]	No children with ≥ 2.5 MenC-memory B cells/million (in %) [median for them]	No children with ≥ 2.5 MenW-memory B cells/million (in %) [median for them]	No children with ≥ 2.5 MenY-memory B cells/million (in %) [median for them]	No children with ≥ 2.5 CRM-memory B cells/million (in %) [median for them]
0 [4 months]	0/4 (0) [0]	1/6 (16.7%) [3.8]	0/4 (0) [0]	0/3 (0) [0]	0/6 (16.7%) [16.3]
7	4/14 (28.6%) [5.1]	4/17 (23.5%) [4.1]	2/15 (13.3%) [17.8]	3/15 (20%) [10.7]	13/17 (76.5%) [13.1]
14	2/9 (22.2%) [6.1]	6/14 (42.9%) [7.6]	5/14 (35.7%) [3.8]	0/9 (0) [0]	9/14 (64.3%) [11.3]
30 [5 months]	18/117 (15.4%) [3.8]	34/170 (20%) [4.2]	19/147 (12.9%) [3.8]	16/123 (13%) [4.2]	85/168 (50.6%) [7.5]
49	3/12 (25%) [3.8]	3/16 (18.8%) [3.8]	4/15 (26.7%) [4.5]	0/13 (0) [0]	9/16 (56.3%) [8.5]
90 [7 months]	1/9 (11.1) [3.4]	6/16 (37.5%) [4.5]	1/15 (6.7%) [4.7]	2/12 (16.7%) [3.3]	9/16 (56.3%) [10.3]
120 [8 months]	2/12 (16.7%) [2.7]	2/16 (12.5%) [4.4]	0/16 (0) [0]	2/14 (14.3%) [2.7]	11/16 (68.8%) [5]

Table 5.3-1: Memory B cell response to a primary course of MenACWY-CRM₁₉₇ vaccine given at 2 and 4 months of age (in ASCs per million cultured lymphocytes). The percentage of children with detectable memory B cells for each Ag (≥ 2.5 cells per million cultured lymphocytes) with the median frequency of Ag-specific memory B cells for these children is reported for each time point.

5.3.4 Natural immunity to N.meningitidis during the first months of life before immunisation with meningococcal vaccines

The concentration of IgG-Ab specific for serogroups C, W135 and Y was low but high for serogroup A in the maternal blood of all mothers at study enrolment (see figure 5.3-1). Most of the mothers had a MenA-IgG concentration above 2 µg/ml, however, the IgG concentration specific for the other serogroups was below 2 µg/ml.

In contrast, most infants at 2 months of age had an IgG concentration below 2 µg/ml for all serogroups of meningococci. The IgG-GMC was lower in the infant blood at 2 months of age, compared to the maternal blood for each serogroup (see Figure 5.3-2 and Table 5.3-2). The ratio between the maternal and infant GMC varied for each serogroup but was higher for MenA than for the other serogroups (Table 5.3-2). There was a strong correlation between the maternal IgG concentration measured during the study enrolment and the infant IgG concentration measured at 2 months of age for all serogroups except for MenW135 (see Figure 5.3-3).

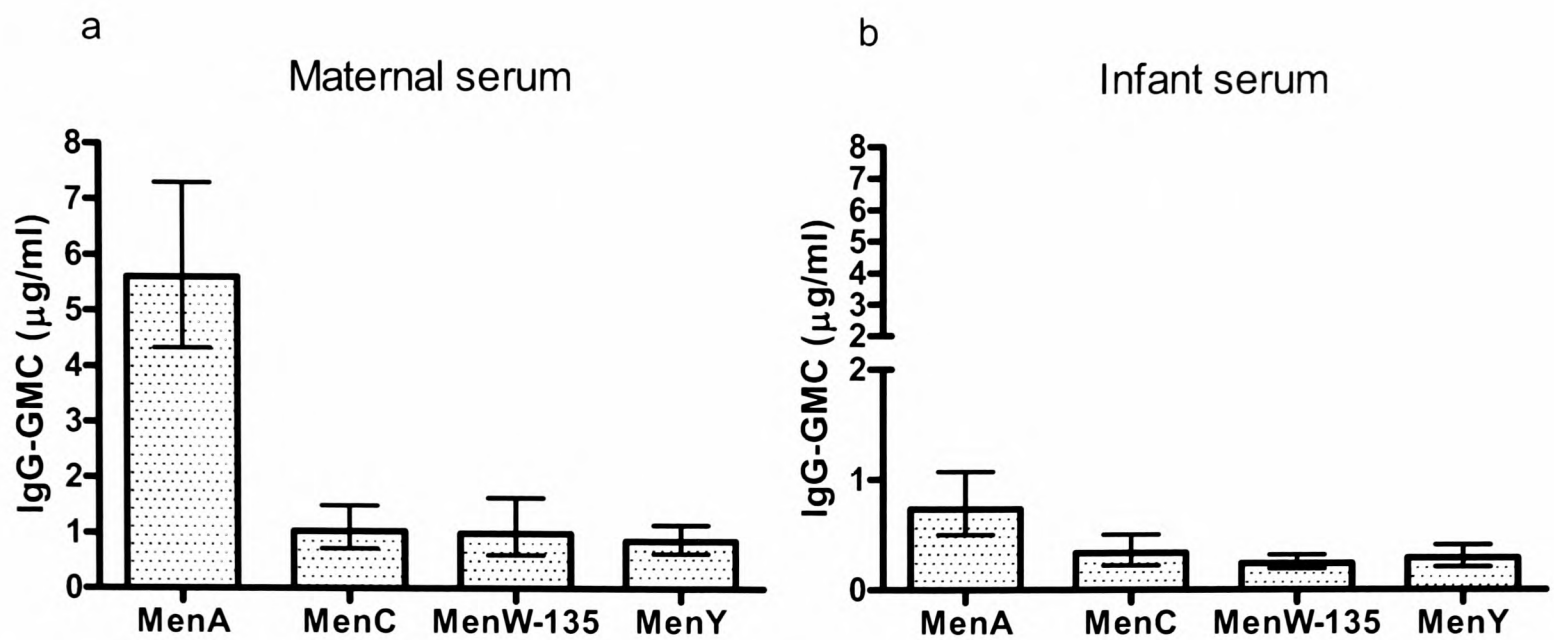


Figure 5.3-2: Meningococcal serogroup-specific IgG GMC with 95% CI in maternal blood at study enrolment (a) and in infant blood at 2 months of age (b).

Serogroups	Maternal blood			Infant blood at 2 months			Ratio		
	GMC	95% CI	n	GMC	95% CI	n	Mean	95% CI	n
MenA	5.61	4.33-7.30	97	0.73	0.5-1.07	51	6.8	5.35-8.73	49
MenC	1.03	0.72-1.49	95	0.33	0.22-0.5	51	4.37	3.58-5.33	49
MenW135	0.99	0.6-1.64	88	0.24	0.19-0.32	51	3.05	2.15-4.35	49
MenY	0.86	0.64-1.15	95	0.29	0.21-0.41	51	3.43	2.68-4.4	49

Table 5.3-2: GMC of serogroup-specific IgG in maternal blood at enrolment and infant blood at 2 months of age, with the 95% CI, and the ratio between Ab present in maternal blood and in infant blood for paired samples with the 95% CI.

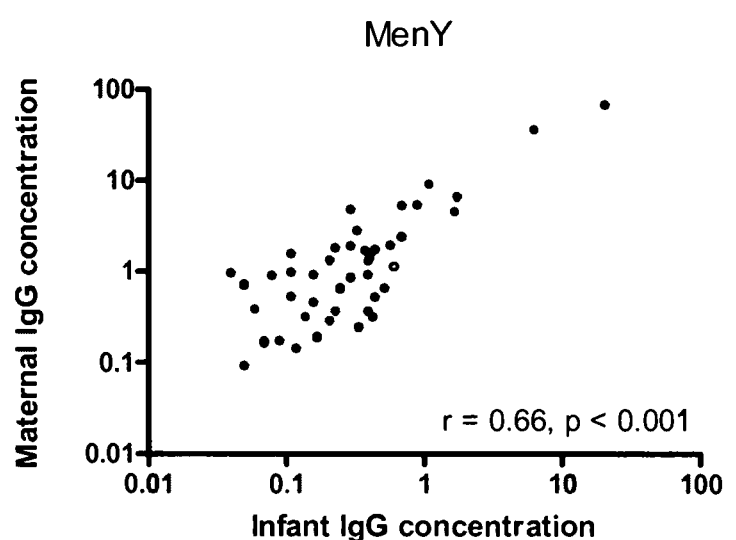
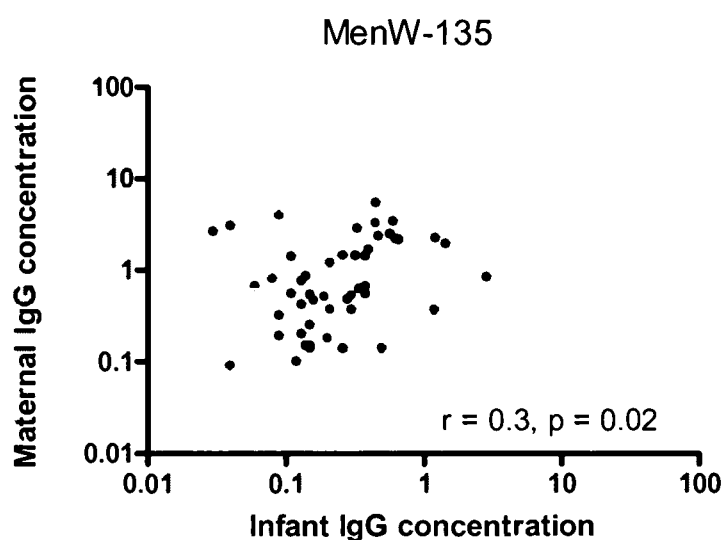
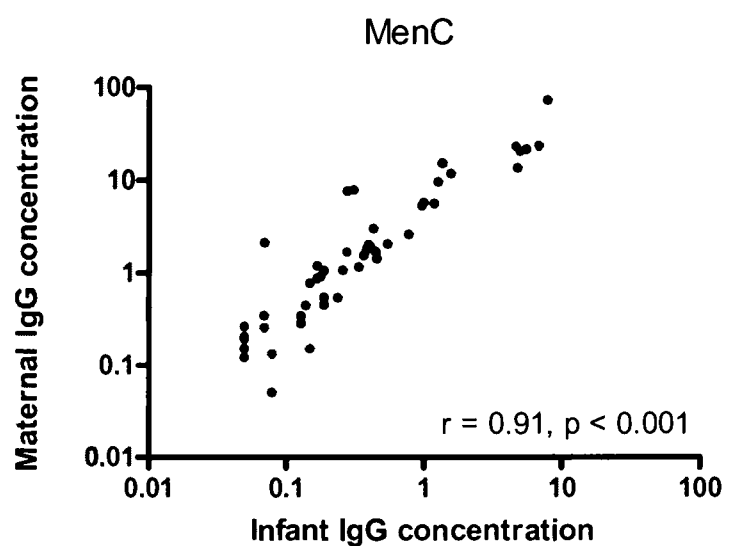
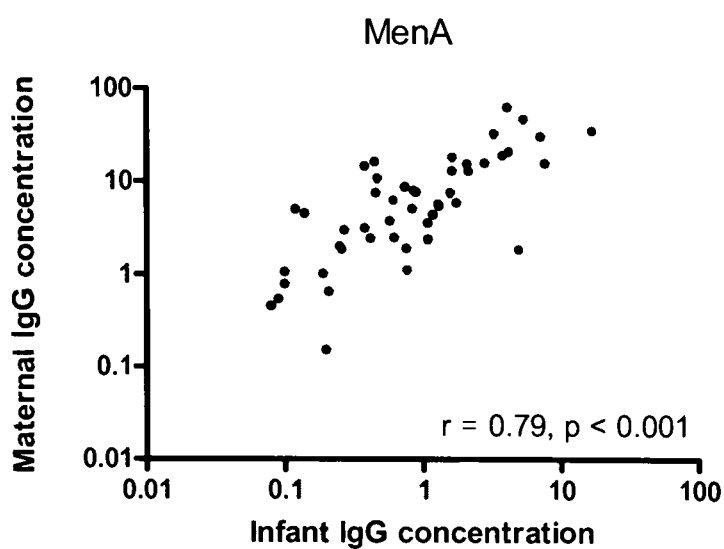


Figure 5.3-3: Correlations between maternal IgG concentration (measured at study enrolment) and infant IgG concentration (measured at 2 months of age) for each serogroup of meningococci, using the Spearman's correlation method. The Spearman's rank correlation coefficients (r) with the corresponding p -values (p) are presented on each graph.

5.3.5 Influence of maternal Ab on the memory B cell response at 5 months of age

There was no significant association between the IgG-Ab measured in infant blood at 2 months of age (before the first dose of MenACWY-CRM₁₉₇ vaccine), and the memory B cell response at 5 months of age (one month after two-dose priming with this vaccine), except a weak negative correlation for serogroup C and a weak positive correlation for serogroup W135

(Figure 5.3-4).

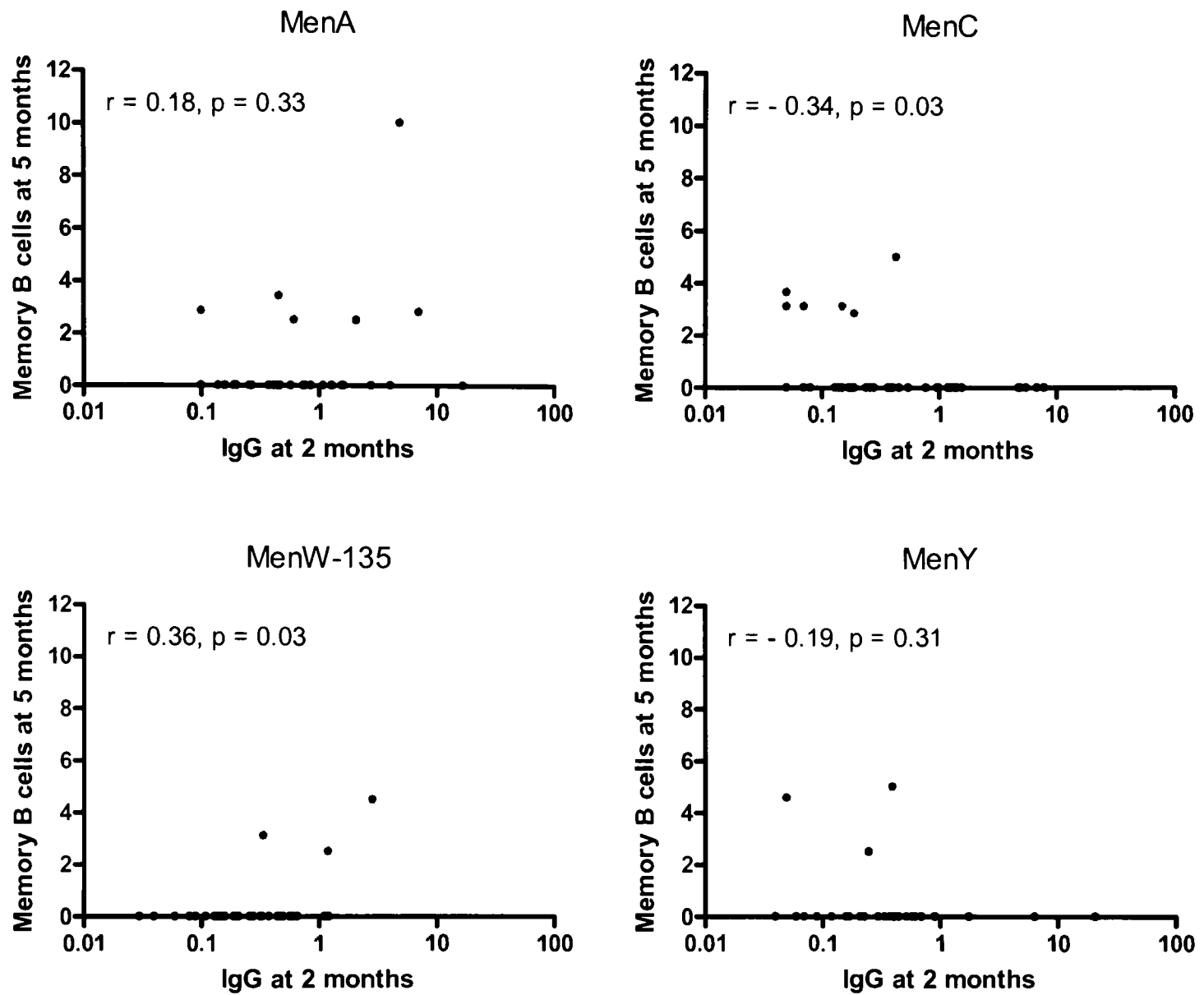


Figure 5.3-4: Correlations between infant Ab at 2 months of age and infant memory B cells at 5 months of age for each serogroup of meningococci, using the Spearman's correlation method. The Spearman's rank correlation coefficients (r) with the corresponding p values (p) are presented on each graph.

5.4 Discussion

Protein-polysaccharide conjugate vaccines have been shown to induce only short-term Ab. In this study, the B cell response following primary immunisation with a new tetravalent meningococcal conjugate vaccine (MenACWY-CRM₁₉₇) was assessed. This vaccine has been shown to be immunogenic since early infancy, but like other conjugate vaccines is associated with rapid waning of Ab in children vaccinated before one year of age (Snape et al., 2008b). In this study, it was observed that only a few children produced memory B cells above the threshold for detection at 5 months of age after two-dose priming with the MenACWY-CRM₁₉₇ vaccine at 2 and 4 months of age. Depending on the serogroups, between 13-20% of the study children produced ≥ 2.5 memory B cells per million cultured lymphocytes. In contrast, the Ab response at 5 months of age following a two-dose primary immunisation (at 2 and 4 months of age) with a similar vaccine, although it was adjuvanted with aluminium phosphate, has been shown to be very high, with $\geq 84\%$ of the study children achieving seroprotection (hSBA titre $\geq 1:4$) for serogroup C, W135, and Y. Of note, the Ab response was lower for serogroup A, with only 60-66% of the study children achieving seroprotection (Snape et al., 2008b). In contrast, in a previous study assessing the Ab and B cell response after a three-dose priming with MenCV at 2, 3, and 4 months of age, 100% of the study children had reached protective bactericidal Ab titres (rSBA $\geq 1:8$) at 5 months of age and 64% had detectable MenC-specific memory B cells (section 4). In the same study, it was observed that by 12 months of age only 41% of the study children had maintained protective bactericidal Ab titre. However, there was a good correlation between the children maintaining protective Ab titre by 12 months of age, and those who had produced memory B cells after priming (section 4). Similarly, the study by Snape *et al.* has shown that after the 2- or three-dose priming with MenACWY-CRM₁₉₇, a substantial decline in

hSBA GMT was observed by 12 months of age for all serogroups (Snape et al., 2008b). After the 2 dose-schedule, there were only 5-8 % of study children maintaining protective hSBA titres against serogroup A, 40-48 % against serogroup C, 56-74 % against serogroup W135, and 52-65 % against serogroup Y (Snape et al., 2008b). However, the persistence of protective bactericidal Ab was higher after a three-dose schedule, with 21-41 % of children maintaining protective hSBA titres against serogroup A, 60-70 % against serogroup C, 81-95 % against serogroup W135 and 86-87% against serogroup Y.

These observations confirm previous findings that short-term Ab responses do not correlate with long-term maintenance of Ab. As suggested in a previous study (section 4), the measurement of memory B cells produced after primary immunisation might better predict long-term protection by persistent Ab. Further protection after one year of age can be induced by giving a booster dose of vaccine. However, understanding the principal factors determining the memory B cell response to priming could help to adapt vaccine schedules and formulations during primary immunisation in order to increase vaccine protection during the first year of life. Additionally, in developing countries a two-dose schedule without booster would be more appropriate for practical and economical reasons if it would achieve similar levels of protection.

In this study, memory B cells were apparent in peripheral blood in a small proportion of the study children at day 7 after the second dose of MenACWY-CRM₁₉₇ and persisted up to 120 days post immunisation (8 months of age). These cells are responsible for the rapid immune response to re-exposure to an Ag and may also participate in the long-term persistence of Ab in serum, together with long-lived plasma cells in the bone marrow. In this study, plasma cells specific for CRM₁₉₇ appeared briefly at day 7 following the second dose of primary

immunisation at 4 months of age. These cells are expected to be produced in GCs together with the memory B cells, and to transit through the circulation to their long-term residence in the bone marrow. The fact that only a small proportion of children produced serogroup-specific memory B cells after priming, in contrast to the high Ab response observed previously with a similar vaccine (Snape et al., 2008b) has several possible explanations. In most children, Abs are only generated by extra-follicular low avidity B cells, which do not survive and do not appear in peripheral blood after immunisation (Smith et al., 1996). The induction of GCs with the production of long-lived plasma cells and memory B cells is very low in early life (Pihlgren et al., 2003), and therefore, Abs are not maintained in the long-term. Alternatively, Ag-specific memory B cells are produced but in frequencies too low to be detected by the ELISpot assay. This small pool of memory B cells might be sufficient to sustain immunological memory but not to maintain protective Ab in serum. This phenomenon has been observed in a mouse study, where the generation of memory B cells during priming was impaired due to a mutation in the *xid* gene, and the persistence of Ab was reduced, although immunological memory was preserved (Ridderstad et al., 1996).

In this study, the maternal Ab, present in infant blood at 2 months of age, did not appear to influence the memory B cell response at 5 months of age. However, the levels of meningococcal Abs were very low at 2 months of age for all serogroups, and might be insufficient for a mechanism of inhibition on B cell activation.

The prevalence of meningococcal Ab in the mothers of the study children was low for serogroups C, Y and W135 but high for serogroup A. Similarly, a previous Dutch study reported low levels of meningococcal serogroup C, Y and W135- specific Ab in maternal blood

and cord blood, but high levels of Ab against serogroup A meningococci (de Voer et al., 2006). This may be due to cross-reactive Ags present in the gut microflora, inducing Ab against serogroup A meningococci (Filice et al., 1985; Robbins et al., 1972). The low levels of meningococcal Ab against serogroup C may be explained by the fact that most European countries had introduced the MenCV in their immunisation schedules in the late 90s, and therefore, most women of childbearing age have not been immunised previously with meningococcal vaccines. Indeed, a study comparing the prevalence of meningococcal Abs before and after (2000-2004) the introduction of MenCV in England has shown an increase in the prevalence of people with MenC-protective bactericidal Abs in the post-vaccination eras only for the age groups targeted by the MenCV vaccination, i.e. < 25 years but no changes for those older than 25 years of age (Trotter et al., 2008). In the present study, only 24/216 mothers had received the MenCV previously, and 4/216 had received the plain polysaccharide MenA/C or ACWY vaccines. Furthermore, carriage of meningococci might not induce serum IgG-Ab but only mucosal IgA-Ab.

However, maternal Abs appeared to wane rapidly after birth. At 2 months of age, only 1/7 to 1/3 of the maternal Ab levels were detectable in infants, depending of the serogroup. Therefore, even in a population where all women of childbearing age would have been previously immunised with meningococcal vaccines, the transmission of maternal Ab to young infants would probably not be sufficient to protect children under one year of age because of rapid waning of these Abs. Many countries, including the Netherlands, where the incidence of meningococcal diseases is low, have introduced the MenCV in their national immunisation program as a single dose after 12 months of age. Protection in the first year of life relies on maternal immunity, herd immunity and natural immunity, however, most mothers in this

population have low levels of circulating meningococcal Abs.

In summary, this study has shown that only a small number of children produced detectable serogroup-specific memory B cells following two-dose priming with MenACWY-CRM₁₉₇ vaccine. This might explain the rapid decline in Ab less than one year after immunisation, despite the majority of children achieving seroprotection at 5 months of age (Snape et al., 2008b). This supports the importance of a booster dose of vaccine at 12 months of age, until we can find a way to influence GC priming during primary immunisation with protein-polysaccharide conjugate vaccine in young infants. Testing the influence of various adjuvants, or protein carriers, or vaccine schedules on the memory B cell response during priming could help to influence long-term Ab persistence. However, genetic factors may also play an important role in influencing the quality of the immune response to priming.

6 Appearance of peripheral blood B cells and Ab in a primary and secondary immune response in humans

6.1 Introduction

The assessment of the immune response to currently licensed vaccines relies on the measurement of Ag-specific Ab produced after immunisation. However, the initial Ab response might not always correspond to the long-term protection induced by immunisation; in young children circulating Abs can drop rapidly (MacLennan et al., 2000) and it has been shown that protective immunity can persist after immunisation when Abs are no longer detectable (Adams et al., 1993; Eskola et al., 1990; Wainwright et al., 1989; West and Calandra, 1996). Long-term humoral immunity may be better represented by the size of the B cell pool induced by immunisation from which long-lived plasma cells and memory B cells are derived.

Plasma cells and memory B cells have been shown to persist in the long-term after their production (Crotty et al., 2003a; Manz et al., 1997; Slifka et al., 1998) and to participate in the maintenance of protective Ab in serum (Bernasconi et al., 2002; Crotty and Ahmed, 2004; Lanzavecchia, 1983). Although memory B cells are detectable in the circulation for several years after Ag exposure in humans (Crotty et al., 2003a; Maruyama et al., 2000), it is not clear whether this population is maintained by memory B cells residing in secondary lymphoid organs (Maruyama et al., 2000; Rajewsky, 1996; Smith et al., 1996; Tangye et al., 1998), in the bone marrow (Osmond, 1986), or whether they continuously recirculate through the blood (Nanan et al., 2001; Salmi and Jalkanen, 1997). It is also unknown whether these cells survive in a quiescent state or are constantly proliferating and self-renewing. Memory B cells are

responsible for the more rapid production of high affinity Ab during a secondary exposure to an Ag, because they are more easily activated than naïve B cells and have already undergone isotype switching and avidity maturation (Liu et al., 1995; Oliver et al., 1999; Tangye et al., 2003; Tangye et al., 1998). However, memory B cells might also contribute to long-term Ab persistence due to their continuous differentiation into plasma cells, which may be Ag-independent and occur by polyclonal stimulation of B cells by microbial products (which stimulate B cells via TLRs), or through stimulation by cytokines secreted by T cells activated by another Ag (bystander effect), or in response to persistent Ag, intermittent Ag exposure or cross-reactive Ags (Bernasconi et al., 2002; Crotty and Ahmed, 2004; Lanzavecchia, 1983). Similarly, it has been shown in mice that some plasma cells persist after their formation in the GC and survive in bone marrow niches independently of memory B cells and of the Ag (Manz et al., 1998). Therefore, the bone marrow is believed to be an important locus of long-term Ab production (Bachmann et al., 1994; Slifka et al., 1995).

Assessing how the measurement of plasma cells and memory B cells produced after immunisation relates to persistent Ab could help in understanding the development of long-term immunity. However, the organs of formation and residence of these cells are not easily accessible in humans (e.g. spleen, lymph nodes and bone marrow). Ag-specific plasma cells are not detectable in peripheral blood at steady state but these cells are thought to use the circulation to reach the bone marrow and they appear transiently in peripheral blood after immunisation. In contrast, memory B cells can be detected in peripheral blood at steady state; however, the majority of memory B cells are likely to reside in lymphoid tissues. An increase in memory B cell frequency is consistently observed shortly after immunisation (Clutterbuck et al., 2006; Crotty et al., 2003a; Kelly et al., 2006; Nanan et al., 2001) and might represent newly

generated memory B cells transiting through the circulation to other lymphoid tissues. The timing of appearance of plasma cells and memory B cells in peripheral blood may vary in a primary as opposed to a secondary immune response and, given the brevity of their detection (particularly for plasma cells), it is important to define the kinetics of their appearance in peripheral blood after immunisation to design further vaccine studies assessing the generation of long-term humoral immunity.

The kinetics of the B cell response during secondary immunisation has previously been studied in humans, and it has been observed that plasma cell response consistently peaks by day 6-7 post secondary immunisation with a rapid return to baseline by day 10-12, and memory B cells rise by the end of the first week and persist for at least one month after immunisation (Clutterbuck et al., 2006; Crotty et al., 2003a; Kelly et al., 2006; Nanan et al., 2001). However, there is very little information on the kinetics of a primary B cell response or on the kinetics of B cell secondary response when booster doses are given close to each other.

In humans, the assessment of the B cell response to a primary exposure to common Ags is complicated by previous exposure to the same Ag, either in the form of symptomatic or asymptomatic infection or by previous immunisation with the Ag. Furthermore, many common pathogens share cross-reacting Ags and can induce natural immunity to other microorganisms. In this study, the inactivated rabies vaccine was chosen as a novel Ag to study a primary B cell response in healthy adults, as rabies virus is a novel Ag for all unimmunised individuals in the UK. In contrast to other non-licensed novel Ags, i.e. α -helix pomatia haemocyanin, keyhole limpet haemocyanin, and bacteriophage ϕ X174 (Korver et al., 1987; Pyun et al., 1989; Weits et al., 1978), rabies vaccine is a licensed inactivated vaccine widely used as a pre-and post-

exposure prophylaxis against rabies virus. Other examples of vaccines that could be used as novel Ags to study a primary B cell response in humans include the hepatitis A vaccine and hepatitis B vaccine (HBV), however, some healthy individuals do not respond well to HBV.

Vaccination against rabies is recommended for travellers to developing countries, people who regularly handle bats, veterinarians, laboratory workers handling the virus, etc. The WHO recommends a primary series of immunisations at day 0, 7, and 28 with 1 ml of rabies vaccine to achieve the recommended protective IgG-Ab titre of ≥ 0.5 IU/ml for pre-exposure prophylaxis (WHO, 1992). However, in this study an alternative, equally immunogenic, schedule of 0, 28 and 56 day immunisation series was used (Nicholson et al., 1987). This was to permit blood sampling at 0, 2,4,7,10,14 and 28 days post vaccination, which is not possible with the normal schedule.

Previous researchers have used rabies vaccine as a novel Ag to investigate both T and B cell primary immune responses (Brinkman et al., 2003; Ghaffari et al., 2001; Korver et al., 1987), however, nobody has characterised the kinetics of the B cell response during priming and boosting.

In this study, the kinetics of the plasma cell, memory B cell and Ab response to a first and third dose of rabies vaccine in immunologically naïve adults is described. Additionally, the results are compared with those from immune individuals vaccinated with rabies vaccine more than 2 years previously, who were given a booster dose of rabies vaccine.

6.2 Materials and Methods

6.2.1 Study population

The study was conducted in Oxford between July and December 2007. Two groups of 10 healthy adult volunteers, aged 18-50 years, were enrolled after written informed consent was obtained. The study was approved by Oxfordshire Research Ethics Committees (approval number BO7/Q1605/29). Group I included 10 volunteers who had not previously received rabies vaccine and group II included 10 volunteers who had received a course of rabies vaccine 2-10 years previously (see table I). Exclusion criteria were: the presence of a disease affecting the immune system; treatment with immunosuppressive drugs; a history of any allergic reaction after previous vaccinations; known hypersensitivity to any vaccine component; a known bleeding diathesis; pregnancy and previous rabies vaccination (group I only).

6.2.2 Vaccine

The rabies vaccine used was the human diploid cell vaccine (HDCV; rabies vaccine BP, Sanofi Pasteur MSD Limited, Lyon, France). The vaccine is prepared using the Wistar Pitman Moore (PM) strain (Wistar PM/WI 38-1503-3M) of rabies propagated on human diploid cells and inactivated by β -propionolactone. HDCV is a whole virus vaccine consisting of a nucleocapsid complex surrounded by a lipoprotein bilayer membrane with glycoprotein projections at the outer surface. The lyophilized vaccine, containing at least 2.5 IU inactivated virus, was stored at

4°C and reconstituted with 1 ml diluent just before use.

6.2.3 Immunisation and sampling protocol

One millilitre of HDCV was given by intramuscular injection into the deltoid region at days 0, 28 and 56 for group I and a single dose was administered at day 0 for group II. After rabies vaccination, the volunteers were observed for at least 15 minutes for any immediate adverse vaccine reactions. Blood samples (20 ml) were taken prior to immunisation and at days 2, 4, 7, 10, 14, 28 after the first and third dose for group I and after the single dose for group II. At the time of blood sampling the volunteers were interviewed to evaluate whether they had experienced any adverse events related to the vaccine. 18 ml of blood were available for PBMCs separation and 2 ml for serum.

6.2.4 Ag for in vitro assay

The dog kidney cell rabies vaccine (DKCV), donated by Department of Paediatrics of the University of Leiden was used as coating Ag in the ELISpot and ELISA assays. The rabies virus PM strain (Wistar PM/WI-38-1503-3M) had been propagated in primary dog kidney cells, concentrated and purified by ultrafiltration and inactivated with β -propionolactone (van Wezel et al., 1978). Each dose of the lyophilised vaccine contained 350 μ g proteins and was dissolved in 1 ml distilled water.

6.2.5 Rabies specific Ab quantification by ELISA

The rabies-ELISA was performed in the laboratories of the Department of Paediatrics of Leiden University Medical Centre in the Netherlands, by myself with the assistance of C.M. Jol-van der Zijde and A. Jansen.

The concentrations of IgG- and IgA- Abs were assessed at days 0, 2, 4, 7, 10, 14, and 28 after primary and secondary immunisation. IgM- Abs were measured at days 0, 7 and 14 post primary and secondary immunisation. IgG subclasses were measured at days 0, 7, 14 and 28 post primary and secondary immunisation. For IgG subclasses only IgG1 and IgG3 were measured as it had been shown previously that IgG2 and IgG4 anti-rabies Abs were not detectable following primary and booster rabies vaccination (Brinkman et al., 2003). The concentrations of IgG, IgG subclasses, IgA and IgM anti-rabies Abs were measured by an ELISA assay following a previously described method (Brinkman et al., 2003). In brief, flat bottom microtiter plates (Costar, Cambridge, MA, USA) with high binding capacity were coated with 10 µg/ml of DKCV in PBS, incubated for 3 hours at 37°C in a humidified atmosphere followed by overnight incubation at room temperature. The plates were then blocked with PBS/0.05%Tween20/2%caseine (PBS/T/C, Sigma, St.Louis, MO, USA) for 1 hour at 37°C in a humidified atmosphere and washed three times with PBS/0.05% Tween (PBS/T). Seven twofold dilutions of the reference serum (starting dilution 1/200 for IgGtot, IgG1 and IgG3 and 1/100 for IgA) and four dilutions of the test sera (starting dilutions 1/50) were made directly in the plate by well-to-well transfer with a multichannel pipette. The second International Standard reference serum of the WHO containing 30 IU/mL of total IgG anti-rabies (Lyng, 1994) (Statens Serum Institut, Copenhagen, Denmark, supplied by NIBSC, Potters Bar, UK) and an in house prepared secondary reference serum (total IgG anti-rabies 3.3

IU/mL) were used to express the IgG data in IU/mL. For IgG subclasses, IgA and IgM anti-rabies Abs no reference sera are available, therefore, the in house reference serum was used as standard, as described previously (Brinkman et al., 2003). After 2 hours incubation at 37°C in a humidified atmosphere, the plates were developed with either goat-anti-human IgG (1:1500) or goat-anti-human IgA (1:1000), conjugated with alkaline phosphatase (Biosource, Camarillo, CA, USA). However, for the detection of IgG1 and IgG3, the plates were incubated for 2 hours with monoclonal mouse-anti-human IgG subclass specific Abs (1:10000) (WHO/IUIS HP 6188, CLB, Amsterdam, The Netherlands and WHO/IUIS HP 6080, Nordic, Tilburg, The Netherlands) and then incubated overnight with rabbit-anti-mouse immunoglobulin conjugated with alkaline phosphatase (RAMAP 1:750; DAKO, Glostrup, Denmark). After incubation with conjugated antisera, the chromogenic substrate 4-nitrophenyl-phosphate disodium salt (PNP, Merck, Darmstadt, Germany) in 0.01 M diethanolamide buffer (DEA, BDH, Poole, England), pH 9.8, containing 150 mg/L MgCl₂·6H₂O (Merck) was added. The reaction was stopped with 3 M NaOH (Merck). The OD of each well was then read at 405 nm using spectrophotometer (Titertek Multiscan, Labsystems, Helsinki, Finland).

IgM anti-rabies was determined using a commercial ELISA kit, i.e. PLATELIA RAGE that contained purified glycoprotein as Ag (Bio-Rad Laboratories, England), according to the manufacturer's instructions. The kit was designed for measurement of IgG anti-rabies and was, therefore, modified as follows for the detection of IgM anti-rabies: the goat-anti-human IgM conjugated with alkaline phosphatase (1:1000; Biosource, Camarillo, CA, USA) was used, followed by substrate and stopping agent as described above.

6.2.6 Ab avidity

Because IgG1 and IgG3 anti-rabies Abs were quantitatively the major IgG subclasses formed, Ab avidity was measured within these subclasses. The avidity of IgG1 and IgG3 anti-rabies was measured by a modified elution ELISA as described previously (Brinkman et al., 2003) at day 0 and 28 after primary and secondary immunisation, when possible (the limit of detection was a concentration ≥ 0.5 IU/ml of IgG1 or IgG3 respectively). In brief, the serum samples, prepared at a dilution calculated to give a final OD of 1, were allowed to interact with DKCV coated on the wells. Subsequently, wells exposed to a distinct sample dilution, were incubated for 15 min with a variable molarity (range 0.5 -4.5 M) of the chaotropic agent sodium thiocyanate (NaSCN) in PBS. The plates were then developed as described above. The relative avidity index is defined as the molarity of NaSCN at which 50% of the amount of IgG subclass Abs remains bound to the coated rabies Ag.

6.2.7 Separation of PBMCs and B-cell ELISpot

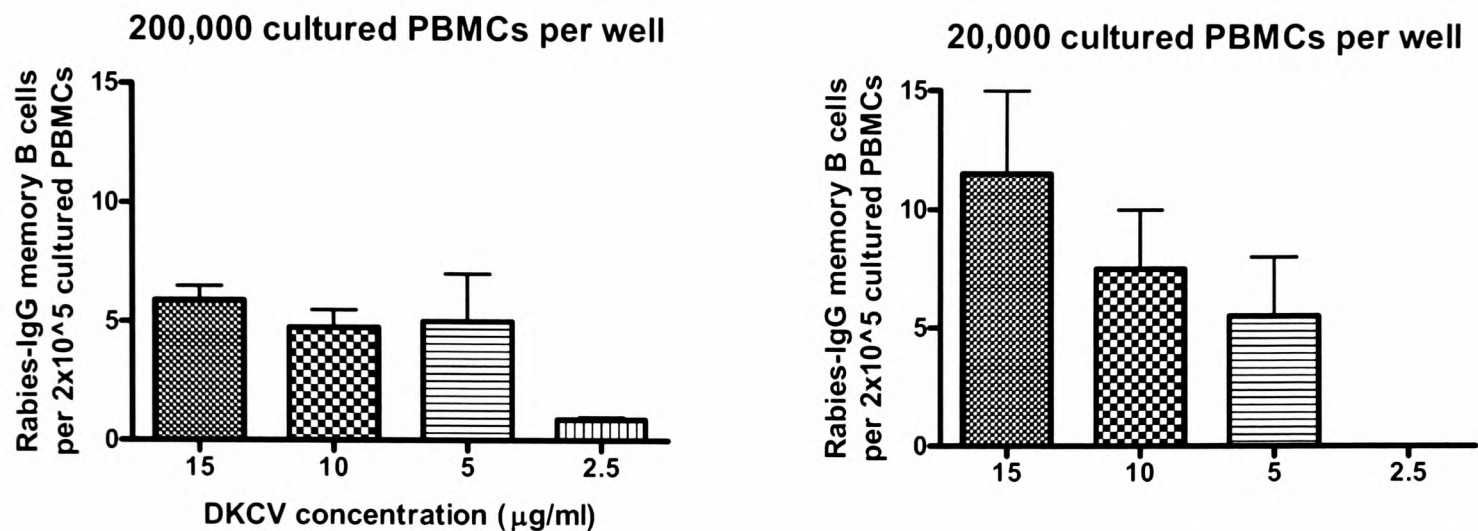
A maximum of 18 mls of heparinised blood was available for the separation of PBMCs, which were separated according to the methods described previously (section 2.2). The frequency of rabies-specific-IgG, IgA and IgM plasma cells and IgG- and IgA-memory B cells were measured at each visit. Rabies specific IgM-memory B cells were not assessed because the baseline level of rabies IgM-memory B cells in the naïve individuals was very high and it was not possible to detect a difference in the frequency of IgM-memory B cells before and after immunisation, even when the cells were diluted. The frequency of IgM-memory B cells in the

blank wells was also very high. These observations suggest that the IgM production was not specific for the vaccine Ag. This may be because SAC, CpG, PWM in the culture medium induced the secretion of low avidity, cross-reactive IgM-Ab by B1 or MZ B cells. ELISpot methods for plasma cells and memory B cells were undertaken according to the methods in section 2.3 and 2.4.

6.2.7.1 Coating of ELISpot plates with DKCV

The effect of different DKCV concentrations for coating rabies-specific-ELISpot wells for the detection of rabies-specific memory B cells was investigated in naïve and immune individuals. Two different PBMC concentrations were assessed (200,000 and 20,000 cultured PBMCs per well) because it had been observed that it was difficult to identify individual spots in immune samples with a high frequency of memory B cells. The results suggest that maximal numbers of rabies-specific memory B cells were detected using DKCV at a concentration of 15 µg/ml for coating rabies-specific-ELISpot wells (Figure 6.2-1). The variation in the number of rabies-specific memory B cells in naïve individuals was similar for the different DKCV coating concentrations.

Immune samples



Naïve samples

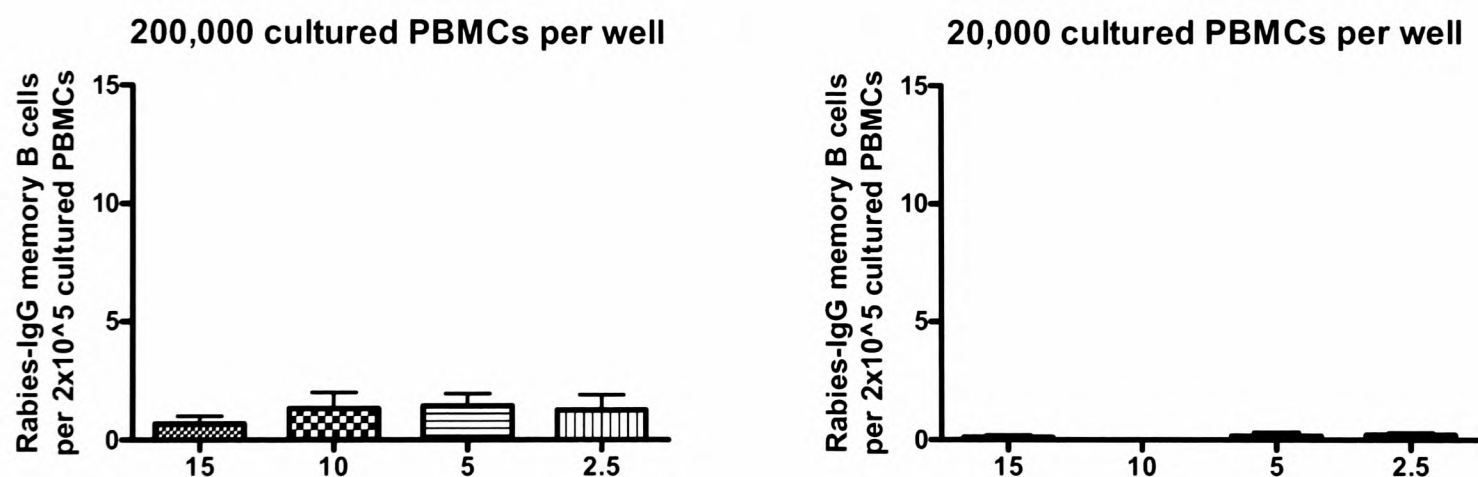


Figure 6.2-1: Determination of the best DKCV concentration for coating of rabies-specific ELISpot wells. Four ELISpot wells for each DKCV concentration were incubated with either 200,000 cultured PBMCs or 20,000 PBMCs of immune or naïve samples. The data represent the mean and standard error of the mean; for immune samples, the results are from two independent experiments in one single individual and for naïve samples the results are from three independent experiments in three different individuals.

ELISpot plate wells specific for rabies were first coated with 100 µl/well DKCV (15 µg/ml) in PBS pH 7.4, incubated for 3 hours at 37°C in 5% CO₂, and then incubated overnight at room temperature. The following day, the remaining ELISpot wells were coated with tetanus toxoid,

goat anti-human immunoglobulin and PBS (see Table 6.2-1). The ELISpot plates were stored at 4°C until use.

6.2.7.2 Assay sensitivity and specificity

Cell counting

The number of cells detected in a well was determined by the use of an automated ELISpot plate reader. Reading involved automated counting using identical settings for all plates. The setting (spot size, spot intensity, spot gradient) was chosen to achieve maximum specificity for rabies-specific spots (Figure 6.2-2).

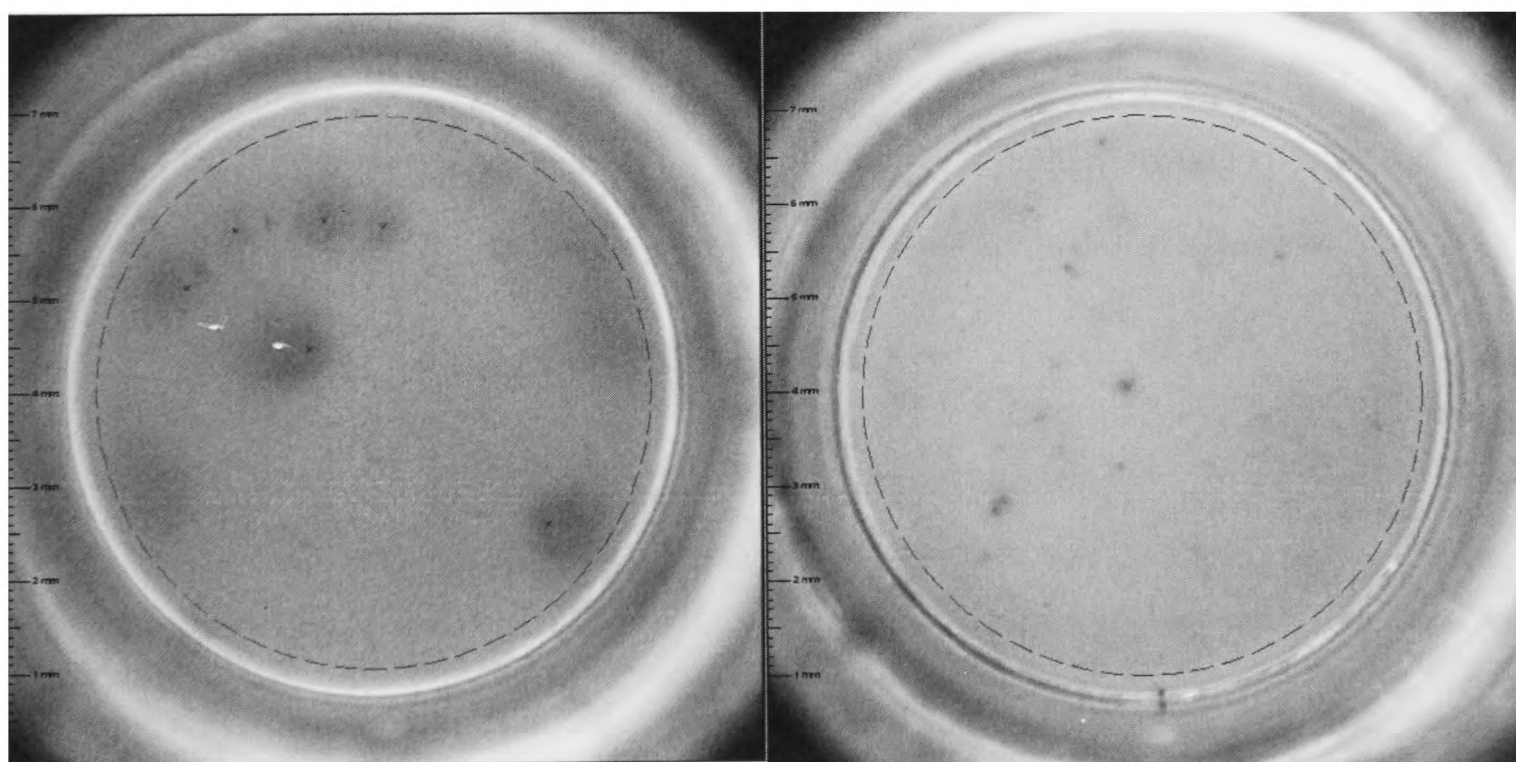


Figure 6.2-2: Photograph of two rabies-specific ELISpot wells demonstrating rabies-specific IgG-ASC detection using plate-reading software. The well on the left was incubated with 200,000 cultured PBMCs from an immune individual (previously immunised for one year ago) and the well on the right with 200,000 PBMCs from a naïve individual. Spots are detected in the two wells; however, the spots detected in the well incubated with the PBMCs of the naïve individual are smaller and less intense. These spots might represent ASCs reacting to cross-reacting Ags present in the vaccine.

The setting of the ELISpot plate reader was chosen to detect only the spots of size and quality seen in immune samples, corresponding to rabies-specific ASCs, and to ignore spots corresponding to *in vitro* artefact or cross-reacting Ags present in the vaccine, such as bovine serum albumin, which is used in HDCV as stabiliser (Deshmukh et al., 2004). With this setting, and using 200,000 PBMCs per well, there was a good discrimination between naïve and immune samples (Figure 6.2-3).

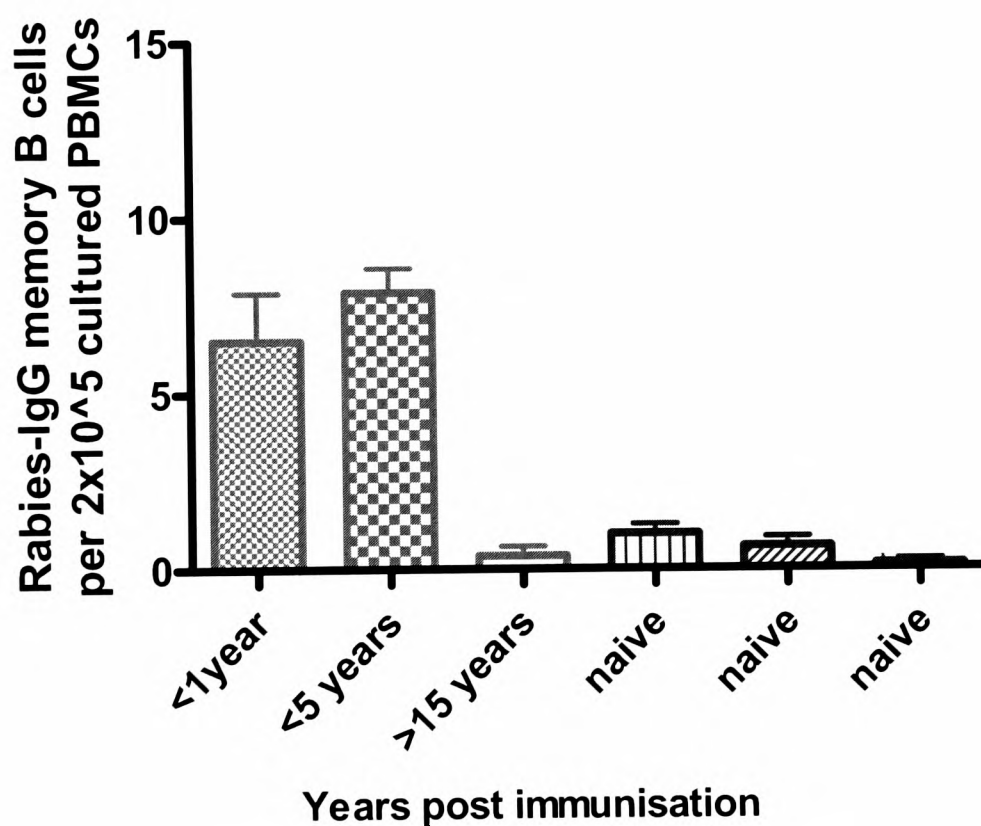


Figure 6.2-3: Rabies-specific IgG-memory B cell frequencies in three immune and three naïve individuals. The data represents the mean and standard error of the mean from 8 replicate ELISpot wells from one individual experiment. The setting of the ELISpot plate reader was set to achieve maximum specificity for rabies-specific spots. The figure shows that, with this setting, there was a good discrimination between naïve and immune samples, except for one individual vaccinated more than 15 years ago. In this sample, the average number of rabies-memory B cells was similar to that obtained from one of the naïve individuals (<1 rabies memory B cell/ 2×10^5 cultured PBMCs in average for 8 replicate wells).

Cell dilution

Cultured PBMCs were added in the rabies-specific ELISpot wells at two different concentrations to allow detection of individual spots in case of high frequency of Ag-specific memory B cells. 200,000 cultured PBMCs/well were added in four separate wells and 20,000 cultured PBMCs/well were added in four other wells (see Table 6.2-1). The highest cell concentration of PBMCs allowed detection of the low frequency of rabies-specific memory B cells, while the lowest cell concentration allowed accurate counting in case of a high frequency of rabies-specific memory B cells.

Subject												
Ig	IgG		IgA		IgM		IgG		IgA		IgM	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Rabies	Ig	Rabies	Ig	Rabies	Ig	Rabies	Ig	Rabies	Ig	Rabies	Ig
B	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
C		Ig		Ig		Ig		Ig		Ig		Ig
D		1:100		1:100		1:100		1:100		1:100		1:100
E	Rabies	Tet	Rabies	Tet	Rabies	Tet	Rabies	Tet	Rabies	Tet	Rabies	Tet
F	1:10	Tet	1:10	Tet	1:10	Tet	1:10	Tet	1:10	Tet	1:10	Tet
G		PBS		PBS		PBS		PBS		PBS		PBS
H		PBS		PBS		PBS		PBS		PBS		PBS

Table 6.2-1: Explanatory diagram illustrating the sample layout and Ag specificity of ELISpot plate wells for the detection of rabies IgG-, IgA-, and IgM- plasma cells: “ex vivo ELISpot”. 200,000 cultured PBMCs were added to each well except where it is stated 1:10 or 1:100. 1:10 = one-tenth dilution of PBMC suspension (i.e. 20,000 PBMCs/well); 1:100 = 1/100 dilution of PBMC suspension (i.e. 2,000 PBMCs/well). PBMCs from one individual at different cell dilutions were placed in 6 columns on the ELISpot plate.

Minimum number of spots for assay acceptance

It has been suggested that the specificity of the ELISpot assay to detect only Ag-specific-B cell responses may be increased if a minimum of 4 spots is used to be certain of an Ag-specific response regardless of the number of wells assayed (D. Kelly, unpublished observations).

Therefore, in this study, ELISpot assays in which fewer than 4 rabies-specific spots were detected in the four replicate wells of 200,000 PBMCs, were treated as though no ASCs were detected. Therefore, as four replicate wells of 2×10^5 PBMCs were used, the minimum sensitivity of the assay was 1 cell per 2×10^5 PBMCs (represented by a dashed line in figures 6.3-1 and 6.3-2).

Positive control for culture B-cell ELISpot

For the cultured B-cell ELISpots, samples were excluded from the analysis in which fewer than 1000 IgG- and 500 IgA- ASCs per 2×10^5 cultured lymphocytes were detected, which corresponded to the lower limit of the 95% CI of the IgG- and IgA- memory B cells values observed in the whole study sample.

6.2.8 Statistical analysis

Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. Geometric means were calculated for the ELISA immunoglobulin concentrations and medians were calculated for the B-cell numbers. Comparisons of B-cell numbers and Ab concentrations at varying time points were made using the Wilcoxon signed ranks test for paired data. Comparison of the magnitude of the B cell and Ab response between the naïve and immune groups were made using the Mann Whitney U test. The correlations between the different variables were compared using the Spearman's rank correlation method, using the log transformed Ab concentration but untransformed B cell numbers.

6.3 Results

6.3.1.1 Recruitment

All 20 adult volunteers completed the study, however, because of unavailability for the visits or assay failures, not all data points were available for each time point (see number of participants for each result in the tables below each graph). A timeline of + or – 5 days was accepted for visits 7 and 14 (day 28 post immunisation), and of + or – 1 day for the other visits.

6.3.1.2 Reactogenicity

In general, the HDCV was well tolerated and only local reactions, such as erythema, pain and induration of the injected arm were reported in both immune and naïve volunteers.

However, 2/10 volunteers in the previously immunised group experienced an “immune complex like” type III hypersensitivity reaction characterised by urticaria and angioedema, with an onset at 8 and 12 days respectively. Both reactions recovered within a week after treatment with corticosteroids and antihistamine.

6.3.1.3 Plasma cell response after primary immunisation in the naïve group

Before immunisation, no rabies-specific plasma cells were detected in peripheral blood of the naïve participants. Following the primary dose of rabies vaccine there was an increase in the proportion of volunteers with detectable rabies-IgG-, IgA-, and IgM- plasma cells and in the number of rabies IgG-, IgA- and IgM- plasma cells in peripheral blood between day 7 and day 14, with a peak of the response by day 10 and a return to baseline by day 28 (although no volunteers were tested between day 14-28) (Figure 6.3-1 a, b, c). However, the number of rabies-plasma cells was significantly higher above baseline by day 10 for IgG-secreting cells ($p = 0.01$) and by day 7 for IgA- ($p = 0.04$) and IgM- ($p = 0.03$) secreting cells (Figure 6.3-1 a, b, c).

By contrast, after a third dose of rabies vaccine given at 56 days after the first dose, there was an increase in the proportion of volunteers with detectable rabies-specific IgG-plasma cells and in the number of rabies-specific IgG-plasma cells in peripheral blood between day 4 and day 10, with a peak of the response by day 7 and a return to baseline by day 14. The number of rabies-specific IgG-secreting cells was significantly higher above baseline by day 7 ($p = 0.008$). However, there was no increase in rabies-specific IgA- and IgM-secreting cells above baseline following the third dose of rabies vaccine, except for 2/10 volunteers by day 4 for IgA-plasma cells (Figure 6.3-1 a, b, c). The IgG-plasma cell response appeared to be of lower magnitude after the third dose than after the first dose, however, this difference was not statistically significant ($p = 0.4$ between d10 post first dose and d7 post third dose) (Figure 6.3-1 a, d).

6.3.1.4 Plasma cell response after secondary immunisation in the immune group

Before immunisation, no rabies-specific plasma cells were detected in peripheral blood of the 10 volunteers previously immunised against rabies (“immune group”). Following a booster dose of rabies vaccine given more than 2 years after last rabies immunisation there was an increase in the proportion of volunteers with detectable rabies-specific IgG- and IgA- plasma cells and in the number of rabies-specific IgG- and IgA- plasma cells in peripheral blood from day 7 to 10, with a peak by day 7 and a return to baseline by day 14. The number of IgG- and IgA- secreting cells was significantly higher above baseline by day 7 post-immunisation ($p = 0.005$ and $p = 0.008$ respectively). However, no increase in rabies-specific IgM-secreting cells was observed. (Figure 6.3-1 d, e, f).

The magnitude of the IgG-secreting cell response following secondary immunisation in the immune group was greater than following primary and secondary immunisation in the naïve group ($p = 0.01$ between d10 post first dose naïve group and d7 post booster dose immune group; $p = 0.0004$ between d7 post third dose naïve group and d7 post booster dose immune group) (Figure 6.3-1 a, d).

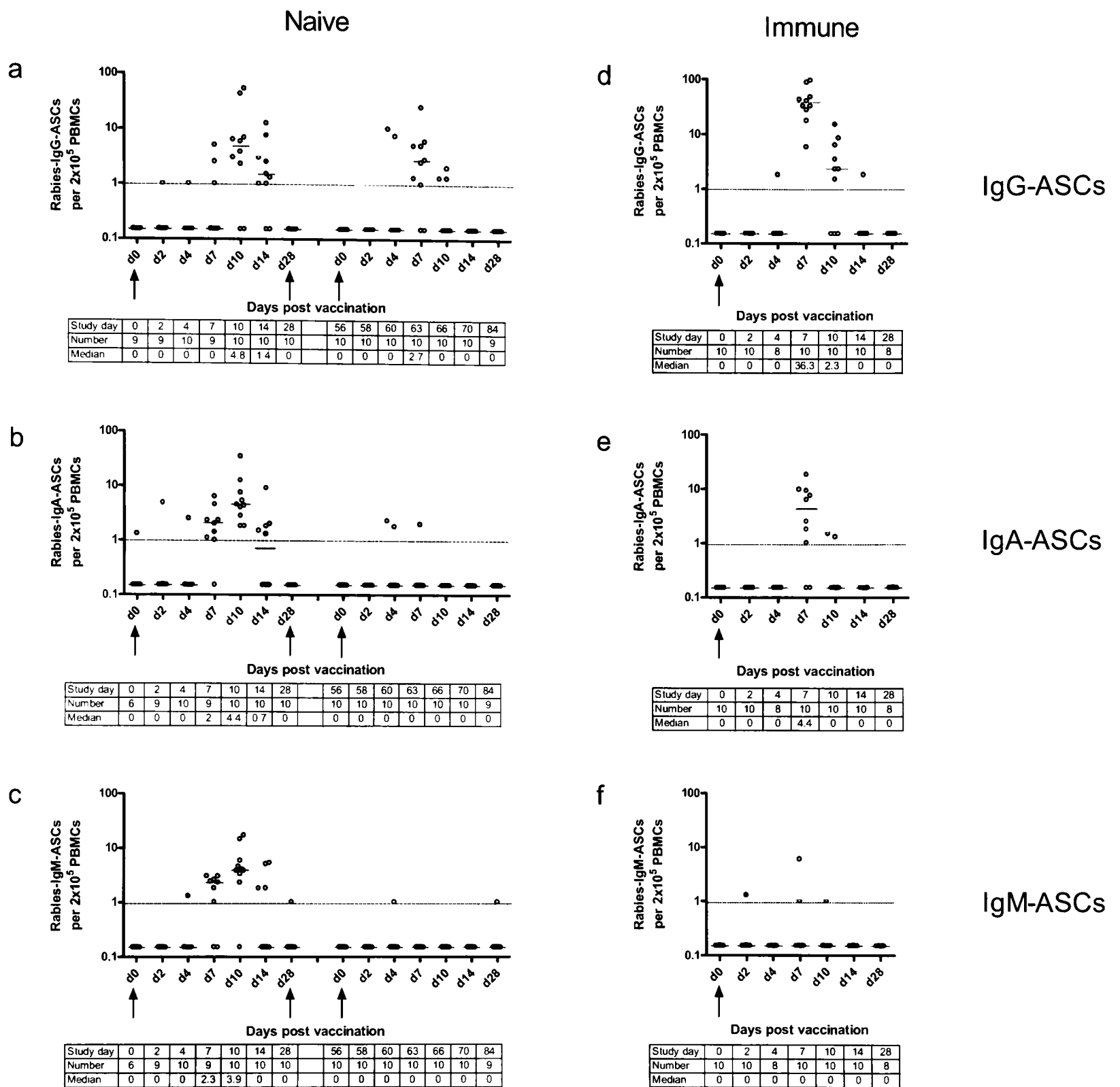


Figure 6.3-1: Rabies- specific IgG, IgA and IgM plasma cell responses to immunisation with rabies vaccine in naïve volunteers (left) and in immune volunteers (right). The naïve volunteers were immunised at day 0, 28 and 56 and the frequencies of rabies-specific IgG- (a, d), IgA- (b, e) and IgM- (c, f) ASCs were measured at various days after the first and third dose of immunisation in the naïve volunteers and after a single dose of rabies vaccine in the immune volunteers. The horizontal bars represent the median number of specific ASCs at each time point. The minimum sensitivity of the assay is plotted as a broken line on the graphs for each figure. The zero values were assigned a value of 0.15 for illustrative purposes.

6.3.1.5 Memory B cell response after primary immunisation in the naïve group

Only IgG- and IgA- memory B cells were assessed because the IgM-memory B cell ELISpot assay was non-specific for rabies-spots (see above section 6.2.7). Before immunisation, the median frequency of rabies-specific IgG- and IgA- memory B cells in peripheral blood of the naïve group was 0. Following the first dose of rabies vaccine there was a small increase in the number of volunteers with detectable rabies-specific IgG-memory B cells and in the number of rabies-specific IgG-memory B cells in peripheral blood between day 10 and day 28. However, the frequency of memory B cells 28 days after first dose of rabies vaccine was not significantly higher than at baseline ($p = 0.09$), although only 6 volunteers were tested at day 28 and 4/6 had detectable memory B cells at this time point (Figure 6.3-2 a).

At day 56, before the third dose of rabies vaccine 8/10 volunteers had detectable rabies-specific IgG-memory B cells and the median frequency was 3.8 rabies-specific IgG-memory B cells per 2×10^5 culture lymphocytes. Following the third dose of rabies vaccine, there was an increase in the proportion of volunteers with detectable rabies-specific IgG-memory B cells and in the number of rabies-specific IgG-memory B cells in peripheral blood from day 4 onwards (10/10 volunteers had detectable memory B cells at day 4). The number of IgG-memory B cells was significantly higher above baseline from day 4 onwards ($p = 0.05$ between d0-d4 post dose 3). There was a significant increase in memory B cells after the third dose of vaccine ($p = 0.009$ between d56-d84) but not after the second dose ($p = 0.8$ between d28-d56) (Figure 6.3-2 a).

By contrast, the rabies-specific IgA-memory B cell response was less clear; there was a transient increase in the proportion of volunteers with detectable rabies-specific IgA-memory B

cells and in the number of rabies-specific IgA-memory B cells in peripheral blood at various days following primary and secondary immunisation, however, the median frequency 28 days post first, second and third dose was not significantly greater above baseline (Figure 6.3-2 b).

6.3.1.6 Memory B cell response after secondary immunisation in the immune group

Before the booster dose of rabies vaccine, 5/10 volunteers of the immune group had detectable rabies-specific IgG-memory B cells and the median frequency of rabies-specific IgG-memory B cells was 1.9 per 2×10^5 cultured lymphocytes. Following immunisation there was an increase in the proportion of volunteers with rabies-specific IgG-memory B cells and in the number of rabies-specific IgG-memory B cells in peripheral blood from day 7 onwards (all volunteers had detectable memory B cells at this time-point). The median frequency 28 days post immunisation was 9.4 rabies-specific IgG-memory B cells per 2×10^5 cultured lymphocytes ($p = 0.006$ between d0-d28). The frequency of rabies-specific IgG-memory B cells at one month post-booster in the immune group was not significantly lower than the frequency in the naïve group one month after the last dose of the three-course priming ($p = 0.97$) (Figure 6.3-2 a, c). Similarly, to the naïve group, there was no change in the IgA-memory B cell frequency following booster dose of rabies vaccine, although there was a small increase in the proportion of volunteers with detectable rabies-specific IgA-memory B cells between day 4 and 28 (Figure 6.3-2 d).

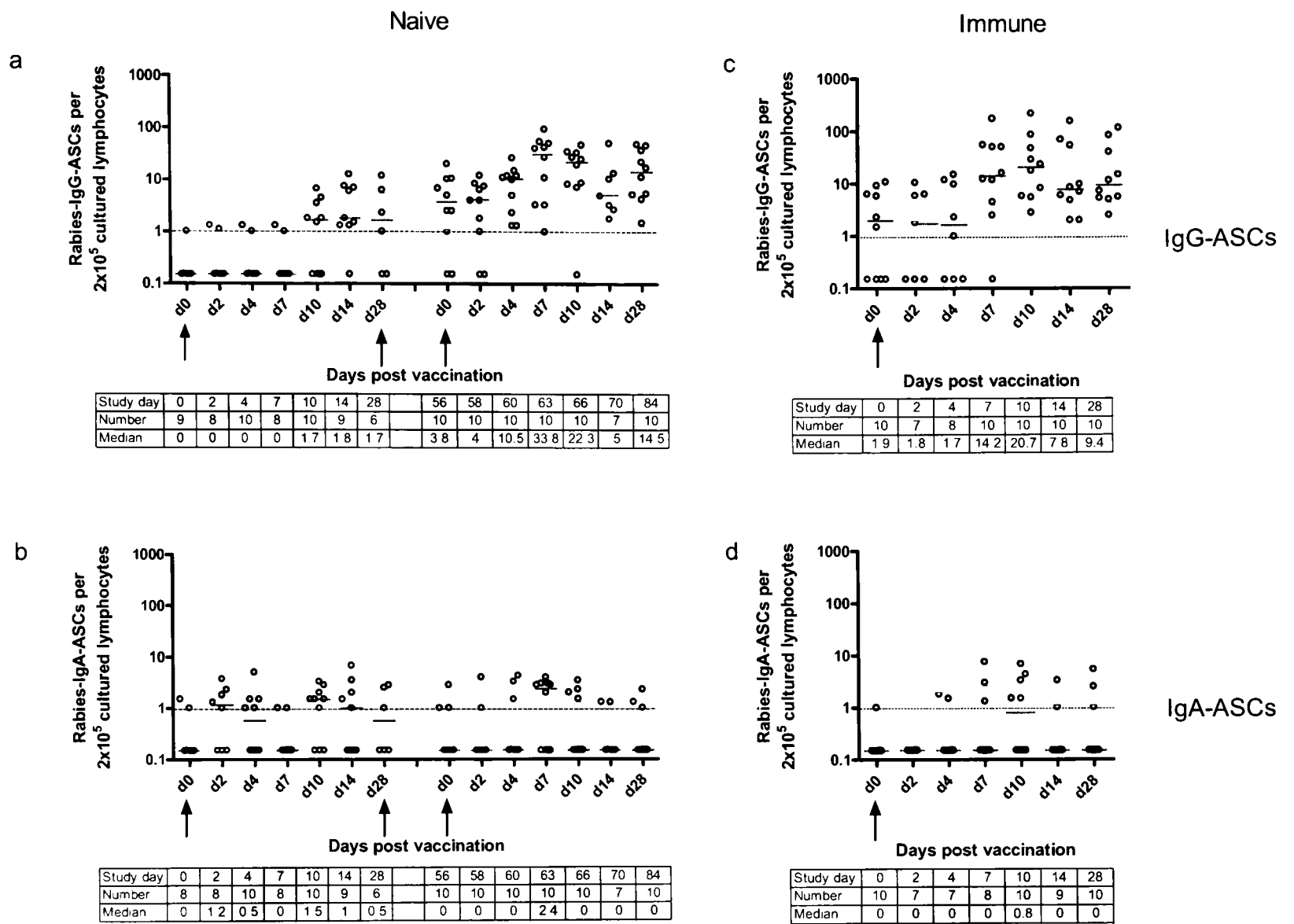


Figure 6.3-2: Rabies-specific IgG- and IgA- memory B cell responses following immunisation of naïve and immune volunteers with rabies vaccine. Using an ELISpot assay after 6 days culture with SAC/CpG/PWM, the rabies-specific IgG- (a, c) and IgA- (b, d) memory B cell frequency was measured at various days following the first and third dose of rabies vaccine in the naïve volunteers and after a single dose of rabies vaccine in the immune volunteers. The horizontal bars on the graph represent the median number of rabies-specific ASCs at each time point. The minimum sensitivity of the assay is plotted as a broken line on the graphs for each figure. The zero values were assigned a value of 0.15 for illustrative purposes.

6.3.1.7 Ab response after primary immunisation in the naïve group (IgG, IgG1, IgG3, IgA, IgM and avidity of IgG1 and IgG3)

Before immunisation, the GMC of rabies-IgG was 0.2 IU/ml for the naïve group. Following the first dose of rabies vaccine the IgG-GMC was significantly greater than baseline from day 10 onwards ($p = 0.005$) and all individuals had a rabies-IgG concentration above the protective level of 0.5 IU/ml from day 14 onwards. At day 28 post first dose of rabies vaccine the IgG-GMC was 1.8 IU/ml with an average of 18.2 fold rise between day 0 and day 28 (figure 6.3-3 a). Following the third dose of rabies vaccine there was a significant increase in IgG at day 7 ($p = 0.005$). There was further significant increase in IgG-Abs after the second ($p = 0.005$ between d28-d56) and third dose of vaccine ($p = 0.005$ between d56-d84), however, the average fold increase was 3.5 between day 28 and day 56 and 1.9 between day 56 and day 84 (Figure 6.3-3 a).

Similarly, there was a significant increase in IgG1 and IgG3 Abs at day 14 post first dose of rabies vaccine ($p = 0.005$ for both subclasses), but not at day 7 ($p = 0.8$ for both subclasses). There was further increase in IgG1 and IgG3 Abs 28 days after the second ($p = 0.005$ for both subclasses between d28-d56) and in IgG1 only after the third dose ($p = 0.007$ for IgG1 and $p = 0.05$ for IgG3 between d56-84) (Figure 6.3-3 b, c). Following the third dose of rabies vaccine the concentration of IgG1 and IgG3 was significantly higher above baseline prior to third dose of vaccine at day 7 ($p = 0.007$ for both subclasses).

Before the first dose of rabies vaccine in the naïve group the mean relative avidity index of

IgG1 and IgG3 was under the limit of detection. At 28 days post first immunisation it was 0.7 for IgG1 and 0.6 for IgG3. After the second and third dose of rabies vaccine there was no increase in the relative avidity index of IgG1 and IgG3 (Figure 6.3-4 a, b).

Before immunisation the IgA-GMC was 0.4 U/ml in the naïve group. Following immunisation the IgA-GMC was significantly greater than baseline from day 10 onwards ($p = 0.007$). At day 28 the IgA-GMC was 0.7 U/ml (Figure 6.3-5 a). There was a further increase in IgA-Abs after the second dose of vaccine ($p = 0.007$ between d28-d56) but not after the third dose of vaccine ($p = 0.4$ between d56-d84) (Figure 6.3-5 a).

The IgM-GMC at baseline was 0.1 U/ml in the naïve group. There was a significant increase in IgM Ab at day 7 after the first dose of vaccine ($p = 0.01$ between d0-d7). However, there was no increase in IgM after the third dose (Figure 6.3-5 b).

6.3.1.8 Ab response after secondary immunisation in the immune group (IgG, IgG1, IgG3, IgA, IgM and avidity of IgG1 and IgG3)

Before immunisation, the GMC of rabies-IgG was 2 IU/ml for the immune group and 8/10 volunteers had an IgG concentration above the protective level of 0.5 IU/ml (Figure 6.3-3 d). Following immunisation there was a significant increase in IgG-Ab above baseline from day 7 onwards ($p = 0.005$) but all the volunteers had an IgG concentration ≥ 0.05 IU/ml at day 2. At day 28 following immunisation the IgG-GMC was 36.1 IU/ml with an average of 10.9 fold rise between day 0 and 28 after the booster dose of rabies vaccine (Figure 6.3-3 d). The concentration of rabies-specific IgG-Ab at one month post-booster in the immune group was

significantly greater than the concentration of IgG-Ab in the naïve group one month after the last dose of the 3 course priming ($p = 0.008$).

Similarly, there was a significant increase in IgG1 and IgG3 Abs at day 7 post booster ($p = 0.005$ for both subclasses) (Figure 6.3-3 e, f).

At baseline, the mean relative avidity index was 1.1 for IgG1 but lower than the limit of detection for IgG3. One month after the booster dose the mean relative avidity index was 1.2 for IgG1 and 1.1 for IgG3 (Figure 6.3-4 c, d).

Before immunisation, the IgA-GMC was 0.6 U/ml for the immune group. Following immunisation the IgA-GMC was significantly greater than baseline from day 7 onwards ($p = 0.005$) (Figure 6.3-5 c).

The IgM-GMC at baseline was 0.2 U/ml. There was no significant change in IgM Ab at day 7 and 14 following immunisation (Figure 6.3-5 d).

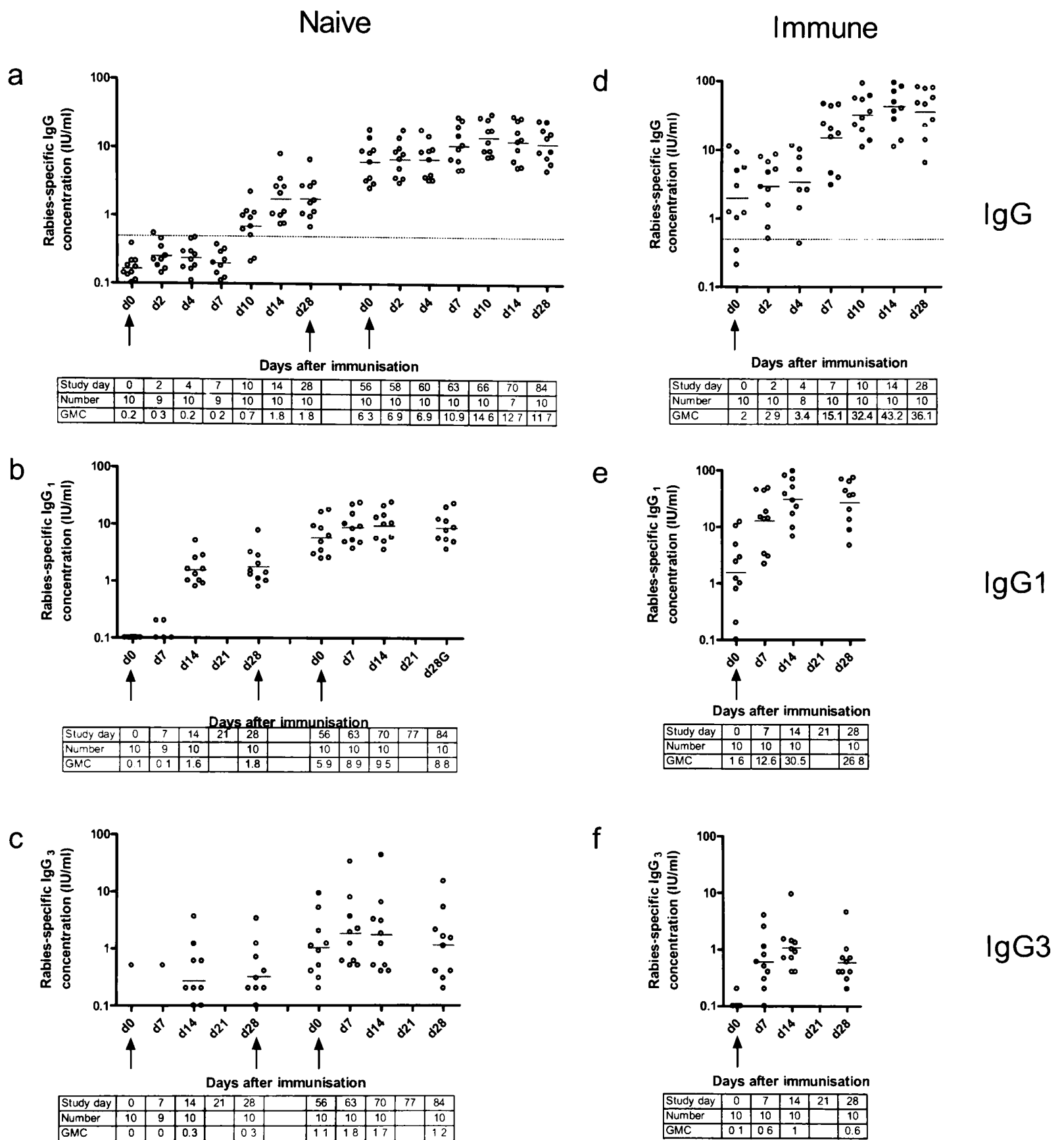


Figure 6.3-3: Rabies-specific IgG-, IgG1- and IgG3- Ab responses to immunisation with rabies vaccine in naïve volunteers (left) and in immune volunteers (right). The naïve volunteers were immunised at day 0, 28 and 56 and the concentration of rabies-specific IgG- (a, d), IgG1- (b, e) and IgG3- (c, f) was measured at various days after the first and third dose of immunisation in the naïve volunteers and after a single dose of rabies vaccine in the immune volunteers. The horizontal bars represent the GMC at each time point. The broken line represents the WHO seroconversion level of 0.5 IU/ml.

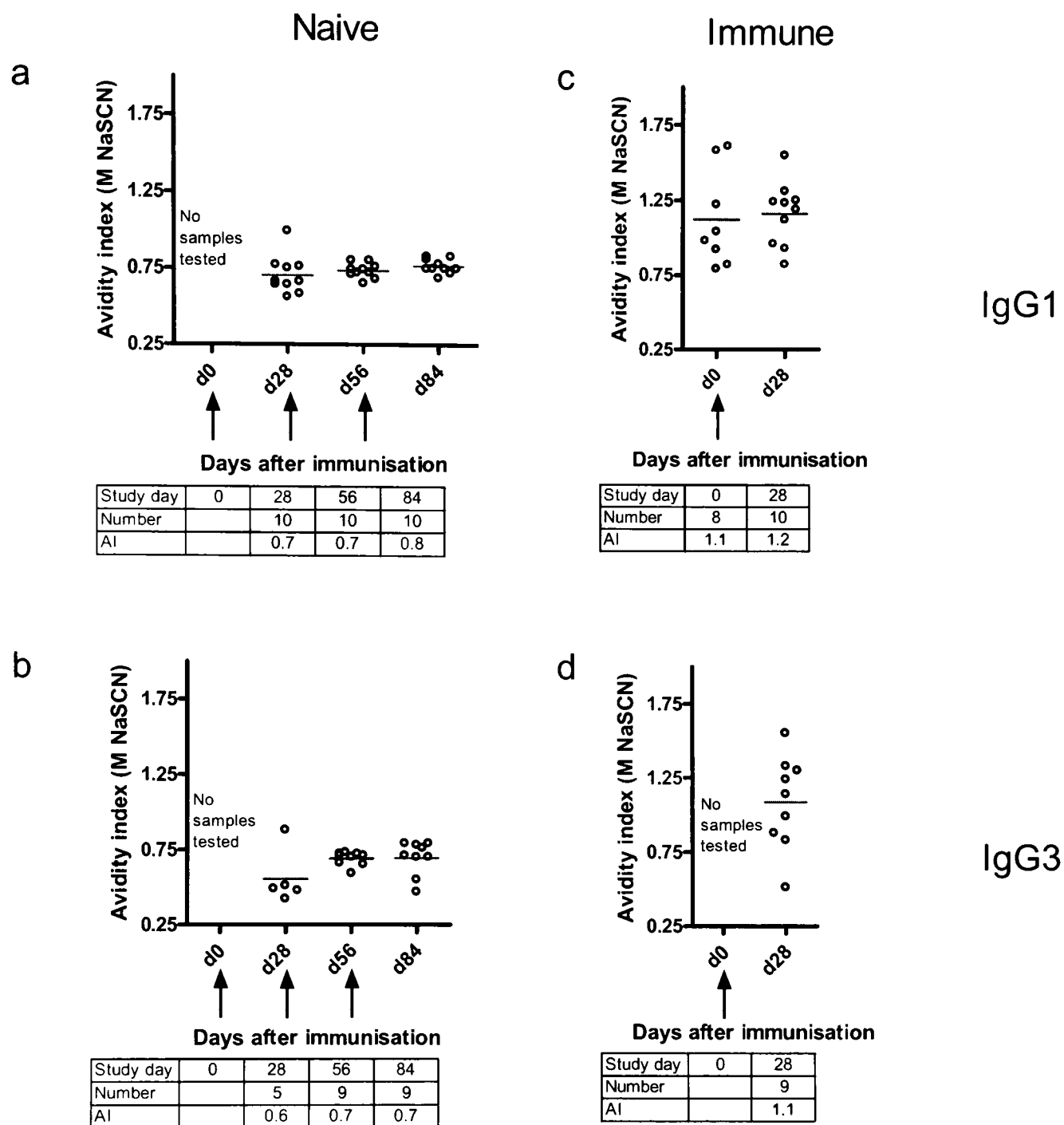


Figure 6.3-4: Relative avidity index (AI) of IgG1 and IgG3 anti-rabies Ab at various days following immunisation with rabies vaccine in naïve volunteers (left) and in immune volunteers (right). The naïve volunteers were immunised at day 0, 28 and 56 and the AI of IgG1 and IgG3 was measured at various days after the first and third dose of immunisation in the naïve volunteers (a, b) and after a single dose of rabies vaccine in the immune volunteers (c, d). The horizontal bars represent the median AI at each time point.

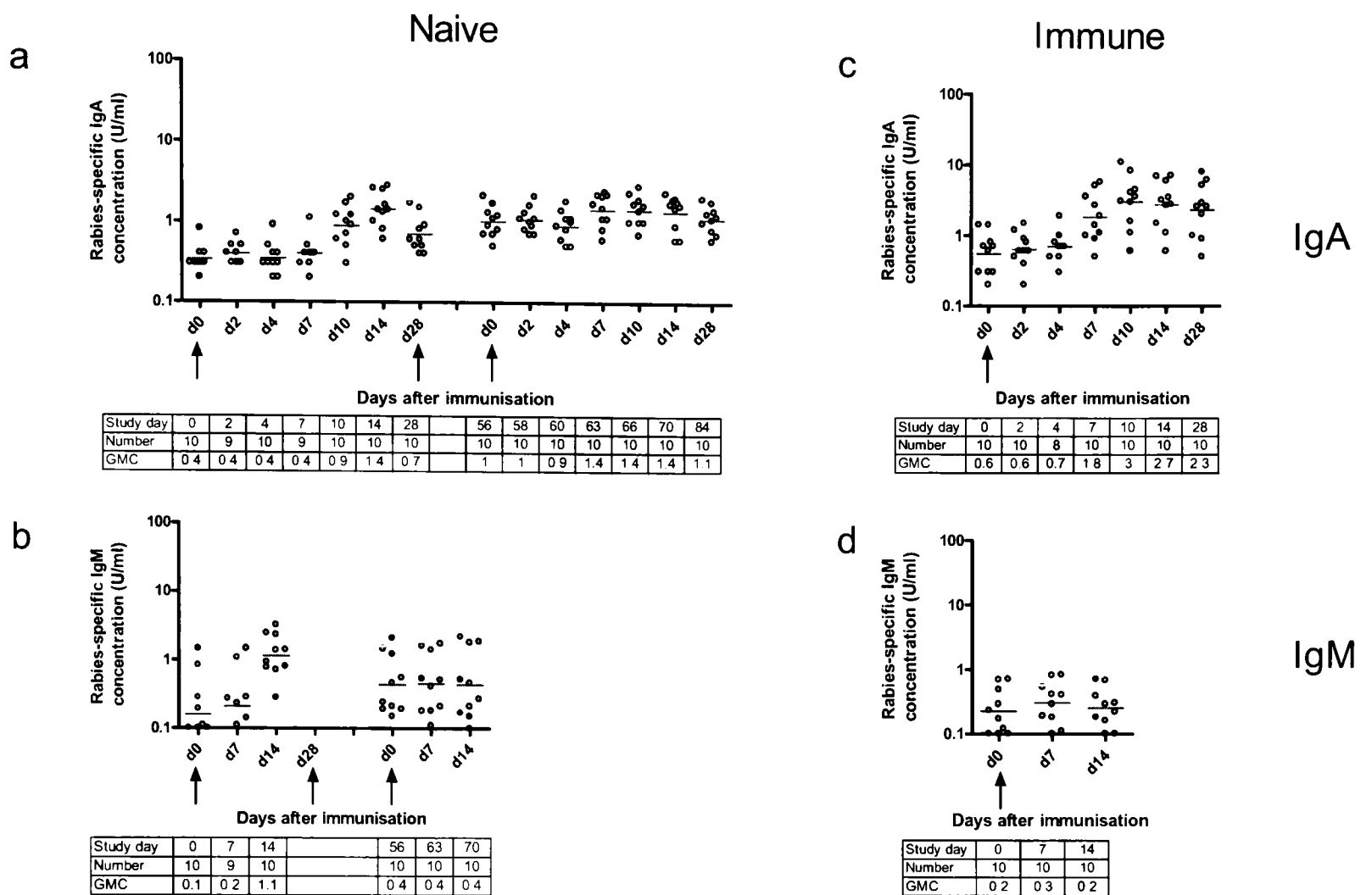


Figure 6.3-5: Rabies-specific IgA- and IgM- Ab responses to immunisation with rabies vaccine in naïve volunteers (left) and in immune volunteers (right). The naïve volunteers were immunised at day 0, 28 and 56 and the concentration of rabies-specific IgA- and IgM- measured at various days after the first and third dose of immunisation in the naïve volunteers (a, b) and after a single dose of rabies vaccine in the immune volunteers (c, d). The horizontal bars represent the GMC at each time point.

6.3.1.9 Correlation between Ab and B cell responses

There was a positive correlation between the IgG-memory B cell frequency and IgG-Ab concentration before (at day 56) and after (at day 84) the third dose of rabies vaccine in the naïve group (figure 6.3-6 a, d). Furthermore, there was a positive correlation between the IgG-memory B cell frequency before the third dose of rabies vaccine and the IgG-Ab concentration 28 days after the third dose of rabies vaccine (figure 6.3-6 b). However, there was no correlation

between the IgG-memory B cell frequency before the third dose of rabies vaccine and the peak of IgG-plasma cell response at day 7 after third dose (figure 6.3-6 c).

There was no correlation between the baseline IgG-Ab concentration and IgG-memory B cell frequency in the immune group (figure 6.3-7 a), however, there was a positive correlation between the frequency of IgG-memory B cells and IgG-Ab concentration 28 days post booster dose in the immune group (figure 6.3-7 d). There was a positive non significant correlation between the IgG-memory B cells before the booster dose and the IgG-Ab concentration after the booster dose ($p = 0.06$) (figure 6.3-7 b). There was no correlation between the IgG-memory B cell frequency before the booster dose and the peak IgG-plasma cell response at day 7 after the booster (figure 6.3-7 c).

Naive

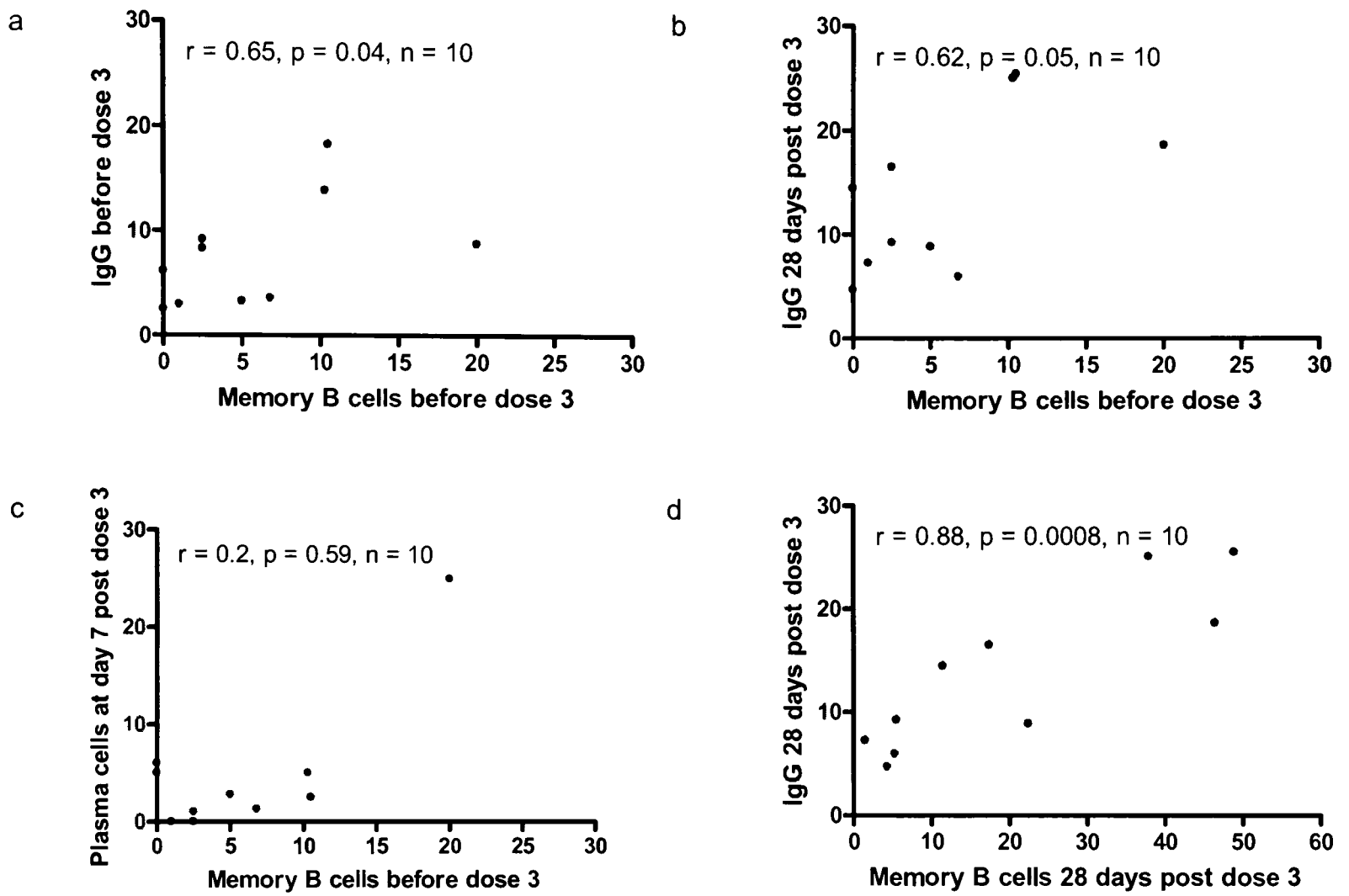


Figure 6.3-6: Correlation between rabies-IgG-memory B cells and IgG-Ab before and after the third dose of rabies vaccine in the naïve group, using Spearman's correlation method. The r and p-values of each correlation are reported on each graph.

Immune

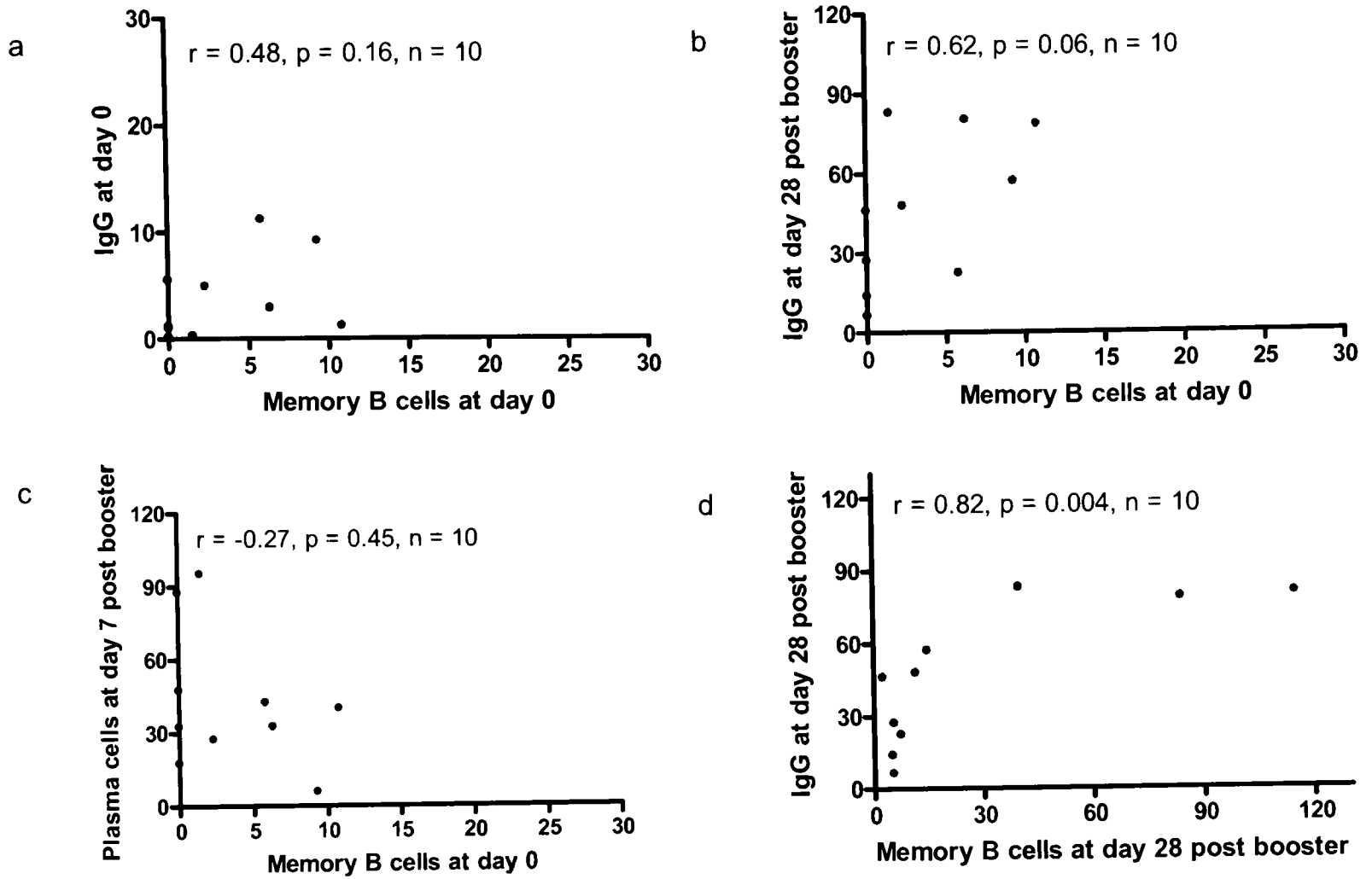


Figure 6.3-7: Correlation between rabies-IgG-memory B cells and IgG-Ab before and after the booster dose of rabies vaccine in the immune group, using Spearman's correlation method. The r and p values of each correlation are reported on each graph.

6.4 Discussion

B cell studies in animal models have provided important information about the cellular basis responsible for the development of long-term humoral immunity after an Ag exposure. However, in humans the measurement of plasma B cells and memory B cells generated after immunisation is complicated because of the inaccessibility of tissues such as bone marrow and lymphoid organs. These cells transit through the circulation to other lymphoid organs rapidly after their production. Therefore, it is important to define the kinetics of appearance of plasma cells and memory B cells in peripheral blood following immunisation in order to determine the optimal time point to measure the generation of these B cell populations by vaccination.

Whereas secondary responses in primed adults and infants have been defined, there is still a lack of information on the kinetics of a primary response in humans. In this study, we describe for the first time the timing of appearance of plasma cells, memory B cells and Abs in peripheral blood following a first and third dose of rabies vaccine in immunologically naïve adults and after a booster dose in immune individuals, and demonstrate important differences between primary and secondary B cell responses. These results provide new information about the generation of long-term humoral immunity in humans.

6.4.1.1 Primary B cell response

In this study, after primary immunisation the appearance of B cells and Abs in peripheral blood was delayed in comparison to a secondary immune response. Furthermore, the magnitude of the B cell and Ab response after the first dose of rabies vaccine was lower than after the third dose of vaccine given to the same individuals 2 months later (except for IgG-plasma cells), and also in comparison to the B cell and Ab response observed in the immune group after a booster dose of rabies vaccine (see below). The plasma cell response was also prolonged in comparison to secondary immunisation, with rabies-specific IgG-, IgA-, and IgM- plasma cells appearing in peripheral blood between day 7 and day 14, with a peak of the response by day 10 and a return to baseline by day 28 (although no volunteers were tested between day 14 and day 28). Memory B cell frequency increased between day 10 and day 28 after immunisation. In comparison, in a secondary immune response, the plasma cell response consistently peaks by day 6-7 after immunisation with a rapid return to baseline by day 10-12, and memory B cells rise by the end of the first week and persist for at least one month after immunisation (Clutterbuck et al., 2006; Crotty et al., 2003a; Kelly et al., 2006; Nanan et al., 2001).

In the present study, the Ab response following the first dose of rabies vaccine was also delayed in comparison to secondary immune response. The IgG- and IgA- Ab concentration started to increase after 10 days, while the concentration of IgM- Ab rose from day 7 onwards. Similarly, a previous study assessing the Ab response after primary immunisation with rabies vaccine has reported an increase in IgG- and IgA- Ab by the second week after immunisation, while IgM- Ab had increased already after one week (Brinkman et al., 2003). In contrast, in primed individuals Ab increased in peripheral blood within 2-5 days of immunisation (Bernasconi et al., 2002; Tsai et al., 2006) (section 4).

The primary B cell response observed in the present study is similar to the response in a study assessing the B cell response to primary immunisation with MenCV in 2 month old infants. In that study there was a small increase in IgG-plasma cells between day 8-16 with a return to baseline by day 30 (although no children were tested between day 16-30), and a small increase in IgG-memory B cells between day 14-30 after the primary immunisation (Kelly et al., 2008). These observations suggest that the kinetics of the B cell response during primary immunisation may be similar in infants and adults, although the infant plasma cell response was of lower magnitude.

Apart from this infant study, there are very few data on the kinetics of appearance of B cells during primary immunisation in humans. After a primary dose of smallpox vaccine in naïve adults, the memory B cells were detected at one month after immunisation, although no earlier time points were assessed (Crotty et al., 2003a).

Murine studies have reported that after primary exposure with an Ag small numbers of naïve B cells are first activated in extra-follicular foci, without direct interaction with T cells. Differentiation of these cells to unswitched plasma cells secreting low avidity Ab occurs from 36-60 hours after antigenic stimulation (Crotty et al., 2003b; Jacob et al., 1991a; Liu et al., 1991; Smith et al., 1996). These extra-follicular foci of activated B cells, apparent during the first week after immunisation, have already disappeared by the second week, due to death of the cells (Jacob et al., 1993; McHeyzer-Williams et al., 1993; Smith et al., 1996). Extrapolating from these murine studies to the present study, it is possible that these cells are responsible for the increase in IgM-Abs observed from day 7 onwards after primary immunisation in our study.

Other mouse studies have shown that these activated B cells or other naïve B cells can also migrate to the follicular area of the spleen and lymph nodes to form GCs, which start to appear in the spleen from day 4 after Ag exposure with peak activity between day 12 and 14. Involution occurs after 3-4 weeks (Crotty et al., 2003b; Jacob et al., 1991a; Jacob and Kelsoe, 1992; Jacob et al., 1993; Liu et al., 1991; Smith et al., 1997; Takahashi et al., 1998). Two distinct subpopulations of B cells are thought to originate from these GC B cells; memory B cells and plasma cells (Smith et al., 1997). While most of these plasma cells survive only a few days, others can migrate to the bone marrow to become long-lived plasma cells and sustain Ab secretion in the long-term (Manz et al., 1997; Slifka et al., 1998). However, the existence of long-lived plasma cells has only been observed in mice, although preliminary studies in humans depleted of CD20⁺ provide further support for their existence in humans (Cambridge et al., 2003; Edwards et al., 2004). In contrast, memory B cells have been shown to persist in humans after their production, and to continuously recirculate through the blood and secondary lymphoid organs (Nanan et al., 2001).

These observations suggest that the increase in plasma cells observed in peripheral blood between day 7 and day 14 following primary immunisation might represent plasma cells produced in GCs and their appearance might correspond to migration to the bone marrow. In contrast, the plasma cells produced in extra-follicular foci do not migrate to the bone marrow and therefore, are probably not seen in peripheral blood (Smith et al., 1996).

In the present study, the memory B cells appeared to be generated and disseminated later than the plasma cells. The reason for the later timing of the appearance of circulating memory B cells in comparison to plasma cells during primary immunisation in infants and adults might be

explained by their later selection in GCs. Other murine studies have observed that memory B cells were formed during the dissolution of GCs (Smith et al., 1997; Smith et al., 1994). The fate of B cells from the GCs appears to depend on co-stimulatory signals from T cells, with CD40-CD154 signalling committing B cells to differentiate into memory B cells, whereas CD27-CD70 signalling induces plasma cell differentiation (Agematsu et al., 1998a; Agematsu et al., 1998b; Arpin et al., 1995). The order in which these signals occur is unclear; in one murine study Ag-specific memory B cells were observed in peripheral blood by day 7 following a first exposure with an Ag (Blink et al., 2005). In contrast, long-lived plasma cells appear to leave GCs earlier than memory B cells (Crotty et al., 2003b; Slifka et al., 1995; Smith et al., 1997).

The Ab observed between days 10-28 after a primary dose of rabies vaccine might be produced principally by long-lived plasma cells, which leave the GC after their production to reside in the bone marrow. Indeed, a murine study has shown that Abs started to appear in serum at 8-15 days after primary Ag exposure and were maintained lifelong (Slifka et al., 1995). The same study has observed that the IgG subclass distribution of bone marrow and splenic plasma cells matched the IgG subclass distribution of Ag-specific Abs detected in serum at different time points after Ag exposure, confirming that both B cells from the spleen and bone marrow contributed to serum Ab production (Slifka et al., 1995). IgM Ab may also be produced by extrafollicular B cells.

6.4.1.2 Secondary B cell response

In this study, the response to the third dose of rabies vaccine in the naïve group was seen much earlier than the response to the primary dose. There was an increase in IgG-plasma cells between day 4 and day 10, with a peak at day 7. However, there was almost no increase in IgA and IgM plasma cells. Similarly, Abs were still present 28 days after the second of two vaccines given one month apart, and after the third dose of vaccine there was a further increase in IgG-Abs but no further increase in IgA or IgM Abs. This might be because following a third dose of rabies vaccine at a short interval after two previous doses most of the plasma cells originate from previously formed memory B cells. However, in the present study, almost no IgA-memory B cells were detected following primary immunisation with rabies vaccine, explaining the low increase in IgA-plasma cells and Ab after the third dose of vaccine.

Before the third dose of rabies vaccine IgG-memory B cells were already detectable in peripheral blood in most of the study participants (8/10), and there was a further increase of IgG-memory B cells in peripheral blood by day 4 after immunisation. This memory B cell response after a third dose of rabies vaccine was much quicker than the memory B cell response observed after a booster dose of rabies vaccine in the immune group (see below).

Similarly, following a third dose of MenCV given in children of 4 months of age after 2 previous doses of the same vaccine at 2 and 3 months of age, IgG-plasma cells (specific for serogroup C and the carrier protein diphtheria toxoid) were already detected in peripheral blood by day 4 and persisted until day 12 after the third dose of MenCV. In these children memory B cells persisted to 4 months of age, and further increased after the third dose of vaccine (Kelly et

al., 2008).

In contrast, following a booster dose of vaccine in the immune group IgG- and IgA-plasma cells were detected in peripheral blood between day 7 and day 10, and there was no increase in IgM-plasma cells. Similarly, IgG- and IgA- Abs increased in peripheral blood after day 7, and there was no increase in IgM-Ab. Furthermore, IgG-memory B cells increased after day 7 and persisted at day 28 after immunisation. The fact that there was no increase in IgM-plasma cells and Abs after booster immunisation in the immune group may be because the secondary immune response was dominated by previously formed memory B cells. However, the increase in IgA- plasma cells and Ab following booster immunisation in the immune group, compared to the response to third dose of rabies vaccine in the naïve group, suggests that there might be a mechanism of negative feedback above a certain level of Ab. Free rabies-IgA-Ab in the naïve group before the third dose of rabies vaccine might inhibit further activation of B cells to differentiate into plasma cells. Similarly, the magnitude of the increase in both the IgG-plasma cells and IgG-Ab was much greater in the immune group than in the naïve group following third dose of rabies vaccine, although in the naïve group the Ab concentration prior to the third dose of vaccine was much higher than the level in the immune group before the booster. Thus, the lower response in the latter could be explained by Abs “masking” Ag presentation to B cells, thereby creating a negative feedback. This mechanism might prevent the formation of too many plasma cells that cannot access the bone marrow. It has been observed that the number of plasma cells in the bone marrow in humans is constant, at around 1-2% of bone marrow cells (Terstappen et al., 1990).

The early appearance of rabies-memory B cells following the third dose of rabies vaccine in the

naïve group, compared to the rise in memory B cells by the end of the first week, which has been observed following booster immunisation in the immune group in the present study or re-immunisation of primed individuals in previous study (Kelly et al., 2006) (section 4), may reflect faster activation of recently generated memory B cells to proliferate and differentiate into plasma cells (either in extra-follicular foci or after re-entry in GCs). It is possible that after repeated doses of vaccine close to each other, memory B cells are more rapidly activated because most GCs would not have totally involuted, or Ag may have persisted in follicular DCs after primary immunisation or may relate to the size of the pool generated after a rapid three-dose schedule, which then wanes. A smaller pool remains after months/years resulting in a smaller B cell response.

However, in general, the appearance of plasma cells and memory B cells in blood was quicker following secondary immunisation than following primary immunisation, even in the participants who did not have circulating memory B cells at baseline in the immune group. This suggests that most of these cells might originate from previously formed memory B cells, which have a lower threshold of activation (Tangye et al., 2003) and which might not need to re-enter GC to differentiate into plasma cells. The fact that the avidity of the IgG1 and IgG3 Abs did not increase after the second and third dose of rabies vaccine in the naïve group suggest that memory B cells may not re-enter GCs during secondary immunisation otherwise memory B cells would be expected to undergo further selection with an increase in the avidity of their immunoglobulin. It is also possible that there is a maximal increase in avidity, in which case they could re-enter GC without a further increase in avidity.

Murine studies have observed that expression of high affinity BCRs on memory B cells

preferentially selects B cells to undergo extrafollicular differentiation into plasma cells, while lower affinity BCRs on naïve B cells cause selection of the B cells for GC development (MacLennan et al., 2003; Paus et al., 2006). However, GC formation has been documented during secondary and higher degree immune responses in murine studies and those responses are of greater magnitude and more rapid than in a primary response (Liu et al., 1991; Maruyama et al., 2000; Vora et al., 1999), due to an increase in T cell help and Ag on follicular DCs (FDCs) (Liu et al., 1991; Miller et al., 1995; Vora et al., 1999). These observations may explain the higher magnitude of the Ab and B cell response during secondary immunisation in the immune group, than during the 3-course priming in the naïve group. However, there was no correlation between the memory B cell frequency before the booster dose and the peak of the plasma cell response post-booster or Ab concentration 28 days after the booster. This may be because at steady state, most memory B cells reside in secondary lymphoid tissues, ready to rapidly proliferate and differentiate into plasma cells upon re-challenge with an Ag.

Finally, the non-correlation between the baseline Ab concentration and baseline memory B cell frequency in the immune group suggests that circulating memory B cells and humoral immunity represent two independent components of long-term immunity. Persisting Ab may principally be produced by long-lived plasma cells in the bone marrow, while circulating memory B cells are responsible for the rapid response to re-challenge with an Ag (immunological memory) (Leyendeckers et al., 1999; Nanan et al., 2001). In contrast, the correlation between the number of memory B cells and Abs in the naïve group 28 days after the second and third dose of rabies vaccine suggests that in the short term, humoral immunity and memory immunity correlate because they both originate from GC B cells. Similarly, the Ab frequency at day 28 after the booster dose correlated with the memory B cell concentration at day 28 after the booster dose in

the immune group.

6.4.1.3 Reactogenicity

The type III hypersensitivity reaction, which occurred in 2 volunteers of the immune group after booster immunisation, has been reported previously after HDCV, principally after booster immunisation (Dreesen et al., 1986). The overall incidence of allergic reaction after HDCV is 0.11%, however, the incidence is 6% after booster immunisation (Fishbein et al., 1993). The majority of the allergic reactions observed after HDCV are the type III hypersensitivity reaction. This allergic reaction may be caused by IgG and IgE Abs to beta-propiolactone (used to inactivate the virus) and human serum albumin (used as stabiliser in the vaccine), which form immune complexes with the albumin (Anderson et al., 1987; Swanson et al., 1987). However, an additional purification step used to remove human albumin may reduce the occurrence of systemic reactions with HDCV, as for the HDCV used in the present study (Fishbein et al., 1989).

In conclusion, this study has shown important differences in the timing of B cell appearance in peripheral blood after primary immunisation, compared to secondary immunisation. These differences are important in interpreting the generation of B cell populations by vaccination.

7 Influence of baseline Ab levels on booster Ab responses in infants after protein-polysaccharide conjugate vaccine

7.1 Introduction

A negative correlation between baseline Ab levels and the Ab increase after a booster dose of vaccine has been described previously (Danilova et al., 2005a; Danilova et al., 2005b; Nicolay et al., 1999; Ronne et al., 2000). This phenomenon is thought to be due to an inhibitory effect of pre-existing serum Ab on the response to the vaccine Ag. This may result from competition for the vaccine epitopes between pre-existing Ab and the BCR, which could impair the activation of B cells (Nanan et al., 2001). These observations have important implications for the recommendations of schedules for re-immunisation. If baseline Ab influence the activation of B cells, it may be important to allow sufficient amount of time between doses of vaccines. Furthermore, this phenomenon could explain why it is commonly observed that a third dose of vaccine during priming does not induce higher Ab levels than are observed after the second dose (Richmond et al., 1999).

Previous studies have used correlation or linear regression to investigate how the absolute or relative increase in Ab following a booster dose of vaccine relates to pre-booster Ab concentration (Barington et al., 1991; Danilova et al., 2005a; Danilova et al., 2005b; Ronne et al., 2000). The results of these studies have been inconsistent. Other studies have investigated the ratio between pre- and post-booster Ab concentration (fold increase) in individuals with different degrees of protection at baseline (using an arbitrary cut-off Ab concentration) and

commonly reported that the increase in the low group was higher than in the high group (Danilova et al., 2005a; Danilova et al., 2005b) (section 4). However, the use of simple correlation or linear regression to assess the relationship between baseline (pre-treatment clinical values) and change (post-treatment clinical values) has long been criticised by statisticians due to the concerns known as mathematical coupling (Hayes, 1988; Tu et al., 2004). Mathematical coupling occurs when one measure directly or indirectly contains the whole or part of the other measure, and the two are then analysed using correlation or regression. This is the case when the Ab increase, derived from the post-booster Ab minus the pre-booster Ab, is correlated with the pre-booster Ab. Testing their relationship using these methods would usually give a correlation because the error term occurs in both the change and initial value (Tu et al., 2004).

Alternative methods to assess the relationship between change and baseline values, avoiding mathematical coupling, include Blomqvist's method, Oldham's method and the variance ratio test. In particular, Oldham's method correlates the change between pre and post-treatment values with the average mean of the pre- and post-treatment values, which has the advantage of preserving the independence between the two measures (Altman, 1982; Mattson et al., 1995; Oldham, 1962). A more sophisticated method was later proposed by means of the multilevel modelling (MLM) approach, which allows considering additional covariates or confounders (e.g., age and gender of study participants) (Gilthorpe et al., 2000). Furthermore, it does not require that each individual is measured on every occasion. The method analyses pre- and post treatment measures as repeated measures within subject and the test of differential baseline effect on the outcome is derived from the covariance between the random intercept and random slope. With correct model specification and without additional covariates, the results are

equivalent to results obtained from Oldham's method.

In the present study, we used the MLM technique to assess the effect of pre-booster MenC-IgG concentration on the relative increase in MenC-IgG concentration following booster immunisation from four studies we previously conducted. The reason for using this method was because it enabled to account for the fact that data were obtained from different studies.

7.2 Materials and Methods

The four studies were all previously conducted in Oxford, UK (Buttery et al., 2005; English et al., 2000; MacLennan et al., 2000) (section 4), in which children primed with MenCV at 2, 3, 4 months of age or HBV, were boosted with MenCV or meningococcal A/C polysaccharide vaccine (MenA/C) at 12 months of age. A total of 8 different vaccine schedules had been used across the four studies (see Table 7.3-1) and the children were separated into 8 groups according to their vaccine schedule. Each dose of MenCV contained 10 µg of *N. meningitidis* group C oligosaccharide and each dose of MenA/C contained 50 µg of *N. meningitidis* group C oligosaccharide, however, only 1/5th of the MenA/C was administered in all studies, except the one reported by MacLennan et al. (MacLennan et al., 2000), where the full dose of MenA/C was used. The MenCV used in each of the four studies used CRM₁₉₇ (a mutant of diphtheria toxoid) as the carrier protein.

The concentrations of MenC-specific IgG-Abs before the booster at 12 months of age and after the booster at 13 months of age were available for the children participating in the four studies. For any particular study, MenC-IgG concentration was measured by ELISA in one of three laboratories (Oxford Vaccine Group, Chiron or Wyeth laboratory). The results were pooled and analysed to assess the effect of pre-booster MenC-specific IgG concentration on the relative increase in MenC-IgG concentration after the booster. The analysis was performed using the MLM technique (Blance et al., 2005) for each group across 4 studies receiving 1 of 8 different vaccine schedules (see table 7.3-1).

For each group a MLM model was fitted using a multilevel software (MLwiN version 2.0.01; <http://www.cmm.bristol.ac.uk>), where the outcome of the model was the MenC-IgG concentration. For groups with more than one study, a 3-level model was fitted with study at level 3, subjects at level 2, and time (i.e. pre- and post booster) at level 1. A 2-level model was used for groups with one study, with subjects at level 2 and time at level 1. Correlation coefficient was derived from the covariance between the random slope and random intercept of the model (Blance et al., 2005), and reflects the strength of the association between pre-booster MenC-IgG concentration and post-booster increase in MenC-IgG concentration.

The correlation between the change in MenC-IgG concentration and pre-booster diphtheria toxoid-IgG concentration was assessed using Pearson's correlation method, for the studies where diphtheria toxoid-IgG concentration was available. For groups with more than one study, a pooled correlation coefficient was derived by means of a meta-analysis method (inverse variance method). Analysis was carried out using Stata (version 9.1, StataCorp, USA). Data of all analyses were log transformed to meet the normal distribution assumption.

7.3 Results

Ab data were available from 643 children, who had been immunised according to 1 of 8 different schedules (table 7.3-1). The children were primed with either MenCV, or a combined 9-valent pneumococcal/meningococcal serogroup C conjugate vaccine (9vPnc-MenCV), or HBV. Boosting was with either MenCV (with or without MMR), or MenA/C. All groups had a pre-booster MenC-GMC below the postulated correlate of protection of 2 µg/ml (Gold et al., 1979; Peltola, 1998), although the 95% CI of the GMC of MenC-Ab of groups 1 to 3 (primed with MenCV) included the value of 2 µg/ml. Following booster immunisation, all groups had a significant increase in MenC-IgG concentration, and all the groups except group 8 had a MenC-GMC above 2 µg/ml (Table 7.3-1).

Using the MLM technique to assess the effect of pre-booster MenC-IgG concentration on the relative increase in MenC-IgG concentration post-booster, a positive correlation was observed when the MenA/C vaccine was used for booster (groups 3 and 8), except for group 5 (primed with 9vPnc-MenCV). In contrast, when MenCV (with or without MMR) was used for booster a negative correlation was observed (statistically significant only for group 1 with the larger sample size), except for group 4 (primed with 9vPnc-MenCV) (Table 7.3-1). However, for the two groups primed with 9vPnc-MenCV, there was no effect of pre-booster MenC-IgG on the post-booster increase in MenC-IgG after a booster dose of MenA/C (group 5), but there was a positive effect of pre-booster MenC-IgG on post-booster increase in MenC-IgG after a booster dose of MenCV (group 4). In contrast, using spearman correlation to assess the effect of pre-booster MenC-IgG concentration on the relative increase in MenC-IgG concentration post-

booster, a significant negative correlation was observed for all the groups (Table 7.3-2).

Using Pearson's correlation method, to assess the effect of pre-booster diphtheria toxoid- IgG concentration on the relative increase in MenC-IgG concentration post-booster, a positive correlation was observed when MenCV was used for booster (groups 1 and 4), except for group 6 primed with HBV. In contrast, there was no correlation when MenA/C was used as booster (groups 3, 5 and 8) (see Table 7.3-3).

Group	Priming at 2, 3, 4 months	Boosting at 12 months	No of studies used	No of participants	Pre-boost MenC-IgG GMC with 95% CI	Post-boost MenC-IgG GMC with 95% CI	MenC-IgG GMR with 95% CI	Correlation coefficient with 95% CI
1	MenCV	MenCV	4	180	1.72 (1.48, 2.00)	34.82 (30.34, 39.90)	20.23 (17.21, 23.82)	-0.16* (-0.3, -0.01)
2	MenCV	MenCV-MMR [#]	1	47	1.97 (1.54, 2.52)	36.12 (29.55, 44.05)	18.06 (13.93-23.39)	-0.21 (-0.47, 0.09)
3	MenCV	MenA/C	3	132	1.87 (1.61, 2.16)	10.72 (8.57, 13.39)	5.70 (4.47, 7.28)	0.21* (0.04, 0.37)
4	9vPnC-MenCV	MenCV	1	47	0.49 (0.40, 0.61)	6.59 (4.69, 9.25)	12.85 (8.76, 18.85)	0.45* (0.18, 0.65)
5	9vPnC-MenCV	MenA/C	1	50	0.39 (0.31, 0.49)	2.35 (1.83, 3.01)	5.62 (4.33, 7.30)	0.09 (-0.19, 0.36)
6	Hep B	MenCV	2	74	0.11 (0.08, 0.13)	6.79 (5.40, 8.53)	61.50 (41.30, 91.56)	-0.03 (-0.26, 0.21)
7	Hep B	MenCV-MMR [#]	1	46	0.08 (0.05, 0.11)	13.46 (10.06, 18.01)	190.99 (145.3, 251.8)	-0.19 (-0.46, 0.1)
8	Hep B	MenA/C	2	51	0.11 (0.09, 0.14)	1.52 (1.11, 2.07)	13.15 (8.22, 21.10)	0.83* (0.72, 0.9)

Table 7.3-1: Correlation between pre-booster MenC-IgG concentrations and relative increase in MenC-IgG concentration post-booster within groups receiving 1 of 8 different vaccine schedules using MLM technique. [#]MenCV given concomitantly with MMR,

^{||} Combined 9 valent pneumococcal and meningococcal serogroup C conjugate vaccine; GMC = geometric mean concentration of MenC-IgG in µg/ml; GMR = geometric mean ratio between post- and pre- MenC-IgG concentration (also known as fold increase), *Coefficient correlation statistically significant for the relationship between pre-booster MenC-IgG concentrations and relative increase in post-booster MenC-IgG concentrations.

Group	Priming at 2, 3, 4 months	Boosting at 12 months	No of studies used	No of participants	Correlation coefficient	p values
1	MenCV	MenCV	4	159	-0.59	<0.001
2	MenCV	MenCV-MMR [#]	1	43	-0.66	<0.001
3	MenCV	MenA/C	3	130	-0.44	<0.001
4	9vPnC-MenCV	MenCV	1	44	-0.44	0.003
5	9vPnC-MenCV	MenA/C	1	44	-0.48	0.001
6	Hep B	MenCV	2	70	-0.87	<0.001
7	Hep B	MenCV-MMR [#]	1	40	-0.47	0.002
8	Hep B	MenA/C	2	50	-0.86	<0.001

Table 7.3-2: Correlation between pre-booster MenC-IgG concentrations and relative increase in MenC-IgG concentration post-booster within groups receiving 1 of 8 different vaccine schedules using Pearson's correlation method. Correlation coefficient (r) with the p-values (p) and the number of subject tested for each association (n). [#]MenCV given concomitantly with MMR, ^{||} Combined 9 valent pneumococcal and meningococcal serogroup C conjugate vaccine.

Group	Priming at 2, 3, 4 months	Boosting at 12 months	No of studies used	No of participants	Pre-boost diphtheria toxoid-IgG GMC	MenC-IgG GMR with 95% CI	Correlation coefficient with 95% CI
1	MenCV	MenCV	4	110	0.32	20.23 (17.21, 23.82)	-0.43* (-0.57, -0.26)
3	MenCV	MenA/C	3	97	0.36	5.70 (4.47, 7.28)	0.06 (-0.14, 0.26)
4	9vPnC-MenCV	MenCV	1	35	0.1	12.85 (8.76, 18.85)	-0.47* (-0.70, -0.17)
5	9vPnC-MenCV	MenA/C	1	38	0.09	5.62 (4.33, 7.30)	-0.04 (-0.36, 0.28)
6	Hep B	MenCV	1	28	0.31	61.50 (41.30, 91.56)	0.29 (-0.1, 0.6)
8	Hep B	MenA/C	1	28	0.36	13.15 (8.22, 21.10)	0.07 (-0.31, 0.43)

Table 7.3-3: Correlation between pre-booster diphtheria toxoid-IgG concentrations and relative increase in MenC-IgG concentration post-booster within groups receiving 1 of 8 different vaccine schedules, using Pearson's correlation method. ^{||} Combined 9 valent pneumococcal and meningococcal serogroup C conjugate vaccine; diphtheria toxoid-IgG GMC = geometric mean concentration of diphtheria toxoid-IgG in µg/ml; MenC-IgG GMR = geometric mean ratio between post- and pre- MenC-IgG concentration (also known as fold increase), *Coefficient correlation statistically significant for the relationship between pre-booster diphtheria toxoid-IgG concentrations and relative increase in post-booster MenC-IgG concentrations.

7.4 Discussion

In this study, using standard correlation methods to assess the effect of pre-booster MenC-Ab levels on the increase in MenC-Abs post-booster, a negative correlation was consistently observed for all the vaccine groups, as previously described (Danilova et al., 2005a; Danilova et al., 2005b; Nicolay et al., 1999; Ronne et al., 2000). However, when using MLM technique it was observed that the pre-booster MenC-specific Ab did not consistently affect the Ab response post-booster. When looking at the two groups with the larger sample size (group 1 and 3), a slight negative correlation was observed when MenCV was used for booster, and a slight positive correlation was observed when MenA/C was used for booster, although the MenC-GMC pre-booster was similar in both groups. This means that higher pre-booster Ab concentration tended to result in a greater increase in Ab concentration following a dose of MenA/C, but resulted in a lower increase in Ab following a dose of MenCV. This could be explained by differences in the processing of these Ags by B cells.

It has been suggested previously that serum Ab is maintained by the differentiation of memory B cells into plasma cells and that there is a correlation between memory B cell numbers and the resting Ab level. Indeed, previous researchers have observed a correlation between circulating memory B cells and Abs at steady state (Bernasconi et al., 2002; Crotty et al., 2003a; Dorfman et al., 2005), although others have not (Leyendeckers et al., 1999). If the Ab levels at baseline reflect the size of the memory B cell pool available, then one might expect greater secondary Ab responses to be associated with higher levels of pre-existing Ab. This was the case in the present study for 2/3 groups after the plain polysaccharide vaccine. Since plain polysaccharides

are T-independent Ags, the vaccine is expected to cross-link the BCR of memory B cells driving them to plasma cells producing Ab. The strongest correlation was observed in group 8, the group primed with HBV and who received the MenA/C vaccine for booster. In this group, the pre- and post-booster MenC-Ab concentrations were low. The presence of pre-booster MenC-Abs in some individuals might have been acquired through natural exposure to non pathogenic strains of the bacteria or other cross-reactive species (Sanchez et al., 2002; Troncoso et al., 2000). It is unknown whether this natural Ab is derived from a TD immune response, with the induction of GCs and production of memory B cells (Donnelly et al., 1990).

By contrast, with the conjugate in most groups the resting level of Ab was associated with a slight negative correlation (which was only significant for group 1 with the larger sample size). Although conjugate vaccines do presumably also stimulate memory B-cells they may in addition stimulate naïve B cells to proliferate, class switch and produce IgG. Naïve B-cells may be more susceptible to inhibition by pre-existing Abs (and their Ag masking effect) than memory B cells, which have a lower threshold for activation than naïve B cells (Tangye et al., 2003). There is also the possibility that pre-formed B cells and T cells specific for the carrier proteins could compete for Ag capture with B cells specific for the polysaccharide, thus inhibiting the immune response to the polysaccharide component of the conjugate vaccine (Barington et al., 1993; Insel, 1995). This phenomenon called “carrier induced epitope suppression” explains possible interactions between vaccines based on the same protein carrier, which are administered simultaneously either at separate sites or as combined vaccines (Buttery et al., 2005; Dagan et al., 1998). In the present study, a moderate negative correlation was observed between baseline diphtheria toxoid-IgG concentration and the increase in MenC-IgG concentration post-booster, when MenCV was used for priming and boosting (groups 1 and 4).

These findings suggest that the level of diphtheria toxoid-specific Ab correlates with the level of diphtheria toxoid-specific B cells, which may compete with polysaccharide-specific B cells for the capture of the MenCV. However, another study has reported that high pre-booster concentration of carrier-specific Ab correlated positively with the increase in Ab specific for the capsule Ag following immunisation with a *H. influenzae* type b conjugate vaccine in adults (Barington et al., 1991). It was suggested by the investigators of this study that carrier-specific Abs correlated with the numbers of carrier-specific helper T cells, which in turn could increase the B cell response against the polysaccharide component of the conjugate vaccine. Therefore, the phenomenon of carrier-induced epitope suppression may depend of several factors. For example, the dose of the carrier may be important. It was observed in a murine study that low-dose carrier priming improved subsequent response to polysaccharides conjugated to the same carrier, while high dose priming suppressed the response (Daum et al., 2001).

Groups 4 and 5, primed with 9vPnC-MenCV had a different response when compared with the other groups. In the original study with this vaccine (Buttery et al., 2005), it was shown that 9vPnC-MenCV interfered with immune responses to concomitantly administered vaccines and also induced lower MenC-Ab responses. It is not clear how these observations relate to the different relationships noted in the response to boosting in the present study. The MenC-Ab levels in the MenCV boosted group were positively correlated with the post-booster Ab increase but the MenA/C boosted group did not show a relationship between pre-booster Ab levels and Ab increase post-booster, observations that are dissimilar to the other groups described above.

In conclusion, using statistical methods that account for mathematical coupling, we have shown that the response in toddlers to the widely used MenCV may be slightly weaker in those with pre-existing capsule-specific and carrier-specific Abs. In contrast, the response to the plain polysaccharide vaccine appears to be preserved or enhanced by pre-existing polysaccharide-specific Ab. These findings indicate differences in the processing of both types of vaccines.

8 Discussion

Protein-polysaccharide conjugate vaccines are widely used to protect children against invasive bacterial diseases. Although these vaccines are immunogenic from early infancy, their effectiveness is of short-duration in infants. It was observed that a two- or three- dose primary schedule before one year of age induced high bactericidal Ab levels, which waned rapidly in most children without a booster immunisation at one year of age. In contrast, Ab persistence improves with increasing age (Snape and Pollard, 2005). Further vaccine protection in young children depends on the induction of immunological memory and herd immunity. Immunological memory can be defined by a rapid rise in high quality Ab following re-exposure to an Ag, which was observed between days 4-6 after challenge in adults and children in the different studies of this thesis (see section 4 and 6). However, it has been shown that *N. meningitidis* can rapidly invade through the mucosal barrier and disease develops within hours. Therefore, persistence of Ab, rather than immunological memory appears to be the principal way to sustain vaccine protection against such rapidly invasive bacterial diseases.

The aim of this thesis was to assess the cellular basis responsible for the low persistence of Ab after immunisation with protein-polysaccharide conjugate vaccines in early infancy. Various aspects of the B cell response to immunisation have been investigated in this thesis.

8.1 Laboratory methods to assess memory B cells in peripheral blood

In this thesis, it has been shown (section 3) that the ELISpot method was more sensitive than the LDA (ELISA-based LDA) method to assess Ag-specific memory B cells of low frequency in small blood volume, such as polysaccharide-specific memory B cells in infants. However, an advantage of the LDA over the ELISpot is that it can directly estimate the frequency of the original Ag-specific memory B cells. In contrast, the ELISpot assesses the frequency of ASCs differentiated from cultured memory B cells but as culture of memory B cells induces proliferation of these cells, before their differentiation, the frequency of Ag-memory B cells is overestimated. However, another method the ELISpot-based LDA, appears to combine the sensitivity derived from the proliferation occurring in culture with the determination of actual cell frequency.

8.2 Kinetics of appearance of B cells in peripheral blood after immunisation

Studies of the B cell responses to immunisation in animal models suggest that the generation of memory B cells and long-lived plasma cells during primary immunisation may be the principal determinant of long-term maintenance of Ab, as these cells have been shown to persist in the long-term and to contribute to Ab secretion (Bachmann et al., 1994; Manz et al., 1998; Smith et al., 1996). However, in humans the organs of formation and residence of these cells are not easily accessible, and B cells can only be assessed in peripheral blood.

Using the ELISpot method, the timing of appearance in humans of plasma cells and memory B cells in peripheral blood after primary and secondary immunisation has been defined. Following

primary immunisation with rabies vaccines in adults (section 6), rabies-specific plasma cells were apparent in peripheral blood between day 7-14 with a peak by day 10, and rabies-specific memory B cells increased from day 10 onwards and still persisted in peripheral blood 28 days after immunisation. In comparison, following primary immunisation with MenCV in 2 month old infants, MenC-plasma cells were apparent in peripheral blood between day 8-16 and MenC-memory B cells increased from day 14 onwards and persisted by day 28 (Kelly et al., 2008). These data suggest that the timing of appearance of plasma cells and memory B cells in peripheral blood after primary immunisation is similar in infants and adults, and after protein vaccines and protein-polysaccharide conjugate vaccines, although the magnitude of the response was slightly lower in infants.

In contrast, following secondary immunisation in infants and adults, the timing of appearance of B cells in peripheral blood was faster (and shorter lasting for plasma cells). Following a booster dose of MenCV at one year of age (section 4), MenC- and diphtheria toxoid- specific plasma cells were apparent in peripheral blood between day 4-9 with a peak at day 6, and memory B cells rose from day 8 onwards and persisted at day 28. After a primary course of MenACWY-CRM₁₉₇ vaccine at 2 and 4 months of age (section 5), memory B cells specific for the four serogroups of meningococci and CRM₁₉₇ were not apparent in peripheral blood at 4 months of age, however, these cells started to appear in a small proportion of children from day 7 onwards and there was no major variation in the percentage of children with detectable memory B cells between day 0 and 120 post-immunisation. Similarly, following booster immunisation with rabies vaccine in primed adults (section 6), rabies-specific plasma cells were detected in peripheral blood between day 7-10 with a peak by day 7, and memory B cells increased from day 7 onwards and persisted at day 28.

In comparison, following a third dose of rabies vaccine given at a short interval after two previous doses (section 6), the B cell response was faster than usually observed in secondary immune response, with plasma cells detected in peripheral blood between day 4-10, and memory B cells rising from day 4 onwards.

It can be extrapolated from murine studies that the transient appearance of plasma cells in peripheral blood observed in these studies after immunisation represents their passage through the circulation to the bone marrow. Therefore, defining the exact timing of appearance of these cells in peripheral blood allows measurement of the pool of plasma cells generated by immunisation, which may contribute to long-term maintenance of Ab.

In contrast, although the frequency of memory B cells did not vary significantly between the first day of their detection (4-10 days after immunisation) to 28 days after immunisation (sections 4, 5, 6), these cells also migrate from the circulation to home in secondary lymphoid organs (Liu et al., 1995; Maruyama et al., 2000). The time period over which memory B cells disappear from peripheral blood appears to vary with the Ag under investigation and the age of the subject. One previous study has assessed the frequency of Ag-specific IgG-memory B cells at day 0, 12 and 90 after booster immunisation with diphtheria toxoid in primed adults and has shown an increase in memory B cell frequency in peripheral blood from day 0 to 12, followed by a small decline by day 90 (Nanan et al., 2001). At one year of age, 8 months after a three-dose priming with MenCV, the median frequency of MenC-memory B cells was 3.5 per million cultured lymphocytes (1/3 of the frequency observed at 5 months of age after priming), while the median frequency of diphtheria toxoid-memory B cells was similar to the one observed at 5

months of age (11.5 per million cultured lymphocytes) (section 4). One year after a booster dose of MenCV in young adults aged between 15-18 years old, the median frequency of MenC-memory B cells was 6.5 per million cultured lymphocytes (1/6 of the frequency observed at one month post booster) and the frequency of diphtheria toxoid-memory B cells was 15 per million cultured lymphocytes (identical to the frequency observed at one month after booster) (Kelly et al., 2006). In contrast, in a group of primed adult volunteers with varied past exposure of rabies vaccine more than 2 years previously, the median frequency of rabies-memory B cells was 9.5 per million cultured lymphocytes (section 6).

In conclusion, these data suggest that memory B cells are kept in the circulation for a certain period following Ag exposure, perhaps with the function of protecting against further environmental exposure in the short term or because of cellular competition (or programmed cell death) making space for new memory B cells in secondary lymphoid organs (Crotty et al., 2003a). Therefore, assessing memory B cells in peripheral blood one month after immunisation might be a good estimate of the memory B cell pool produced, although a part of memory B cells may be kept in secondary lymphoid organs with the Ag retained on FDCs (MacLennan, 1994) and not re-circulate immediately after their production.

8.3 Association between primary B cell response and persistence of Ab

An important finding of this thesis is that the long-term maintenance of Ab depends on a good plasma cell and memory B cell response during primary immunisation. Indeed, the study described in section 4 showed a strong association between the levels of Ab and memory B cells measured at 5 months of age (one month after three-dose primary immunisation with

MenCV), and the persistence of Ab by one year of age. This may be due to the long-term persistence of long-lived plasma cells or memory B cells produced in the GCs, and their role in the continuous production of Ab. In murine studies, long-lived plasma cells have been shown to survive in bone marrow niches and to continue to secrete Ab after the Ag exposure for months (Slifka et al., 1998). Memory B cells are assumed to persist for years after the Ag exposure in humans, and are expected to contribute to long-term persistence of Ab by continuously differentiating into plasma cells, in response to polyclonal stimuli (such as microbial products or cytokines secreted by activated T cells), or Ag-dependent stimuli (through persisting Ag or cross-reactive Ag) (Bernasconi et al., 2002; Crotty et al., 2003a; Ochsenein et al., 2000; Sprent and Tough, 1994).

8.4 Magnitude of B cell response in children, compared to adults

The lack of persistence of Ab in infants given protein-polysaccharide conjugate vaccines can be explained by lower generation of long-lived plasma cells and memory B cells in GCs, in comparison to adults. Indeed, it has been discussed previously that a primary B cell response was delayed and of lower magnitude in comparison to secondary response (section 6). However, when comparing a primary B cell response in infants and adults, although the kinetics of the B cell response was similar, the magnitude of the response was lower in children (section 6) (Kelly et al., 2008). Furthermore, it was observed that there was no difference between the level of MenC-memory B cells reached at 5 months of age after three-dose priming with MenCV, and the level of MenC-memory B cells reached one month after the 12-months booster dose of MenCV (section 4). However, the median frequency of MenC-memory B cells observed at 5 months of age, one month after 3 dose-priming with MenCV (section 4), was

lower than that observed in primed adolescents one month after a booster dose of MenCV (Kelly et al., 2006). Similarly, murine studies have demonstrated a limited induction of the GC in early life, associated with a slow maturation of FDC networks (Pihlgren et al., 2003), and the inability of B cells to induce T cell activation (Marshall-Clarke et al., 2000).

The children producing memory B cells during priming also maintained Ab in the long-term, while all children were able to mount a secondary immune response after booster immunisation by one year of age (section 4). It might be that only a large pool of memory B cells can sustain Ab persistence, while a small pool of memory B cells may only be sufficient to produce a secondary immune response. This phenomenon has been observed in a murine study, where the generation of memory B cells during priming was impaired due to a mutation in the *xid* gene, and the persistence of Ab was reduced, although immunological memory was preserved (Ridderstad et al., 1996). It is possible that all children produce a small number of memory B cells during priming with MenCV or MenACWY-CRM₁₉₇ vaccines (section 4 and 5), but only higher frequencies of memory B cells were detectable by the ELISpot assay. This would explain why the detection of memory B cells at 5 months of age was associated with the persistence of Ab at one year. In the children where no memory B cells were detected at 5 months of age, there might be enough cells to produce a primed immune response but not enough to maintain Ab persistence.

In conclusion, these findings together with the observations in murine studies that the survival of long-lived plasma cells is poor in infants, because of a reduced capacity of infant bone marrow to sustain plasma cell survival (Pihlgren et al., 2006; Pihlgren et al., 2001), suggest that the maintenance of polysaccharide-specific Ab in children might principally depend on memory

B cells.

8.5 The presence of maternal Ab in infant blood may explain the lower magnitude of the B cell response after infant immunisation

The presence of maternal Ab has been evoked to explain the lower Ab response to immunisation in early life, compared to older ages. In this thesis (section 5), the influence of maternal Ab specific for serogroups A, C, Y and W135 meningococci in peripheral blood at 2 months of age, on the memory B cell response to primary immunisation with MenACWY-CRM₁₉₇ has been investigated. It was found that anti-meningococcal Ab present in infant blood at 2 months of age did not affect the memory B cell response to a primary course of the MenACWY-CRM₁₉₇ given at 2 and 4 months of age, although the anti-meningococcal Ab level was very low in these children.

8.6 Relationship between peripheral blood B cells and Ab at steady state and after immunisation

It was observed that at steady state, circulating memory B cells and Abs do not always correlate. In primed adult volunteers before a booster dose of rabies vaccine (section 6), there was no correlation between circulating rabies-Ab and rabies-memory B cells ($r = 0.48$, $p = 0.16$, $n = 10$) (section 6). In comparison, in primed children before a booster dose of MenCV at one year of age, there was a correlation between the circulating memory B cells and Abs specific for MenC ($r = 0.65$, $p = 0.004$, $n = 18$) but not for diphtheria toxoid ($r = 0.25$, $p = 0.27$, $n = 22$) (section 4). Similarly, previous researchers have observed a correlation between circulating

memory B cells and Abs at steady state for measles (Bernasconi et al., 2002), tetanus toxoid (Bernasconi et al., 2002; Dorfman et al., 2005), smallpox (Crotty et al., 2003a); plasmodium falciparum (Dorfman et al., 2005), although others have not for diphtheria toxoid (Nanan et al., 2001) and tetanus toxoid (Leyendeckers et al., 1999; Nanan et al., 2001).

In contrast, there was a correlation between memory B cells and Ab, after three dose priming with MenCV at 5 months of age ($r = 0.74$, $p = 0.0001$, $n = 21$) (section 4), and after three dose priming with rabies vaccine in immunologically naïve adults ($r = 0.88$, $p = 0.0008$, $n = 10$) (section 6). Furthermore, one month after booster immunisation with MenCV at one year of age, there was a weak correlation between circulating memory B cells and Abs specific for MenC ($r = 0.54$, $p = 0.006$, $n = 25$) but not for diphtheria toxoid ($r = 0.25$, $p = 0.27$, $n = 22$) (section 4), and there was a strong correlation between these two parameters in adults after booster immunisation with rabies vaccine ($r = 0.82$, $p = 0.004$, $n = 10$) (section 6).

These results suggest that circulating memory B cells and humoral immunity may represent two independent components of long-term immunity. Persisting Ab may principally be produced by long-lived plasma cells in the bone marrow, while circulating memory B cells are responsible for the rapid response to re-challenge with an Ag, although they may also contribute in renewing the population of long-lived plasma cells in the bone marrow (Crotty and Ahmed, 2004). The role of memory B cells in sustaining Ab in the long-term might depend on several factors, such as the pool of memory B cells available (discussed above), the persistence of Ag or cross-reactive Ag, number of previous exposure with the Ag. Indeed, it was reported previously that the capacity of B cells to expand diminish with every Ag re-challenge (Nanan et al., 2001). This was explained by more mature B cells being more susceptible to apoptosis and

having a lower proliferative capacity (Liu et al., 1995) or the increasing level of Ab with each-Ag exposure competing with the activation of B cells.

In contrast, after primary immunisation humoral immunity and memory immunity correlate because they both originate from GC B cells.

8.7 Influence of baseline Ab on booster Ab response

In this thesis, the influence of baseline MenC-Ab on the Ab response to booster immunisation with MenCV or MenA/C at one year of age was investigated in four studies previously conducted by Oxford Vaccine Group, using MLM technique. It was observed that in general, pre-booster MenC-Abs had no effect or a slight negative effect on the Ab response to booster immunisation with MenCV at one year of age, however, carrier-specific-Abs appeared to impair the MenC-Ab response. However, these results are not strong enough and it is necessary to do further research to see if we can extend these findings using additional clinical studies. Defining how pre-booster polysaccharide- or carrier- specific Abs influence the Ab response to booster immunisation with protein polysaccharide conjugate vaccines may be important in determining the optimal timing of administration of booster doses of vaccine to maintain Ab levels, but also for the introduction into childhood vaccination programs of multiple vaccines sharing common protein carriers.

8.8 Conclusion

In conclusion, the results of the different studies in this thesis suggest that priming with

polysaccharide-protein conjugate vaccines in early infancy may generate GCs of various efficiency in different children. Thus, the children who generate the more efficient GCs during priming, identified by higher production of memory B cells, plasma cells and Abs after primary immunisation, best maintain protective Ab levels in the long-term. In contrast, the children who generate less efficient GCs will have a smaller B cell pool and lower Ab response during priming, and might not maintain protective Ab levels in the long-term. Therefore, a better understanding of the factors that determine the production of adequate GCs during priming in early infancy may be the key to improve short and longer-term protection induced by protein-polysaccharide conjugate vaccines that are so important to protect young children against diseases caused by polysaccharide encapsulated bacteria.

8.9 Future work

It would be useful to define the exact phenotype of the cells participating in the immune response to polysaccharide-protein conjugate vaccines in early infancy, in comparison to older children and adults. This type of analysis would involve flow cytometric phenotyping methods associated with the development of polysaccharide specific B cell labelling techniques, using for example MHC-tetramer, which is used to quantitate Ag-specific T cells. However, because polysaccharide Ags bind to all scavenger receptors, which are expressed on many types of cells (DCs, macrophages, etc.), it would be necessary to first isolate the B cells from the rest of the PBMCs. Then, we would gate the Ag-specific B cells and look at different markers expressed on these cells to identify the exact B cell subpopulation. This would allow identification of age differences in the B cell population responding to polysaccharide-protein conjugate vaccines, which could explain the lack of Ab persistence in children. This

It would also be important to conduct genetic studies looking at the differences between those children, who generate high numbers of polysaccharide-specific memory B cells during primary immunisation with polysaccharide-protein conjugate vaccines, in comparison to those who do not, to provide insight into the genetic control of early life responses. For example, during the study of the B cell response to MenACWY-CRM₁₉₇ vaccine (section 5) DNA has been extracted from the cellular plug remaining after serum centrifugation. These DNA samples contributed to a DNA bank pooling samples of different Oxford Vaccine Group studies, which have assessed the Ab response to primary immunisation with diverse meningococcal protein polysaccharide conjugate vaccines. Using these DNA samples, we could assess the association between gene single-nucleotide polymorphism (SNPs) and the magnitude of the immune response to primary immunisation, looking either at candidate genes [e.g. TLR, human leukocyte antigen (HLA)] (Docherty et al., 2007; Kimman et al., 2008; Ovsyannikova et al., 2006) or high density SNP analysis covering the whole genome (Butcher et al., 2004).

An investigation of the factors influencing the activation of T helper cells by immunisation with protein-polysaccharide conjugate vaccines, such as various types and doses of carrier protein Ags, and analysis of their effect on B cell activation during immunisation could help to increase vaccine protection in children. For example, we could compare the B cell and T cell responses following primary immunisation with various serogroup C meningococcal vaccines conjugated to different carrier proteins. It would also be useful to assess the B cell and T cell responses following concomitant or separate administration of various protein-polysaccharide conjugate vaccines.

Similarly, the influence of circulating Ab on B cell response to immunisation should be further investigated. This is especially relevant when maternal Ab is present in peripheral blood, but also later on, when repeated doses of vaccines are administered close to each other resulting in high levels of Ab. Finally, the potential of combined vaccines to decrease polysaccharide-specific B cell activation should also be assessed.

The different studies in this thesis have defined the optimal time point to measure B cell responses during primary and secondary immunisation in children and adults. This should help to design further vaccines studies to assess how these different factors might influence production of adequate GCs during priming in early infancy.

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