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Une étude de la réponse des cellules B mémoires au vaccin
polysaccharidique conjugué contre le méningocoque du sérogroupe C

Blanchard Rohner, Géraldine

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Thèse préparée sous la direction de la Professeure Claire-Anne Siegrist

**UNE ETUDE DE LA REPONSE DES CELLULES B MEMOIRES AU
VACCIN POLYSACCHARIDIQUE CONJUGUE CONTRE LE
MENINGOCOQUE DU SEROGRUPE C**

Thèse
présentée à la Faculté de Médecine
de l'Université de Genève
pour obtenir le titre de Docteur en Médecine

par

Géraldine Blanchard

de Soral (GE)

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Table des matières

<u>REMERCIEMENTS</u>	<u>2</u>
<u>TABLE DES MATIÈRES</u>	<u>3</u>
<u>RÉSUMÉ</u>	<u>6</u>
<u>LISTE DES ABRÉVIATIONS</u>	<u>7</u>
<u>LISTE DES FIGURES</u>	<u>8</u>
<u>LISTE DES TABLEAUX</u>	<u>9</u>
<u>CHAPTER 1: INTRODUCTION AU TRAVAIL</u>	<u>10</u>
<u>1.1 EPIDÉMIOLOGIE</u>	<u>10</u>
<u>1.2 IMMUNITÉ</u>	<u>11</u>
<u>1.3 CONTRIBUTION DES ANTICORPS ET DE L'IMMUNITÉ MÉMOIRE</u> <u>À LA PROTECTION</u>	<u>12</u>
<u>1.4 RÉFÉRENCES</u>	<u>14</u>
<u>CHAPTER 2: LITERATURE REVIEW</u>	<u>15</u>
<u>2.1 NEISSERIA MENINGITIDIS</u>	<u>15</u>
2.1.1 STRUCTURE AND CLASSIFICATION	15
2.1.2 EPIDEMIOLOGY	16
2.1.4 MENINGOCOCCAL CARRIAGE	17
2.1.5 VIRULENCE FACTORS	17
2.1.5 PATHOGENESIS	18
2.1.6 MARKERS OF IMMUNOGENICITY IN PROTECTION AGAINST MENINGOCOCCAL DISEASE	19
2.1.7 MENINGOCOCCAL DISEASES	20
2.1.7 DIAGNOSIS	22
2.1.8 MONITORING AND TREATMENT	24
<u>2.2 REVIEW OF THE IMMUNE SYSTEM</u>	<u>24</u>
2.2.1 INNATE IMMUNITY	24
2.2.2 ADAPTIVE IMMUNITY	25
2.2.3 SECONDARY IMMUNE RESPONSE AND MEMORY IMMUNITY	26
2.2.4 LONG TERM PROTECTIVE IMMUNITY	26

<u>2.3</u>	<u>ANTIMICROBIAL VACCINES.....</u>	<u>28</u>
2.3.1	KILLED OR INACTIVATED VACCINES.....	29
2.3.2	LIVED VACCINES.....	30
2.3.3	FUTURE VACCINES.....	30
2.3.4	VACCINE AGAINST T-INDEPENDENT ANTIGENS: ENCAPSULATED BACTERIA.....	30
<u>2.4</u>	<u>FACTORS THAT DIMINISH THE VACCINE EFFICACY IN VERY YOUNG INFANTS</u>	<u>31</u>
2.4.1	DEFICIENCIES OF NEONATAL B CELL RESPONSES TO T-DEPENDANT ANTIGENS	31
2.4.3	ANTIBODY PRODUCTION IS OF SHORT DURATION	32
2.4.4	QUALITATIVE DIFFERENCES IN ANTIBODY RESPONSES	33
2.4.5	INTERFERENCE OF PERSISTING MATERNAL ANTIBODIES	33
2.4.6	SUBOPTIMAL T CELL RESPONSES	33
<u>2.5</u>	<u>APPLICATION TO MENINGOCOCCAL GROUP C GLYCOCONJUGATE VACCINE</u>	<u>34</u>
2.5.1	MENINGOCOCCAL VACCINES.....	34
2.5.2	IMMUNISATION CAMPAIGNS WITH MENC GLYCOCONJUGATE VACCINE	35
2.5.3	EFFECT OF MENC INTRODUCTION	36
2.5.4	OPTIMAL MENC SCHEDULE.....	39
<u>CHAPTER 3: DETERMINATION OF MEMORY RESPONSE TO MENC CONJUGATE VACCINE</u>		
<u>IN CHILDREN OF ONE YEAR OF AGE, AFTER PRIMING AT 2, 3 AND 4 MONTHS.....</u>		<u>40</u>
<u>3.1</u>	<u>RATIONALE</u>	<u>40</u>
<u>3.2</u>	<u>STUDY DESIGN.....</u>	<u>40</u>
<u>3.3</u>	<u>MATERIALS AND METHODS</u>	<u>41</u>
3.3.1	PREPARATION OF PBMCs FOR CULTURE OR FOR EX VIVO ELISPOT	41
3.3.2	ACTIVATION OF MEMORY B CELLS DURING 5 DAYS FOR MEMORY B CELL ELISPOT.....	42
3.3.3	DETERMINATION OF ANTIGEN-SPECIFIC-PLASMA CELLS AND MEMORY B-CELLS BY ELISPOT ..	43
<u>3.4</u>	<u>RESULTS</u>	<u>46</u>
3.4.1	PERSISTENCE OF IGG-SPECIFIC MEMORY B CELLS AND PLASMA CELLS SPECIFIC FOR MENC AND FOR OTHER ANTIGENS PRIOR TO VACCINATION	46
3.4.2	KINETICS OF THE RESPONSE OF MENC-SPECIFIC PLASMA CELLS AFTER BOOSTER DOSE OF MENC- AND HIB- CONJUGATE VACCINES, AND IN COMPARISON TO OTHER ANTIGENS	48
3.4.3	KINETIC OF THE RESPONSE OF MENC-SPECIFIC MEMORY B CELLS AFTER BOOSTER DOSE OF MENC- AND HIB- CONJUGATE VACCINES, AND IN COMPARISON TO OTHER ANTIGENS	51
<u>3.5</u>	<u>DISCUSSION.....</u>	<u>54</u>
3.5.1	PERSISTENCE OF IGG-SPECIFIC MEMORY B CELLS FOR MENC AND FOR OTHER ANTIGENS PRIOR TO IMMUNISATION AND KINETICS OF MEMORY B CELL RESPONSE FOLLOWING IMMUNISATION	54
3.5.2	PERSISTENCE OF PLASMA CELLS SPECIFIC FOR MENC AND FOR OTHER ANTIGENS PRIOR TO VACCINATION AND KINETICS OF PLASMA CELL RESPONSE FOLLOWING IMMUNISATION	57
3.5.3	COMPARISON OF THE RESULTS OF THE PRESENT STUDY WITH THE ONE PERFORMED ON THE	

SAME INFANTS, DURING PRIMING AT 2, 3 AND 4 MONTHS OF AGE	60
3.5.4 WHY CRM197-SPECIFIC B CELL FREQUENCY WAS LOWER THAN DIPHTHERIA TOXOID-SPECIFIC B CELL FREQUENCY?	63
3.5.5 EXPRESSION OF THE MEMORY B CELL FREQUENCY AS A PERCENTAGE OF TOTAL IGG ASCs	63
 <u>CHAPTER 4: CONCLUSIONS</u>	<u>64</u>
<u>REFERENCES</u>	<u>65</u>

Résumé

Neisseria meningitidis est encore une cause importante de maladies invasives chez les enfants. En Angleterre, le vaccin polysaccharidique conjugué contre le sérogroupe C fait partie du plan de vaccination de routine. Chez les enfants de moins d'une année, 3 doses de vaccin induisent une montée importante des anticorps bactéricides, qui ne persistent cependant pas au-delà d'une année.

Les cellules B mémoires semblent essentielles pour protéger ces jeunes enfants primo-immunisés comme nourrissons. Dans cette étude, j'ai évalué la persistance dans le sang des plasmocytes producteurs d'anticorps et des cellules mémoires spécifiques chez des enfants de 12 mois primo-immunisés à 2, 3, et 4 mois. Les résultats montrent que ces cellules, généralement non détectables, réapparaissent à jour 6 (plasmocytes) ou 8 (cellules B mémoires) après une dose de rappel de vaccin conjugué. Ces données permettent de mieux comprendre la participation de l'immunité mémoire à la protection contre les infections invasives à *N.meningitidis*.

Liste des Abréviations

Ab : antibody	Mat-ab : maternal antibodies
Ag : antigen	MCV : quadrivalent polysaccharide meningococcal vaccine conjugated to diphtheria toxoid
APC : antigen presenting cell	MD : meningococcal disease
ASC : antibody-secreting cells	MenC : meningococcal serogroup C
BCR : B cell receptor	MenCV : MenC-CRM ₁₉₇ glycoconjugate vaccine
CFR : case fatality rate	MLEE : multilocus enzyme electrophoresis
CRM : cross reactive materials	MLST : multilocus sequence typing
CRM₁₉₇ : mutant peptide related to diphtheria toxoid	MZ : marginal zone
CSF : cerebrospinal fluid	OMP : outer membrane proteins
CTL : cytotoxic lymphocytes	OMV : outer membrane vesicles
DC : dendritic cells	PBMC : peripheral blood mononuclear cells
dH₂O : distilled water	PBS : phosphate buffered saline
DIC : disseminated intravascular coagulation	PCR : polymerase chain reaction
DISC : defective infectious single-cycle	PSV-4 : quadrivalent plain polysaccharide vaccine
ELISA : enzyme linked immunosorbent assay	PWM : Pokeweed mitogen
ELISpot : enzyme linked immunoSPOT	RBC : red blood cell
ET : electrophoretic type	SAC : Staphylococcus aureus Cowan strain
FACS : fluorescence-activated cell sorter	SBA : Serum bactericidal activity (hSBA with human complement; rSBA with rabbit complement)
FDCs : follicular dendritic cells	TCR : T cell receptor
FMS : fulminant meningococcal sepsis	TD : T-dependent
GC : germinal center	TH : T-helper cells
GMT : geometric mean titre	TI : T-independent
Hib : <i>Haemophilus influenzae</i> de type B	TLR : Toll like receptor
Ig : immunoglobulin	TNF : tumor necrosis factor
IL : interleukin	
INF : interferon	
ISCOM : immune stimulating complex	
LOS : lipooligosaccharides	
LPS : lipopolysaccharides	
LT : lymphotoxin	

Liste des figures

Figure 1: Structure de la paroi de <i>N. meningitidis</i>	10
Figure 2: Worldwide serogroup distribution (2006)	16
Figure 3: Serum bactericidal antibody assay.....	20
Figure 4: Gram stain of CSF showing <i>N.meningitidis</i>	23
Figure 5: TH cell-regulated B cell memory development	28
Figure 6: Types of active vaccines	29
Figure 7: Persistence of Ag-specific IgG memory B cells prior to vaccination	47
Figure 8: Persistence of Ag-specific IgG plasma cells prior to vaccination.	47
Figure 9: Kinetics of the response of MenC-specific IgG plasma cells after MenC- and Hib- conjugate vaccines.....	48
Figure 10: Kinetics of the response of CRM ₁₉₇ -, diphtheria toxoid-, Hib-, tetanus toxoid- specific IgG plasma cells after booster dose of MenC- and Hib- conjugate vaccines.	49
Figure 11: Kinetics of the response of total IgG-plasma cells after booster dose of MenC- and Hib- conjugate vaccines.....	50
Figure 12: Kinetics of the response of MenC-specific IgG memory B cell response after booster dose of MenC- and Hib- conjugate vaccines	51
Figure 13: Kinetics of the response of CRM ₁₉₇ -, diphtheria toxoid-, Hib-, tetanus toxoid- specific IgG memory B cells after booster dose of MenC- and Hib- conjugate vaccines.....	52
Figure 14: Kinetics of the response of total IgG memory B cells after booster dose of MenC- and Hib- conjugate vaccines	53
Figure 15: Secondary immune response after booster dose of diphtheria vaccine	55
Figure 16: Diphtheria- and TT- specific memory B-cells during the first 90 days after a booster dose of diphtheria toxoid vaccine.....	55
Figure 17: Longevity of smallpox vaccine-specific B cell memory	55
Figure 18: Kinetics of the response of plasma cells specific for tetanus toxoid and for unrelated antigens after a booster dose of tetanus toxoid vaccine	57
Figure 19: Memory B cell and plasma cell differentiation	59
Figure 20: Mechanisms that sustain serum antibody levels following boosting	59
Figure 21: Plasma cell response and memory B cell response at different days after immunisation with 3 doses of MenCV at 2, 3, 4 months of age	61
Figure 22: MenC-plasma cell and memory B cell response following immunisation with MenCV in teenagers previously primed with MenCV.....	62

Liste des tableaux

Table 1: Virulence factors in <i>N. meningitidis</i>	18
Table 2: Indicators of FMS and a poor prognosis	24
Table 3: New UK vaccination schedule since 2006	36
Table 4: Effectiveness of MenC vaccine in UK (1)	37
Table 5: Effectiveness of MenC vaccine in UK (2)	38
Table 6: Schedule of visits for blood sampling	41
Table 7: ELISpot plate layout (1)	44
Table 8: ELISpot plate layout (2)	45

Chapter 1: Introduction au travail

1.1 Epidémiologie

La méningite bactérienne est encore aujourd'hui une cause importante de maladie invasive chez les enfants. Elle est causée par différents groupes de bactéries encapsulées, telles que *Neisseria meningitidis*, *Hemophilus influenzae* et *Streptococcus pneumoniae*. *N. meningitidis* est la seule capable de générer des épidémies de méningite.

N. meningitidis est l'une des principales causes de méningite bactérienne et de septicémie chez les enfants, en particulier entre 6 et 24 mois. Son taux d'incidence, entre les épidémies et dans les pays industrialisés, varie entre 1 et 3/100 000 habitants, avec un taux de mortalité de 10%. Dans les pays en voie de développement l'incidence dans les années non épidémiques est de 30/100000 habitants, 10 fois plus élevées que dans les pays industrialisés. Pendant les épidémies, le taux d'attaque peut atteindre 1000/ 100000 habitants avec un taux de mortalité entre 10 et 15% [1, 2]. Entre 10 et 20% des survivants développent des séquelles permanentes comme l'épilepsie, un retard mental ou un déficit neural sensoriel.

N. meningitidis est entouré d'une capsule polysaccharidique, qui est un facteur majeur de virulence. Les méningocoques sont classés en sérogroupes, sérotypes et séro sous-types sur la base de différences antigéniques portant respectivement sur leurs polysaccharides capsulaires et sur leurs protéines de membrane externe. Des 12 sérogroupes existants, la plus grande partie des infections est causée par les sérogroupes A, B, C, W135.

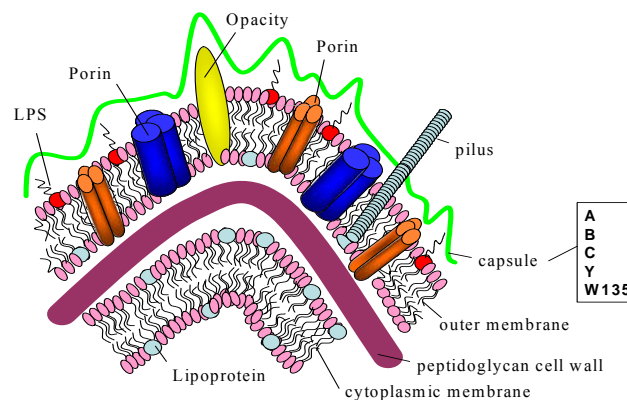


Figure 1: Structure de la paroi de *N. meningitidis*

Le séro groupe A cause des épidémies à large échelle dans les pays en voie de développement mais reste rare en Europe et en Amérique du Nord. Il est la cause majeure des méningites épidémiques et endémiques dans l'Afrique sub-saharienne. Les trois quarts des cas touchent les jeunes de moins de 15 ans. Les épidémies à séro groupe A surviennent tous les 5-12 ans, durent 2-3 ans, avec des pics en mars et avril, à la fin de la saison sèche, et disparaissent pendant la saison des pluies.

Le séro groupe B est la cause la plus importante de méningite endémique dans les pays industrialisés, responsables de 30-40% des cas en Amérique du Nord et 30-80% des cas en Europe. Le reste des cas est causés par le séro groupe C en Europe et C et Y en Amérique du Nord [1, 2].

1.2 Immunité

La protection contre les méningocoques repose sur la présence d'anticorps bactéricides induits par le portage de souches apparentées, non pathogènes. L'acquisition lente et progressive de ces anticorps explique la vulnérabilité particulière des enfants. Des anticorps bactéricides peuvent également être induits par la vaccination. Il existe actuellement 2 types de vaccins : les vaccins polysaccharidiques et les vaccins conjugués.

Vaccins polysaccharidiques contre les méningocoques

Une immunité à court terme contre les infections à méningocoques des sérogroupes A, C, Y et W135 peut être obtenue par des vaccins à base de préparations polysaccharidiques.

Le vaccin confère une protection de courte durée (maximum 3 ans) et est très peu immunogénique chez les enfants de moins de 18 mois, qui constituent 25% de tous les cas.

La situation épidémiologique de certains pays, comme l'Angleterre, l'Irlande, l'Espagne, la Hollande, la Belgique, l'Australie, le Canada, et le sud de la France au début des années 90, qui ont connu une augmentation de l'incidence des maladies méningococciques associée à de nouveaux clones du séro groupe C, a justifié l'instauration de campagnes de vaccination systématique contre le séro groupe C. Le vaccin contenant la capsule polysaccharidique pure du méningocoque du séro groupe C (un antigène T-indépendant), a d'abord été utilisé dans ces campagnes, mais il n'induisait pas de protection chez les enfants, ni de mémoire immunologique.

L'ontogenèse de la réponse immunitaire aux polysaccharides est encore mal comprise. C'est un processus tardif qui n'est terminé que vers l'âge de 24 mois. Au contraire, la production d'anticorps contre les antigènes protéiques est effective dès les premiers mois de vie, avec des taux d'anticorps contre les antigènes des vaccins contre le tétanos ou la diphtérie, qui après plusieurs doses de vaccin avoisinent les valeurs adultes mêmes chez les jeunes enfants. Les processus connus, caractérisant la maturation différée de la réponse immunitaire aux antigènes polysaccharidiques par rapport aux antigènes protéiques sont [3] un taux réduit de l'expression du récepteur au complément CD21/CR2 sur les lymphocytes B des enfants, une limitation de la production des anticorps spécifiques contre les antigènes polysaccharidiques (IgG2) pendant les 2 premières années de vie, une faible activité du complément limitant le dépôt de C3d sur les polysaccharides, et l'immaturité de la zone marginale de la rate.

Vaccins polysaccharidiques conjugués contre les méningocoques

Pour améliorer les réponses vaccinales, la capsule polysaccharidique a été conjuguée à une protéine porteuse, devenant ainsi un antigène T-dépendant capable d'induire une réponse immune dès l'enfance et de produire une immunité mémoire.

Le vaccin méningococcique du séro groupe C conjugué induit des anticorps anti-polysaccharidiques spécifiques qui semblent corrélés avec la protection contre la maladie. Le corrélat de protection est un taux géométrique moyen de l'activité bactéricide sérique ≥ 8 utilisant le complément du lapin et ≥ 4 utilisant le complément humain [4].

La protection à long terme induite par les vaccins polysaccharidiques conjugués résulte de facteurs divers, tels que la réduction de la circulation des bactéries liée au phénomène de l'immunité de groupe (« herd immunity »), la persistance des anticorps anticapsulaires dans le sérum, la mémoire immunologique qui est caractérisée par l'induction d'une réponse sérologique secondaire

rapide lors de la nouvelle rencontre avec l'antigène spécifique.

Des études de surveillance de la persistance de la protection vaccinale, réalisées après l'introduction du vaccin conjugué contre le sérotype C en Angleterre, ont suggéré que l'efficacité du vaccin était excellente (>90%) pendant un an. Cependant cette efficacité est faible déjà une année après une immunisation primaire réalisée (avec une série de 3 doses) pendant la première année de vie, ou après un peu plus d'une année pour les enfants plus âgés ayant reçu une seule dose de vaccin dans leur seconde ou troisième année de vie [5, 6]. D'autres études ont également rapporté une chute rapide de la protection sérologique et du taux d'anticorps bactéricide, malgré la persistance d'un priming immunologique dans une période de temps similaire [6, 7].

Malgré la chute rapide du taux des anticorps sériques dans le sang, la mémoire sérologique - définie par le développement rapide d'un taux élevé d'anticorps de haute affinité après une dose de rappel de polysaccharides - peut être observée jusqu'à plusieurs années après une primo-immunisation.

Cette mémoire immunologique a été considérée critique pour une protection prolongée et pour cette raison, la preuve d'une mémoire immunologique est un pré-requis régulateur pour l'enregistrement des vaccins conjugués.

1.3 Contribution des anticorps et de l'immunité mémoire à la protection

La compréhension des contributions relatives de la persistance des anticorps et de la mémoire immunologique, pour la protection à long terme induite par les vaccins polysaccharidiques conjugués est essentielle pour améliorer le choix des formules et des calendriers vaccinaux.

Des études sur l'efficacité du vaccin conjugué anti-Hib ont démontré que certains enfants développaient des infections à *H. influenzae* malgré une mémoire immunologique évidente, par la production rapide des anticorps spécifiques lors d'une maladie invasive à *H. influenzae*, indiquant que la mémoire immunologique n'était pas suffisante pour la protection à long terme de certains individus [8, 9].

Différentes études sur la cinétique de la montée des plasmocytes et des anticorps après une rencontre secondaire avec un antigène ont démontré que lors d'une réponse immune secondaire, les plasmocytes mettaient environ quatre jours avant d'apparaître dans le sang. De ces observations, on comprend que lorsqu'un individu primo-immunisé acquiert une souche invasive de *N. meningitidis*, la protection apportée par les anticorps nécessite quelques jours, alors que le méningocoque peut envahir le sang et la maladie se développer en quelques heures seulement. Dès lors, la protection doit dépendre avant tout des mécanismes de l'immunité innée plutôt que des mécanismes de l'immunité mémoire.

Les processus cellulaires qui se cachent derrière ces observations ne sont pas bien définis chez l'homme et ont très peu été étudiés chez l'enfant. Les anticorps sont produits par les plasmocytes qui ont migrés des centres germinaux à la moelle osseuse après une stimulation antigénique naturelle ou par un vaccin. Les plasmocytes peuvent survivre jusqu'à plusieurs mois dans des « niches » spécifiques de la moelle osseuse et continuent à sécréter des anticorps pendant une durée de temps variable selon les études. Les cellules B mémoires sont produites après une stimulation antigénique T-dépendante, qui induit une réaction des centres germinaux. Elles circulent ensuite entre le sang et les organes lymphoïdes secondaires jusqu'à nouvelle rencontre avec l'antigène, où elles se différencient rapidement en plasmocytes, capable de sécréter des anticorps de haute affinité pour neutraliser le pathogène. Nous sommes capables de détecter les plasmocytes brièvement dans le

sang périphérique après immunisation avec des vaccins [10], cependant il n'est pas clair si le taux basal des anticorps est produit exclusivement par les plasmocytes à longue durée de vie localisés dans les niches de survie de la moelle osseuse, ou si les plasmocytes sont régulièrement remplacés par le turn over des cellules B mémoires.

Chez l'humain, les cellules B mémoires peuvent être détectées dans le sang périphérique jusqu'à plusieurs mois à années après l'administration de certains vaccins [11-13] et elles sont considérées comme source de base de la réponse immune secondaire à un antigène et de la persistance des anticorps [14-16]. Cependant pour tout antigène vaccinal il demeure incertain comment la réponse des cellules B mémoires dans le sang périphérique reflète la persistance des anticorps ou le priming [16]. Les cellules B mémoires apparaissent après les vaccinations et une sélection de clones de cellules B à haute affinité apparaît après des semaines à mois dans le pool mémoire. Il demeure incertain si la magnitude de la réponse des cellules B mémoires après une immunisation reflète l'importance et la durée de protection, ou si il y a une relation directe entre le nombre des cellules B mémoires produites pendant le priming, et la magnitude, la vitesse et la qualité de la réponse à la dose de rappel, une année plus tard.

Les cellules B mémoires ne sécrètent pas d'anticorps, et il est difficile de mesurer les cellules B mémoires spécifiques à des antigènes sans premièrement induire leur activation et différenciation en plasmocytes. Les analyses de laboratoire consistent dès lors en une stimulation polyclonale des cellules B mémoires durant un certain nombre de jours, en utilisant des combinaisons variées de mitogènes, pour induire leur différenciation en cellules productrices d'anticorps. Par la suite, il est possible d'identifier les cellules productrices d'anticorps spécifiques d'un antigène par la méthode ELISpot (enzyme linked immunospot assay).

Le but de ce travail est donc :

- de revoir la littérature et de résumer les connaissances actuelles concernant les méningocoques, leur pathogénèse, les maladies qu'ils provoquent, l'immunité qu'ils induisent, celle qu'il est possible d'induire au moyen des vaccins récemment devenus disponibles, les limitations des réponses vaccinales des jeunes enfants et les problèmes que cela pose, quant au choix des calendriers de vaccination en particulier;
- d'investiguer la persistance des plasmocytes producteurs d'anticorps et des cellules B mémoires spécifiques des méningocoques du séro groupe C, chez des enfants âgés d'une année ayant été primo-immunisés à l'âge de 2, 3 et 4 mois avec un vaccin conjuguant les polysaccharidiques méningococciques du séro groupe C à la protéine porteuse CRM₁₉₇;
- d'étudier la cinétique de la réponse immune secondaire des plasmocytes et des cellules B mémoire réactivées après une dose de rappel du vaccin conjugué, et d'observer comment les cellules induites par la dose de rappel persistent dans le sang, jusqu'à un mois après immunisation.

1.4 Références

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Chapter 2: Literature review

2.1 *Neisseria meningitidis*

2.1.1 Structure and classification

Neisseria 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.

The outer membrane contains lipooligosaccharide (LOS), which is composed of lipid A and a core oligosaccharide, but lacks the O-antigen polysaccharide found in most Gram-negative rods.

The organism is encapsulated with polysaccharide and antigenic differences in the polysaccharide capsule is the basis for serogrouping the bacterium. The structure of the oligosaccharide capsule of the serogroup A meningococci is composed of N-acetyl mannosamine-1-phosphate, whereas the serogroup B, C, Y and W135 capsules are composed of N-acetyl neuraminic acid (NANA). The serogroup B NANA is α 2-8 linked and serogroup C is α 2-9 linked. The serogroup Y capsule is modified with glucose and the serogroup W135 capsule with galactose.

Traditionally, meningococcal strains have been classified by serological typing based on antigenic variation of the capsular polysaccharide, identifying the sergroup; the PorB outer-membrane protein (OMP), identifying the serotype; the PorA OMP, identifying the serosubtype and the lipopolysaccharide (LPS), giving the immunotype [17]. Each of these characteristics can be determined using antisera and monoclonal antibodies.

Molecular methods of classification include multilocus enzyme electrophoresis (MLEE), that assesses the electrophoretic mobility of various, relatively conserved, cytoplasmic enzymes. Meningococci sharing identical MLEE profiles are identified as an electrophoretic type (ET). Alternatively at a genomic level, DNA multilocus sequence typing (MLST) assesses fragments of genes that code for cytoplasmic enzymes.

N. meningitidis has been divided into 13 serogroups: A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z. 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. In contrast, approximately 50% of the strains isolated from carriers lack capsule and therefore cannot be serogrouped [18]. Meningococci without capsules may possess the genes for capsule synthesis since capsule production in meningococcal strains can switch on and off at a high frequency [19]. Alternatively, the capsular operon may be absent - capsule null strains do not have the capacity to produce a polysaccharide capsule [18].

Population genetic studies using MLEE [20] have shown that the majority of invasive *N. meningitidis* strains worldwide belong to a limited number of clonal complexes, of which ET-5 and ET-37 complexes have played an important role in the northern hemisphere during the last two to three decades [21].

Later MLEE has been replaced by MLST, as the reference standard for characterisation of meningococci [22].

Genetic recombination takes place between meningococci, resulting in the emergence of new strains [23].

2.1.2 Epidemiology

Worldwide serogroup distribution

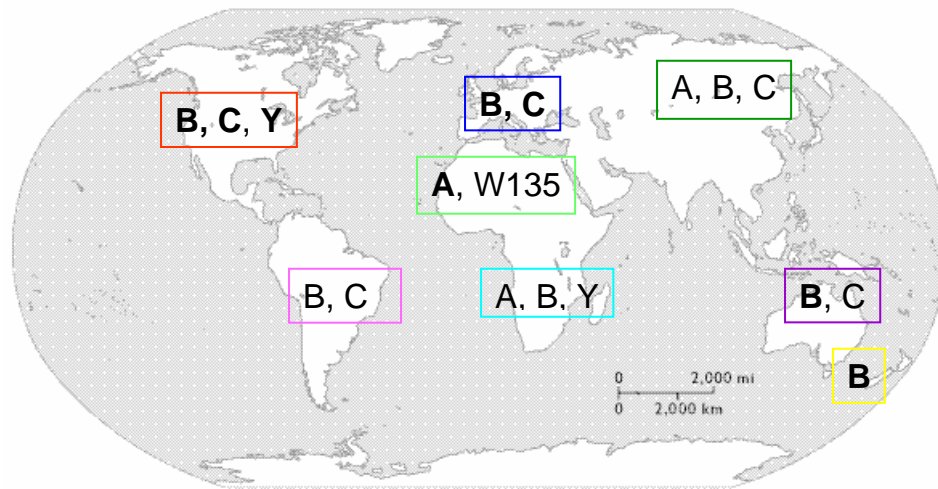


Figure 2: Worldwide serogroup distribution (2006)

Endemic meningococcal disease occurs worldwide, and epidemics are common in developing countries. Epidemics of meningococcal disease (MD) occur after the introduction of a new, virulent strain into an immunologically naïve population. Pandemics of disease have not been seen in developed countries since World War II.

Of the 12 serogroups, almost all infections are caused by serogroups A, B, C, Y, and W135. Serogroup A and W135 are epidemic in Sub-Saharan Africa. W135 has been associated with the annual Hajj pilgrimage. Serogroup Y is prevalent in USA. In Europe and the Americas, serogroups B, C, and Y predominate.

The incidence of MD varies from 1-3/100000 in most industrialized nations to 10-25/100000 in some resource poor countries. However, an incidence of 1000/100000 may be reached during severe epidemics in sub-Saharan Africa. These different attack rates reflect the different pathogenic properties of *N. meningitidis* strains and different socioeconomic, environmental, and climatic conditions. The case fatality rate (CFR) is also variable, between 5-10% in industrialized countries and 10-15% in developing countries.

In the late 90s, surveillance of meningococcal infection in Europe reported overall incidence of meningococcal disease of 1/100000 population. Northern European countries had a higher incidence of disease and an increasing predominance of serogroup C cases. Also, the age group tended to shift from younger children to teenagers. The CFR was 8.3% and the most common serosubtypes were B and C [24].

Following the introduction of a systematic MenC immunisation campaign in November 1999, surveillance of meningococcal disease in England, Wales and Northern Ireland indicated a fall in the incidence of MD from 9.2 to 8.0 per 100000 population with a decrease of cases caused by serogroup C and an increase of cases caused by serogroups B and W135. Between these 2 periods, the overall CFR was 5.8%, being higher for serogroup C (13.5%) than B (5.8%) [25].

2.1.4 Meningococcal carriage

Meningococci are obligate commensals in man and colonize the nasopharyngeal mucosa without affecting the host, a phenomenon known as carriage [26]

Humans are the only natural carriers of *N. meningitidis*. Exposure to *N. meningitidis* occurs by aerosol spread and mostly results in asymptomatic nasopharyngeal carriage. Studies of asymptomatic carriage of *N. meningitidis* have shown a carriage rate of about 1% of the human population [27] with variation in its prevalence, from less than 1% to almost 40% (in 20-to-24-year-old males) [28]. Around 10% of adults and up to 30% of teenagers carry this bacterium at any one time [29, 30]. Carriage is less common in infants and young children [28]. Infants more commonly carry *Neisseria lactamica*, a related but non-pathogenic organism. Also, infants acquire immunity to the meningococcus through repeated exposure to cross-reactive antigens present on this organism and other commensal bacteria.

The carriage rates are highest for school-age children, young adults, for lower socioeconomic populations (caused by person-to-person spread in crowded areas), and under conditions where people from different regions are brought together, such as military recruits, pilgrims, boarding-school students, or prisoners. Colonisation is also increased among active and passive smokers [28].

Carriage does not vary with the season, even though disease is most common during winter. The duration of the carrier state varies; it may be chronic, lasting for several months, intermittent or transient [29] and may be strain specific. Carriage is typically transient, with clearance occurring after specific antibodies develop.

Studies of the clones in asymptomatic carriers and in patients with meningococcal diseases have shown that hyperinvasive meningococci, like the ST-11 complex, were almost only found in meningococci causing disease and were very rare in carried meningococci [31].

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.

2.1.5 Virulence factors

N. meningitidis possesses several surface located or secreted molecules which influence adhesion, invasion and survival of the bacteria in human host. The capsule is thought to protect the meningococcus from dessication during transmission between hosts and also from antibody-mediated phagocytosis in the bloodstream. The pili and Opacity associated proteins mediate adherence to and invasion of host cells. 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. Antigenic variation or changes in expression of genes encoding outer membrane molecules also mediates meningococcal resistance against host immune defences.

Component	Function
Capsule	against host defence (complement -dependent bacteriolysis and phagocytosis)
Outer membrane protein	
<u>Porins (PorA /Por B)</u>	form pores or channels for nutrients to pass into the cell and waste products to exit; promotes intracellular survival by preventing phagolysosome fusion, resist to complement-mediated serum killing
<u>Opacity-associated proteins</u>	adherence to host cells
<u>Opa/Opc</u>	
Lipooligosaccharide LOS	lipid A has endotoxin activity; during rapid cell growth, the bacteria release outer membrane blebs, which enhance toxicity
Pili	attachment to host cells, transfer of genetic material, motility, and interference with neutrophil killing
IgA protease	against host defence (degrade IgA)
Transferrine binding protein	mediate acquisition of iron for bacterial metabolism (by direct link to human transferrine)

Table 1: Virulence factors in *N. meningitidis*

2.1.5 Pathogenesis

There are a number of conditions necessary for invasive disease; exposure to a pathogenic strain, colonisation of the naso-oropharyngeal mucosa, passage through that mucosa, and survival of the meningococcus in the bloodstream [32]. These processes are influenced by bacterial properties, climatic and social conditions, preceding or concomitant viral infections, and the immune status of the patient [2].

2.1.5.1 Colonization of the naso-oropharyngeal mucosa

Colonization occurs on the exterior surface of the mucosal cell and subsequently, intra- or subepithelially. Local or systemic immune status influenced by concurrent viral infections or damage to the integrity of the mucosae, perhaps by active or passive smoking or preceding, may also influence colonisation.

Pili are the major adhesins that permit the attachment of the bacteria to mucosal 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 [33]. Adhesion of meningococci to host cells leads to a transient up-regulation of PilC production and down-regulation of capsule synthesis [34] and removal of sialic acid from LOS, which seems essential for meningococcal interaction with host cells [26]. After initial attachment mediated by pili, closer binding to the host cell occurs via the class 5 OMPs Opa and Opc [2].

2.1.5.2 Invasion or penetration of the naso-oropharyngeal mucosa

Meningococci pass through the mucosal epithelium via phagocytic vacuoles as a result of endocytosis. During invasion, several bacterial factors change the metabolism of the mucosal cell. PorB moves into host cell membranes and affect the maturation of phagosomes [35] and IgA1 proteases inactivate specific IgA1 [36].

2.1.5.3 Survival of the meningococcus in the bloodstream

Meningococci can survive and proliferate in the bloodstream via bacterial virulence factors or deficit of the host defence. For example, the bacteria acquire iron from human transferrin by transferrin binding proteins [37]. The polysaccharide capsule protects against complement-mediated bacteriolysis and phagocytosis [38].

Host defence after meningococcal invasion depends on humoral and cellular responses of the innate and adaptive immune systems. Specific complement-fixing antibodies provide entire protection. However, because production of antibodies takes at least 1 week after colonization, the initial defence depends on the innate immune system [2]. Early innate defence are complement-mediated bacteriolysis and opsonophagocytosis [2].

In healthy people, the incidence of MD is reciprocally related to the titre of specific antibodies, with the highest incidence of disease occurring from 6 months, after which maternal antibodies have waned, to 24 months of age [4, 39].

Throughout life, specific antibodies are induced by the repeated and intermittent carriage of different meningococci and *N. lactamica*. Certain enteric bacteria have a capsule that is structurally and immunologically identical to the capsular polysaccharide of meningococci (for example the capsule from *E. Coli K1* is identical to the serogroup B capsule). These bacteria participate in the defence against meningococci by the induction of cross-reacting antibodies [40].

In conclusion, growth of the meningococcus in the blood-stream can occur when intravascular killing is impaired, either because of special properties of the meningococcus or because of a naïve or defective immune system of the host (for example deficit in one of the terminal complement factors or variants of mannose binding lectin) [2].

2.1.6 Markers of immunogenicity in protection against meningococcal disease

The original correlate of protection was established by using a Serum Bactericidal antibody Assay (SBA) with human complement (hSBA). The SBA titre represents a measure of bacterial killing by functional antibody and is expressed as a dilutional factor, with titres $\geq 1:4$ predicting protection [4]. Today, because of a greater availability and degree of standardization, human complement has been replaced by rabbit complement [41] and a rSBA titre $\geq 1:8$ predicts protection. rSBA titres $< 1:8$ predict susceptibility and for licensing MenC vaccine, rSBA titre should be more than 1:16. Protection correlates with an rSBA $\geq 1:128$ [41].

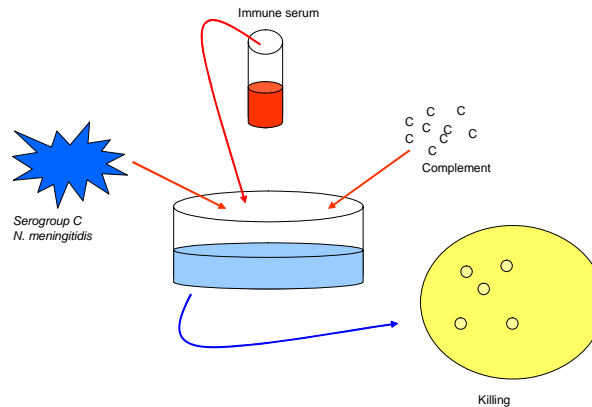


Figure 3: Serum bactericidal antibody assay

The importance of serum bactericidal antibodies in protection against group C meningococcal disease was first demonstrated during an epidemic among military recruits in the 1960s. Group C bactericidal antibodies were present in baseline sera of about 82% of the recruits. The subjects with detectable bactericidal antibodies in serum became carriers of the epidemic strain but did not develop MD, while all cases of disease occurred in the 18% of individuals whose baseline sera lacked bactericidal activity (hSBA titres < 1:4) [4, 39]

The approach adopted in the 1960s by Goldschneider *et al.* was the individual based correlate of protection. They measured pre-exposure antibody levels in all vaccinated subjects and looked at the relation of these levels to whether the subjects subsequently develop MD, to identify a threshold level in the individual that predicts protection.

In a vaccine efficacy trial, a population-based correlate of protection is used. It looks at the level of antibody acquired by the majority of vaccinated individuals and not acquired by the majority of unvaccinated individuals to establish a cut off of antibody level to predict protection. The rSBA cut-offs $\geq 1:16$ have been established in the UK using this approach in a vaccinated population [41].

The presence of SBA is a reliable marker of protection against MD 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 antibodies at concentrations that are insufficient to elicit bactericidal activity but that are sufficient to confer protection via opsonophagocytosis [42]. The presence of immunologic memory is also important, as illustrated both by the response to a polysaccharide challenge and by the maturation of antibody avidity in the months following primary immunisation [43].

2.1.7 Meningococcal diseases

2.1.7.1 Clinical manifestations

Different disease manifestations can develop after meningococci have reached the blood stream; transitional asymptomatic bacteraemia, focal infection of the central nervous system, joint, bone or heart, septic shock (meningococemia), or purpura fulminans (fulminant meningococcal sepsis) [2].

Sepsis (severe infection with evidence of systemic response, characterised by tachycardia, tachypnoea, hyperthermia or hypothermia) progresses into sepsis syndrome (sepsis plus evidence of altered end-organs perfusion with at least one of the following: acute changes in mental status, oliguria, elevated lactate and hypoxaemia) and finally into septic shock (sepsis syndrome with hypotension requiring fluid resuscitation and/or vasopressor support) [44].

The two principal manifestations of MD are rapid onset meningitis or severe sepsis (meningococemia). The mortality rates are very high, and range from 4-40%. Meningitis has a mortality rate of 4-6%, and septic shock of up to 40%. [45]. 10-20% of survivors develop permanent sequelae, such as deafness, mental retardation, and amputation [46]. Rare manifestations of MD are septic arthritis, pneumonia, purulent pericarditis, conjunctivitis, otitis, sinusitis, and urethritis. A rare syndrome, chronic meningococemia, may present as fever, rash, joint aches and headache during several weeks [46].

In patients with a low level of bacteraemia, meningococci are cleared spontaneously. This transient meningococemia is characterized by a short febrile flu-like episode.

2.1.7.2 Systemic inflammation response

The role of the systemic inflammatory response in patients with MD is to neutralize the bacteria and their toxic products, but it can also overreact and provoke serious tissue damage to the host. Three pathways characterize the intravascular inflammatory response. The first is the complement system, which contributes to phagocytosis of the bacteria, and induces the inflammatory reaction via C3a and C5a. The second is the coagulation and fibrinolysis pathway. Both pathways are stimulated in MD and result in prothrombotic state. The third is the inflammatory response mediated by different cytokines and chemokines, among which tumor necrosis factor (TNF) α and interleukin (IL) 1 β have a central role [45].

Pro-inflammatory mediators

During growth and lysis of meningococci, endotoxin is released as vesicular outer membrane structures (blebs) consisting of up to 50% of LOS, and OMPs, lipids, and capsular polysaccharides [47, 48] The level of cytokine produced in response to endotoxin varies among individuals.

The principal proinflammatory cytokines produced are TNF- α and IL-1 β , which act by different mechanisms; induction of other cytokines, activation of neutrophils and leukocytes, increased adherence of PMN and monocytes to endothelium, generation of prostaglandins and production of nitric oxide. The release of other mediators (IL-6, IL-8, IL-12 and IFN- α , GM-CSF, IL-10 and IFN- γ) is triggered by LPS, TNF- α , and IL-1 β [49]. In general cytokine levels correlate with disease severity and risk of death [50].

Complement

The complement system plays a key factor in defense against the meningococcus, by lysis of the bacteria, increase of phagocytosis by monocytes or Polymorphonuclear leucocytes (PMNs) or neutralization of endotoxin [51]. In the early stage of invasive disease, complement is activated principally through the alternative pathway and partly by the classical or mannose-binding lectin pathway. The degree of complement activation correlates with the severity of shock [52].

Endothelial damage and capillary leakage

Hypovolemia seems to be the most important event creating shock and results from increases in vascular permeability. The immune response induced by meningococci in the bloodstream provokes a major increase of permeability of the endothelium in all vascular beds via circulating mediators of inflammation (TNF- α , IL-1, IL-8, PAF, leukotrienes, thromboxane etc.) and the adherence of neutrophils and platelets. Increased vascular permeability leads to profound interstitial edema with diffuse parenchymal cell damage and hypovolemia. [44].

The hallmark of invasive meningococcal disease are skin hemorrhages. Microscopically, these lesions are characterized by endothelial damage and haemorrhages around small vessels that contain microthrombi. These lesions reflect the vasculitis induced by endotoxin and cytokine. Clinically, they are the visual manifestations of disseminated intravascular coagulation (DIC) and consumption coagulopathy. Even if DIC affects all organs, the adrenals are more vulnerable. Adrenal haemorrhages, diagnosed post-mortem as Waterhouse-Friederich syndrome, can provoke a transitory adrenal insufficiency.

2.1.7.3 Meningitis

The mechanisms by which meningococci invade the meninges and pass across the blood-brain barrier are poorly understood. Once present in the subarachnoid space, as humoral and cellular host defence are absent, meningococci proliferate in an uncontrolled way. Liberation of endotoxin provokes the activation of pro-inflammatory cytokines, which increase the permeability of the blood-brain barrier and contribute to the development of clinical manifestation of meningitis.

The major difference between sepsis and meningitis is that in meningitis the inflammatory response is localized 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. 8-20% of survivors suffer from neurological sequelae, varying from sensorineural deafness, mental retardation, spasticity, and/or seizures to concentration disturbances.

Since the skull cannot expand, cerebral oedema results in increased intracranial pressure, impeded cerebral perfusion and can lead to brain stem herniation. The 1-5% mortality rate associated with meningococcal meningitis is mostly caused by this rapidly fatal complication.

2.1.7.4 Fulminant meningococcal sepsis

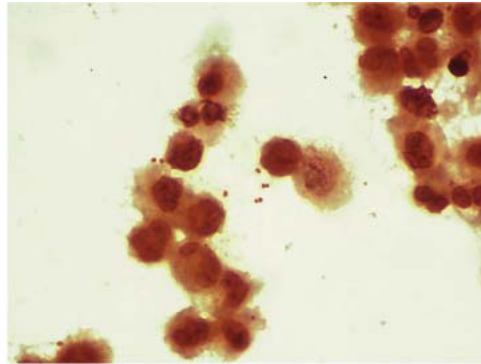
Within a few hours, fulminant meningococcal sepsis (FMS) may develop without signs of meningitis; this condition is characterised by high concentrations of endotoxin and cytokines in plasma. FMS is characterized by shock and DIC. Shock results from decreased vascular tone and hypovolaemia from capillary leak. The mortality rate of FMS is high and varies from 20-80% in different studies. Clinical deterioration is rapid and half of the patients who die will do so within 24 hours after the first clinical manifestation.

2.1.7 Diagnosis

Microscopy

Gram stain of cerebrospinal fluid is sensitive and specific but is of limited value for blood specimens, because most patients have too few organisms in their blood (except those with

overwhelming sepsis). Diagnosis in patients with FMS is also possible with a Gram stain of a skin lesion biopsy specimen. In patients with meningococcal meningitis, skin lesions rarely reveal the bacteria and only CSF samples are positive. Positive result of Gram stain can provide evidence of infection, even if cultures are negative [53].



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Figure 4: Gram stain of CSF showing *N.meningitidis*

Culture methods

N. meningitidis is generally present in large numbers 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. When the patient is already under antibiotic therapy, only cultures of skin biopsy specimens will reveal the bacteria. Definitive identification of *N. meningitidis* from the different *Neisseria* species is based on the mode of oxidation of carbon-hydrates. Various carbonhydrates (glucose, maltose, lactose, and sucrose) are added to different culture medium and as different members of *Neisseria* produce acid from different carbonhydrate, the production of acid from oxidation of glucose and maltose will allow the identification of *N. meningitidis* [53, 54]

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 [54].

Serological assays

Antisera for the principal disease-causing serogroups of *N. meningitidis* are available. Agglutination will occur with the serogroup corresponding to the antisera. A negative control is performed with saline and no 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 enough specimens to give reliable results [54].

Nucleic Acid Amplification

Polymerase chain reaction (PCR) assays to detect meningococcal DNA in blood or cerebrospinal fluid (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. Sensitivity and specificity of PCR assays are high (91%), however false-positive results can occur due to contamination (assay design is therefore of

paramount importance) and false negative results can be seen with presence of inhibitor in CSF. PCR assays allowing determination of capsular status are also available [55, 56].

2.1.8 Monitoring and Treatment

During the first few hours, patients should be monitored closely because shock may develop after the start of antibiotic treatment. The early signs of shock development are low diastolic blood pressure, delayed capillary refill, cold extremities, and tachycardia.

The progression of DIC can be monitored by the change in the number and size of skin lesions and by the platelet count.

General agreements concerning therapy are:

- Early recognition.
- Early start of parenteral antibiotic therapy, which should not be delayed by diagnostic procedures.
- Repeated prognostic evaluations.
- Rapid fluid resuscitation, start of mechanical ventilation, and transfer to an intensive care unit for patients with poor prognostic signs or shock (Table 2).

the extremes of age
a short period between onset of disease and admission
the absence of meningitis
progressive or diffuse skin lesions
shock (characterized by slow capillary refill, cold acra, hypotension, or metabolic acidosis)
the absence of leucocytosis, the presence of thrombocytopenia, DIC, and hypofibrinogenemia
a normal C-reactive protein concentration in serum or moderately elevated

Table 2: Indicators of FMS and a poor prognosis

However, treatment of shock and the use of glucorticoid, fresh-frozen plasma, plasma exchange, and other immunomodulating or adjuvant therapies all are subject to debate.

Normally, penicillin is used in the treatment of meningococcal disease and in occasional cases, a broad-spectrum cephalosporin or chloramphenicol can be used.

There is no way to eradicate carriage of *N. meningitidis*. For this reason, efforts have been concentrated on the prophylactic treatment of close contacts of diseased patients and on the induction of immunity to the five serogroups most commonly associated with disease.

Sulfonamides and penicillin are ineffective in eliminating the carrier state, chemoprophylaxis for contact is with rifampicin, ciprofloxacin, or ceftriaxone.

2.2 Review of the Immune system

2.2.1 Innate immunity

Innate or natural immunity is the first mechanism of defence against microbes. Components of natural immunity, which include physical and chemical barriers, complement proteins and various phagocytes and natural killer cells, are in place before infection and are stimulated by structures

shared by groups of microbes. Innate immunity does not induce memory.

Marginal zone (MZ) B cells also contribute to innate immunity. MZ B cells are a distinct subset of B cells that respond mainly to polysaccharides and produce IgM. Macrophages located in the MZ of lymphoid follicles in the spleen are particularly efficient at trapping polysaccharides which persist for prolonged periods on their surface and are recognized by MZ B cells [57]. MZ B cells express high levels of CD21 that help them to attach to complement coated polysaccharides [58]. MZ B cells have been functionally linked to immune responses against T-independent antigens because the spleen appears to be essential in these responses [59]. Patients having undergone a splenectomy have an increased susceptibility to encapsulated bacteria and in children younger than two years, the immune response against encapsulated bacteria is not effective because their marginal zone is immature.

It has been observed that generation of somatic mutations in splenic MZ B cells does not seem to require exposure to the antigen, because mutations are already present in very young infants. It has been proposed that MZ B cells in humans appear first as naïve non-mutated B cells, but then rapidly diversify by somatic mutation, either in response to environmental antigens or by a spontaneous developmental process [57, 60]. In contrast to rodents, human MZ B cells appear to re-circulate, accounting for 10 to 30% of the B-cell pool in blood and in spleen and contain mutated immunoglobulin genes. It is believed that immune responses to TI antigens do not generate immune memory or affinity maturation and protection only lasts for approximately 6 months. MZ B cells can switch after activation and differentiate rapidly into plasma cells that last for a few months, representing an immediate line of defence without the characteristics of immune memory.

Natural antibodies might be considered as a component of innate immunity. They are made in response to TI-antigens and are apparently produced without requiring exposure to antigens. Most natural antibodies are low affinity anti-carbohydrate antibodies, that could be produced by MZ B cells and/or B-1 peritoneal B cells (stimulated by bacteria that colonise the gastrointestinal tract) [61]. Plasma cells of the B1 lineage produce antibodies that bind with low affinities to multiple antigens, often microbial structures shared by a variety of pathogens [62]. These antibodies are usually of the IgM, IgA, or IgG3 subclass. Prenatal induction of natural antibody secretion contributes to the innate component of serum antibody levels, which seems to be maintained throughout life. Two mechanisms are thought to drive B1 lymphocytes to differentiate into plasma cells, stimulation by microbial antigens and /or mitogens of the gut flora and stimulation by autoantigens. The postulation of the continuous activation of B1 cells by bacterial antigens, mitogens and autoantigens imply that plasma cells of the B1 lineage should be short-lived and constantly replaced by newly formed plasma cells [63].

2.2.2 Adaptive immunity

Adaptive immunity is characterised by a higher specificity, the induction of memory and the ability to respond more vigorously to repeated exposure to the same antigen. Adaptive immunity is composed of humoral immunity and cell-mediated immunity. We distinguish two types of antigens: T-dependent (TD) antigens which are principally protein antigens and require the contribution of T helper (TH) cells to activate B cells and T-independent (TI) antigens, which are non protein antigens, such as polysaccharides and lipids and activate B cells in the absence of TH cells but which are unable to induce immunological memory. Antibodies produced in the absence of T cell help are of low affinity and consist mainly of IgM with limited isotype switching to some IgG subtypes.

The most important TI antigens are polysaccharides, glycolipids, and nucleic acids. These

antigens cannot be processed and presented with MHC molecules, and therefore cannot be recognized by TH cells. Most TI antigens are polyvalent, in that they are composed of multiple identical antigenic epitopes that cross-link multiple membrane immunoglobulins (Ig) molecules on a B cell, leading to activation without T cell help. In addition many polysaccharides activate the complement system by the alternative pathway, generating C3d which binds to the antigen and provides another signal for B cell activation.

TI antigens can be divided into two classes, TI-1 or TI-2 [64].

TI-1 antigens, such as bacterial lipopolysaccharide, are potent B-cell mitogens and produce a non-specific, polyclonal activation of B cells. At high concentrations these molecules induce the proliferation and differentiation of most B cells in the absence of specific antigen binding to surface immunoglobulin. At low concentrations, only B cells specific for the TI-1 antigen bind to it and become activated. TI-2 antigens consist of highly repetitive structures such as capsular polysaccharides from bacteria. Unlike TI-1 antigens, these antigens can only activate mature B cells because immature B cells are inactivated by repetitive epitopes. This could explain why infants do not make antibodies efficiently against polysaccharide antigens, as most of their B cells are immature. The molecular mechanism of the immune response to TI-2 antigens is not completely understood. It is known that certain subjects do not respond well to TI-2 antigens, such as asplenic patients, HIV seropositive patients, children (<2 years of age) and the elderly (adults >65 years of age) [65].

2.2.3 Secondary immune response and memory immunity

Immunological memory is the ability of the immune system to respond more rapidly with high avidity switched antibodies, to a second challenge with the same organism or vaccine than to the first challenge. The induction of a secondary antibody response after revaccination with the same antigen is named immunological priming. Like immunological memory, immunological priming can only be induced by T-dependent antigens.

Primary and secondary antibody responses to protein antigens differ qualitatively and quantitatively. Primary responses result from the activation of naïve B cells whereas secondary responses are due to stimulation of expanded clones of memory B cells. Therefore, the secondary response occurs more rapidly and large amounts of antibodies are produced. Heavy chain isotype switching and affinity maturation also increases with repeated exposure to protein antigens. Antibodies are produced within 10-14 days after vaccination in the primary immune response and within 4-6 days in the secondary immune response [66, 67]. The primary immune response requires the presence of an antigen, optimally with adjuvant (for protein antigens), but secondary immune responses can be induced with lower doses of antigens, and possibly without adjuvant.

2.2.4 Long term protective immunity

In the absence of antigen, memory B cells carrying somatically mutated Ig genes survive in secondary lymphoid organs, such as the lymph nodes and spleen, in the absence of Ag and mediate secondary immune responses upon re-challenge. About 40% of all circulating human B cells are memory B cells. Plasma cells are terminally differentiated non-dividing cells that home to the spleen and bone marrow and are the main source of antibodies which they secrete at a high rate.

Lymphocyte recirculation and migration to particular tissues, depends on cell surface adhesion molecules on lymphocytes, endothelial cells, the extra-cellular matrix and chemokines produced in the endothelium and in tissues. Survival of plasma cells is dependent on signals provided in a limited number of survival niches situated in the bone marrow. Stromal cells found in the bone marrow provide these signals and protect plasma cells from apoptosis. The number of these survival niches seems to be limited because the frequencies of plasma cells in bone marrow are constant at about 0.5% throughout life and in different species [63].

Long-lasting specific antibody responses come from antibodies produced by ASCs differentiated from long-lived memory B cells upon re-exposure to a pathogen and by long lived plasma cells homing in bone marrow survival niches [68].

The presence of pre-existing neutralising and/or opsonising antibody is the only way to prevent an infection and is the most important protective mechanism against many viral and most microbial infections. Following the introduction of routine Hib or MenC immunisation in infants, it was clear that some individuals with low or undetectable antibody concentrations were still protected against disease and this protection was attributed to herd immunity and priming [67]. However, occasional failures of protection have been reported in primed individuals with low baseline antibody level [5, 7], demonstrating that low or undetectable levels of specific antibody may leave an individual susceptible to rapid invasion before the primed response develops.

The T-dependent activation of memory B cells progress in a cascade of cellular development: [14] (Figure 5) that includes 5 phases :

- Phase I: MHC-II complexes presented on activated antigen presenting cells (APCs) are critical for effective TH cell selection, clonal expansion, and effector TH cell function development.
- Phase II: Cognate effector TH cell-B cell interactions then promote the development of either short-lived plasma cells (PCs) or germinal centres (GCs).
- Phase III: These GCs expand, diversify, and select high-affinity antigen-specific B cells for entry into the long-lived memory B cell compartment.
- Phase IV: Upon antigen re-challenge, memory B cells rapidly proliferate and differentiate into PCs under the cognate control of memory TH cells.

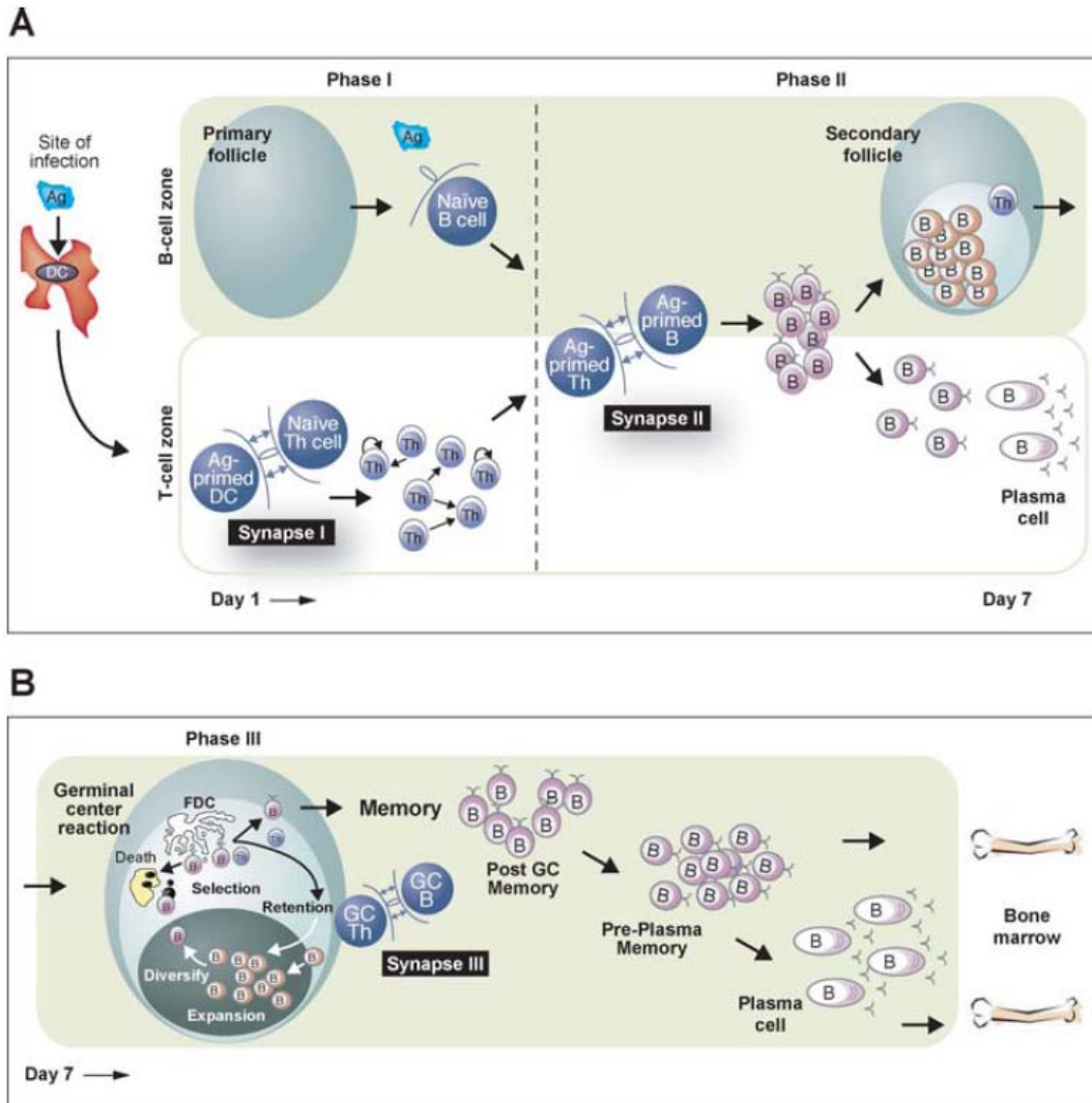


Figure 5: TH cell-regulated B cell memory development [14]

2.3 Antimicrobial Vaccines

The immunisation of a subject can prevent or diminish the serious symptoms of disease by blocking the spread of a pathogen at its target organ or at the site of infection. The immunisation of a population, like individual immunity, also stops the spread of the infectious agent by reducing the number of susceptible hosts: herd immunity. Active immunisation is characterised by the induction of an immune response. It can be stimulated after contact with an environmental pathogen (natural immunisation) or through exposure to microbes, or their antigens contained in vaccines (artificial immunisation). Active immunisation has the potential to induce memory immunity. Therefore, with each re-exposure to the pathogen, the immune response is faster and more effective in neutralizing the microbe and increased serum antibody level. Classical vaccines can be subdivided into two groups including live vaccines, or inactivated-subunit-killed vaccines (Figure 6). These groups differ in the way they stimulate the immune system.

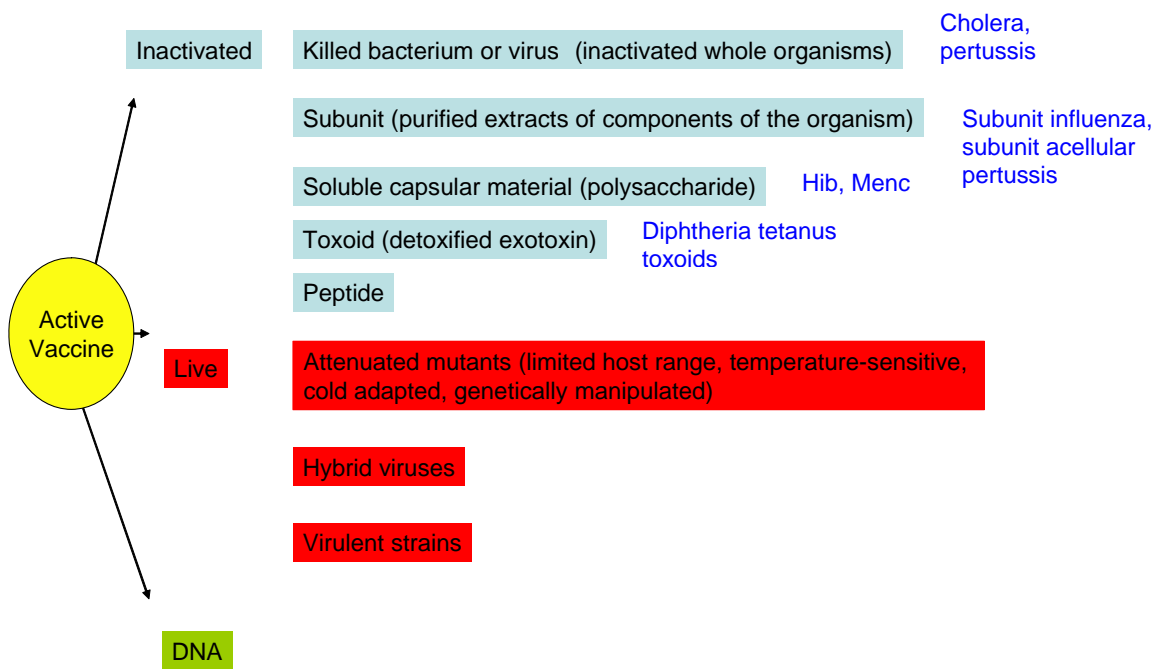


Figure 6: Types of active vaccines

2.3.1 Killed or inactivated vaccines

Inactivated or killed vaccines require a large amount of antigen to produce an antibody response. These vaccines are produced by inactivation of the pathogen either by chemical (e.g. formalin) or heat, or by purification or synthesis of the components of subunits of the infectious agents. They are usually administered with an adjuvant. Inactivated vaccines are used against bacteria and viruses that cannot be attenuated, or that can cause recurrent infection, or have oncogenic potential. Peptides representing part of an antigen can also be used as a vaccine. Their advantage is that the product is chemically defined, stable, safe, and contains only B cell and T cell specific epitopes. However, it is difficult to manufacture peptides with exact conformational epitopes and once in the body, peptides are very sensitive to proteolysis. Further, peptides are poor immunogens and induce only weak humoral and cellular responses [69].

Inactivated vaccines are generally safe, except in people who are allergic to products contained in the vaccine. For example, many antiviral vaccines are produced in eggs and therefore cannot be given to people who are allergic to eggs. The disadvantages of inactivated vaccines are that they only induce humoral immunity and they do not stimulate cell-mediated immunity and therefore are not able to generate memory immunity. As a result they require frequent booster immunisations and these vaccines cannot stimulate a local IgA response. However, with inactivated vaccines, there is no risk of reversion to the wild type infectious agent.

2.3.2 Live vaccines

Live vaccines are prepared using organisms that are limited in their capacity to replicate (e.g., avirulent or attenuated organisms). Live vaccines are very useful for protection against enveloped viruses, because they stimulate cell-mediated immune responses. Immunisation with a live vaccine mimics natural infection and therefore induces humoral, cellular, and memory immune responses. However, there are 3 major problems with these vaccines: the vaccine may still be dangerous for immuno-suppressed subjects or pregnant women who do not have the immune potency to combat even a weak infection. Further, the vaccine may revert to a virulent form, and the viability of the vaccine is difficult to maintain.

Live viral vaccines can be created with less virulent (attenuated) mutants of the wild-type virus or with viruses from other species that share antigenic determinants. Using genetic engineering, there is also the possibility to create vaccines with viruses that lack the virulence properties. Wild-type viruses are attenuated by growth in embryonated eggs or in tissue culture, at no physiologic temperatures (32°C to 34°C) and away from the selective pressure of the immune system. These conditions select mutant strains that are less virulent because they grow poorly at 37°C (temperature-sensitive strains and cold adaptive strains), cannot proliferate in human cells (host-range mutants), and cannot resist immune defences.

2.3.3 Future vaccines

Molecular techniques are being used to develop new vaccines. By genetic engineering, new live vaccines can be generated by the induction of mutations to delete or inactivate genes encoding virulence factors. These new techniques appear to be more reliable than random attenuation of the virus by passage through tissue culture. Hybrid virus vaccines can be formed when genes from infectious agents that cannot be easily attenuated can be inserted into safe viruses. A defective infectious single-cycle (DISC) virus vaccine is formed by a virus with a deletion of an essential gene that is grown in a tissue culture cell that expresses the defective gene.

In DNA vaccines, the genes coding for a protein that express an important B- and T-cell-specific viral or bacterial epitopes, is inserted to a plasmid, permitting the protein to be expressed in eukaryotic cells. Plasmid DNA is injected into muscle or skin, then taken up by dendritic cells, where the cDNA is transcribed and the immunogenic protein expressed, permitting the induction of a cell-mediated and humoral immune response. Attenuated viruses or bacteria, such as *E.Coli* may be used as vectors containing the plasmid.

Reverse vaccinology, which utilises genomic sequence data, is a new approach for the development of vaccines against the meningococcus [70]

2.3.4 Vaccine against T-independent antigens: encapsulated bacteria

Multi-serotype, polysaccharide-based vaccines have been used for more than 15 years against *S. pneumoniae* and *N. meningitidis*. These vaccines are able to protect against encapsulated bacteria by inducing complement-mediated bactericidal antibody and opsonophagocytosis [65]. The plain polysaccharide pneumococcal or meningococcal vaccines prevent invasive disease in immune-competent adults but not in children younger than two years of age. They do not induce high affinity antibody production or memory B cell formation because of the TI-2 nature of these vaccines. To resolve the problems associated with the TI nature of PS antigens, conjugate vaccines have been developed to convert the TI-2 immune response to a TD immune response. Other

strategies, namely anti-idiotypic antibodies, bacteriophage display libraries or DNA vaccines are being studying [65].

Conjugate vaccines

Conjugate vaccines are composed of purified capsular polysaccharide or oligosaccharide antigens covalently linked to a carrier protein, changing the polysaccharide to a TD antigen and increasing its immunogenicity. It is postulated that PS-specific B cells internalize the PS-carrier and that proteolysis of the carrier protein generate peptides that are presented in association with MHC-II molecules [71]. There is therefore an activation of T-cells and a germinal centre reaction, able to generate PS-specific plasma cells and memory B cells [72].

The first conjugate vaccine introduced for use in humans was against *Haemophilus influenzae* type B (Hib) in 1990 [73]. It induced an important fall of Hib disease that was attributed both to direct protection and to its capacity to reduce colonization of the nasopharynx by the bacteria. Therefore, it also reduced transmission in non-immunized subjects [74].

Vaccines against *Streptococcus pneumoniae* are complicated by the fact that there are over 90 different serotypes, each with a distinct capsular polysaccharide, and the fact that anti-capsular antibodies are serotype specific and therefore do not cross-react with other serotypes. However, a small number of serotypes cause the majority of pneumococcal disease. The first pneumococcal conjugate vaccine was licensed in humans in 1999 and this conjugate vaccine included seven serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F, and was conjugated to the mutant diphtheria toxoid protein CRM₁₉₇. The pneumococcal conjugate vaccine induced priming and subsequent boosting with the plain polysaccharide vaccine was able to generate high avidity IgG antibody responses [75]. Further, the pneumococcal conjugate vaccine reduces carriage of vaccine serotypes [76].

Conjugate vaccines exist for particular serogroups of meningococci and these are detailed below. Other conjugate vaccines are being developed against Group B Streptococci, *Salmonella Typhi*, *Escherichia Coli*, *Shigella sonnei* and *Shigella flexneri*.

2.4 Factors that diminish the vaccine efficacy in very young infants

With the exception of BCG, all infant vaccines require several doses to induce protection when they are administered before 6 months of life. This delayed induction of immune protection by vaccine is directly linked to the progressive maturation of their immune system [3].

2.4.1 Deficiencies of neonatal B cell responses to T-dependant antigens

In early life, studies in mice have demonstrated limited induction of the germinal centre, that was associated with a slow maturation of follicular dendritic cell (FDC) networks [77].

The reduced neonatal responses to TD antigens appear to be caused by the lack of an appropriate anatomical microenvironment for T-B cell interaction [63].

Even if T and B cells and white pulp of the spleen appear very early in human foetal development, the phenotype of the cells in the marginal zone remain immature until about two years old [78]. Lymphotoxin α (LT- α), LT- β and tumour necrosis factor have been identified as important signalling molecules for the development of the microarchitecture of secondary lymphoid organs

and for the formation of FDC networks [79]. Furthermore, B cells have been identified as the cells responsible for inducing the FDC network by secreting membrane LT [79]. The formation of GCs seems to depend on the interaction of B cells, T cells, FDC, and other APCs [80]. Molecules participating in the development of GCs include intercellular signalling proteins CD40 and CD40L, CD19, CD28, and B7-2. These molecules act at the level of the B cell/T cell interactions which are required for the maturation of B cells into GC cells.

Splenic B cells of neonatal mice are unable to upregulate expression of MHCII after BCR ligation, although this response is induced by IL4 or CD40 ligation. Therefore, neonatal B cells seem to give a weaker signal to T cells via their TCR, which is probably too low to activate T cells. In consequence, there is no induction of CD40 ligand (CD40L) and no functional T-B cell interaction can occur [81]. Also, there is no induction of the expression of B7.2 (CD86) on neonatal B cells, by BCR ligation and neonatal B cells are unable to give the costimulatory signals to T cells via CD28 [81].

In conclusion, neonatal B cells appear to be ineffective APCs. Furthermore in neonatal mice, macrophages and DCs are rare in the spleen and do not acquire antigen processing and presentation capacity until later in life.

2.4.2 Poor response to T-independent antigens

In infants and toddlers, antibody responses to most bacterial capsular polysaccharides are weak.

Some polysaccharide antigens can activate the complement cascade by the alternative pathway. The role of complement for protection against encapsulated bacteria is essential, and seems to act by directing the marginal zone B cells or APCs toward the antigens on the surface of the bacteria or by increasing B cell activation by inducing co-ligation of the BCR and CD21 [81]. However, there is a reduced expression of CD21/CR2 complement receptor on infant B cells, that impairs B-cell activation [82]. Furthermore, human neonates have low levels of C3, which only attain adult levels after one year of life [83]. These two limitations, with a low production of IgG2 antibody [84] and the immaturity of the splenic marginal zone [78] contribute to the poor response to T-independent antigen during the first two years of life.

It has also been demonstrated that the complement component C3 is important for the IgG response and for generation of GCs [85]. Therefore, low levels of C3 in neonates might contribute to the reduced induction of isotype switching and memory immunity that are observed in their response to TD and TI antigens [81].

2.4.3 Antibody production is of short duration

Several studies on early immunisation in the first year of life have shown that antibodies are induced but do not last, even if high titres have been produced after repeated doses of vaccine [5]. Recent studies in murine models suggest that this could be linked to the fact that bone-marrow homing of long-lived plasma cells is lower in early life [86]. Several studies have 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 BM surviving niches [86, 87]. Activation of plasma cells in bone marrow niches are dependent on stromal cells, which express adhesion molecules including CXCL-12, V-CAM-1 and BAFF and secrete different cytokines [88].

2.4.4 Qualitative differences in antibody responses

Qualitative differences characterise early life antibody responses compared to those generated later in life. IgG and IgA responses to viral and bacterial infections are very low in the first year of life. As a result, immunisation during the first year of life can increase IgG antibody response [3]. Furthermore, there is a predominance of IgM, caused by defective isotype-switching, due to limited T cell help. However, IgG rather than IgM antibodies are generated after vaccination, suggesting that repeated doses of vaccine are able to induce enough T cell help to activate B cell isotype switching. This switch is characterized by high levels of IgG1 and IgG3 antibodies, and low levels of IgG2 [84].

2.4.5 Interference of persisting maternal antibodies

Maternal antibodies also contribute to the reduced antibody response to vaccine in infants.

Mat-abs are able to inhibit the replication of live vaccine and to hide epitopes of inactivated vaccines impairing their recognition by B-cells. The infant response is also influenced by the “ratio Mat-ab/ag”. The effects of maternal antibody on the infant response to live and inactivated vaccines are influenced by the levels of these antibodies present at immunisation and the use of different vaccines in mothers and infants [89, 90].

Studies in mice and human infants have demonstrated that T cell responses and priming are not inhibited by Mat-ab [89] and therefore repeated doses of vaccine will induce infant antibody responses as soon as the Mat-ab titre is below the infant response threshold [90]. They have shown that high levels Mat-ab specific for the carrier protein, inhibit antibody responses to the carrier protein but do not interfere with T-cell helper responses and with the antibody response to polysaccharides [89].

Infant T cell responses are less affected by Mat-ab than B-cell responses. It is known that the introduction of a vaccine in a recipient with pre-existing passive antibodies induces formation of immune complexes of antigen-antibody. When a vaccine is introduced in a child who has pre-existing Mat-ab, these will bind to specific B-cell epitopes on the vaccine antigens and will impair the recognition of these epitopes by infant B cells. These antigen-antibody immune complexes will be taken and processed by infant APC and peptide will be presented on the surface of APC, inducing CD4/CD8 priming, despite the inhibition of B-cell responses.

The development of novel antigen delivery systems, such as biodegradable polymer microspheres or DNA vaccines, could prevent the inhibition of infant antibody responses by Mat-ab by prolonging immune stimulation. This could occur either by a progressive release of the vaccine antigen or by a continuing synthesis of the immunogenic antigen, allowing the Ma-ab to decrease and infant B cells to bind to the newly released or formed antigen.

2.4.6 Suboptimal T cell responses

It has been observed in several studies in mice that early life murine T-cells develop preferentially towards the TH2 pathway, i.e they respond better to viral/protein antigen. Studies on the *in vitro* maturation of T cells of umbilical cord blood have suggested that the TH2 bias of the neonatal response was not due to intrinsic properties of neonatal T cells [91]. The activation and maturation of neonatal CD4⁺T cells depends on the CD28-mediated co-signal, which influences the response to IL-12. High levels of stimulation by CD28 favour the production of type 1 cytokines such as IL-2, IFN- γ and TNF β , but diminishes the production of type 2 cytokines, such as IL-4 and

IL-13, by neonatal T cells.

Adkins *et al.* has suggested that neonatal T cells exposed to the antigen under particular conditions are able to generate adult-like TH1 responses. In mice this is associated with high levels of co-stimulatory signals, use of strong TH1-promoting agents and the reduction of infectious doses [92]. Other studies have demonstrated that both TH1 and TH2 cells were primarily generated in the neonate in lymph nodes, but differences with adults concerned kinetics of cytokine production and responses to adjuvant [93]. Neonates appear to be able to generate adult-like primary TH1 responses but the maintenance of TH1 cells seems impaired in early life and secondary immune response is therefore dominated by TH2 cells [93].

Clinical observations of disease caused by intracellular pathogens in early life suggest that both innate (NK cells, IL-12, IFN- γ production) and adaptive (CD8 T cells, CD4 TH1 cells) immunity, responsible for pathogen clearance and for elimination of infected cells, are impaired [3]. It has also been proposed that the weak T-cell responses could be due to the low number of T cells in the neonatal spleen compared with lymph nodes, where T-cell maturation seems to be faster [93].

2.5 Application to Meningococcal serogroup C (MenC) glycoconjugate vaccine

2.5.1 Meningococcal vaccines

2.5.1.1 MenC/MenA vaccines

The first meningococcal vaccines to be developed were the meningococcal plain polysaccharides against serogroups A and C in the late 1960's. These were immunogenic in adults and used in outbreak control but were poorly immunogenic in infants less than 2 years old and did not induce immunological memory (T cell independent response) [94]. Meningococcal conjugate vaccines were developed throughout the 1980s and the first human trials were conducted in 1991 [95] using meningococcal A and C capsular oligosaccharides conjugated to the mutant diphtheria toxoid protein CRM₁₉₇ (MenAC). This conjugate vaccine generated T cell helper responses, and was therefore able to induce isotype switching, affinity maturation and immune memory [96, 97].

2.5.1.2 MenC conjugate vaccine

The emergence of the ST11 clone bearing the C serogroup capsule concentrated efforts on the development of a serogroup C conjugate vaccine. A monovalent MenC-Crm197 glycoconjugate vaccine (MenCV) was therefore developed and in 1999, the UK was the first country to introduce MenC into its immunisation schedule where it was offered to all people younger than 18 years.

2.5.1.3 MenB vaccines

Serogroup B *N. meningitidis* causes around 50% of meningococcal disease cases worldwide [98], and is the only serogroup whose infection cannot be prevented by CPS (capsular polysaccharide) -based vaccines. Serogroup B CPS is a polymer of $\alpha(2-8)$ -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 [98]. An attempt to produce a conjugated vaccine using group B capsular PS, was developed by replacing the N-acetyl groups of the sialic acid residues with N-propionyl groups. However, the antibodies induced by this vaccine had no functional activity [99].

Therefore, because of the risk of induction of autoimmunity and because of the lack of immunogenicity, commercial development of PS-based group B vaccines was abandoned.

Alternative approaches have focused on other antigens, such as those present in the outer membrane vesicles (OMVs). Native OMVs consist of intact outer membrane and contain OMPs and LOS. One of the OMPs, PorA, was identified as a major inducer of serum bactericidal antibodies. This protein is expressed by almost all meningococci. However, there are a large number of PorA proteins which elicit variant specific antibodies that do not confer protection against meningococci with heterologous PorA variants[100].

Other OMPs, such as the transferring-binding protein [101], PorB, Opa, etc., are also being explored as possible meningococcal vaccine candidates. The successful development of a broadly protective Group B vaccine may come as a consequence of the sequencing of the meningococcal genome which will certainly contribute to develop a highly effective Group B vaccine, by helping to identify new potential antigens to be included in vaccine candidates [102].

2.5.1.4 Quadrivalent (group A, C, Y, W-135) vaccines

The quadrivalent plain polysaccharide vaccine (PSV-4) (GSK and Sanofi-Pasteur) has long been used for travellers over two years old in epidemic or endemics areas, and for professionals working with suspensions of meningococci. However, this vaccine is poorly immunogenic in young infants and children less than 2 years old and does not induce immunological memory. A quadrivalent polysaccharide meningococcal vaccine conjugated to diphtheria toxoid (MCV-4) (Sanofi Pasteur) has been developed and has been licensed in the US since January 2005 for use in adolescents and adults. Comparison of MCV-4 and PSV-4 demonstrated that they were both highly immunogenic and induced a greater increase in serum bactericidal antibody to the 4 serogroups. However, a three-year follow-up study reported that persistence of SBA and booster responses to MCV-4 was only observed in participants previously vaccinated with MCV-4 but not in those who had previously received PSV-4 [103]. Another study in the US tested the MCV-4 vaccine in infants, receiving 3 doses at 2, 4 and 6 months of age. In this study MCV-4 was only modestly immunogenic in infants (after the third dose, between 54 and 92% of infants had an rSBA titre of ≥ 1 in 8, depending on the serogroup and dosage given). Further, GMTs of rSBA against all serogroups elicited titres lower than those reported after a primary series of other conjugate C and AC meningococcal vaccines conjugated to CRM₁₉₇ and adjuvanted with Al(OH)₃. However, it appeared to induce priming in most infants, given three doses in infancy [104]. Quadrivalent conjugate vaccines are also being developed by Novartis vaccines and Glaxosmithkline biologicals.

2.5.2 Immunisation campaigns with MenC glycoconjugate vaccine

In the early 1990s, an increase in incidence rates and reported outbreaks due to serogroup C meningococci in some industrialised countries (North America, Canada, Spain, England and Wales, Scotland and Ireland, etc.) led to immunisation campaigns with MenC glycoconjugate vaccine in the UK and many other countries. The UK was the first country in 1999 to introduce MenC glycoconjugate vaccine and, immunisation was offered to all people younger than 18 years. The uptake rate was more than 70%. 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 toddlers (5-11 months age group) in a catch up campaign and a single dose in older children and adolescents (1-17 years) [6].

The UK vaccination schedule in 2005 was 3 doses of MenC glycoconjugate vaccine at 2, 3 and 4

months with a booster dose between 20 and 24 months old.

In 2006, this schedule has been modified as follows:

Birth	1 month	2 months	3 months	4 months	12 months	13 months	3-5y	11-12y	13-18y
		DTaP-IPV-Hib	DTaP-IPV-Hib	DTaP-IPV-Hib	Hib-MenC		dTaP-IPV		dT-IPV
		MenC	MenC						
						MMR	MMR		
	Pnc7		Pnc7			PnC7			

Table 3: New UK vaccination schedule since 2006 [105]

Ireland and Spain introduced MenC immunisation in 2000 with incorporation into their routine 2, 4, and 6 months schedule. The Netherlands, Belgium, and Australia introduced a single dose of MenC into the routine schedule at 12-14 months in 2002-2003. In Canada, the primary immunisation regimen varies from province to province; in Saskatchewan, only high-risk individuals are immunised; in Alberta, all infants are immunised at 2, 4, and 6 months of age; in the rest of the provinces, there is a 12-month single dose regimen. British Columbia did not introduced MenC with a mass immunisation campaign but added MenC to the routine immunisation schedule to coincide with pre-existing immunisations at either year 6 (age 11-12 years) or year 9 (age 14-15 years) [6]. Other countries, such as France, introduced MenC only in regions with higher incidence. In Switzerland, MenC is not obligatory, but is a complementary recommended vaccine. It should be administered at 12 months (1 dose) and between 11 and 15 years of age (1 dose). MenC is particularly recommended for high-risk groups, i.e. persons with underlying conditions as asplenia, immunodeficiency, diabetes, etc. It is also recommended for military recruits or close contacts to a suspected case of meningococcal infection

2.5.3 Effect of MenC introduction

2.5.3.1 Herd immunity and carriage after MenC introduction

A study by the UK Meningococcal Carriage Group [106] aimed to establish whether the mass immunisation campaign could result in herd immunity. They compared meningococcal carriage in students before vaccination (in 1999) with that of students of the same age, 1 year after mass vaccination. It was shown that carriage of serogroup C meningococci was reduced by 66%, indicating that the vaccine induced sufficient mucosal immunity to inhibit carriage of meningococci expressing the serogroup C polysaccharide [106]. A herd immunity effect has also been demonstrated by a 67% reduction in serogroup C MD for unvaccinated children when comparing 1998/1999 with 2001/2002 [107]. It is not known how long herd immunity will last and whether the reduction in nasopharyngeal carriage may eventually result in an increased individual susceptibility to serogroup C invasive meningococcal disease because of a lack of natural boosting of immunological memory through reduced carriage in older adolescents [106].

2.5.3.2 Efficacy of MenC vaccine

Since the introduction of MenC in the UK in 1999, a marked decrease in the incidence of group C meningococcal disease has followed. The efficacy of the vaccine was approximately > 90% in all groups targeted for immunisation [43]. In all other countries where MenC was introduced the incidence had also fallen: in Ireland, by 96%; in Spain by 58%, in Belgium 55% and in Netherlands by 73% [6]. The effectiveness of glycoconjugate vaccines such as MenC is determined by the association of functional antibody titre, memory immunity and herd immunity. Functional antibody titre, measured by SBA, is the only validated correlate of protection from invasive meningococcal disease [108]. Immunological memory and herd immunity have been invoked to predict persistence of vaccine effectiveness despite waning SBA [6]. Presence of immunological memory can be demonstrated by characteristics of secondary immune response after challenge with plain polysaccharide vaccine. Low incidence of diseases in age groups with low SBA titres, implied that protection is due to this memory response or herd immunity [6].

2.5.3.3 Duration of immunity

Trotter *et al.* assessed surveillance data from the 4 years since introduction of the MenC vaccine programme in the UK. They reported that vaccine effectiveness was maintained in children vaccinated in the catch-up campaign (aged 5 months to 18 years), but for children vaccinated in the routine infant immunisation programme, the effectiveness of the vaccine fell to low levels after only 1 year (table 4).

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)				
Juniors 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: MCC vaccine effectiveness in immunised cohorts to end of March, 2004

Table 4: Effectiveness of MenC vaccine in UK (1) [5]

A study by Snape *et al.* reported that SBA titres wane rapidly, and were below the protective titre of 1 in 8 between 2–4 years after immunisation, depending on the age and on the schedule (Table 5). In the first year after routine infant immunisation at 2, 3 and 4 months vaccine effectiveness had fallen to 93%. More than 1 year after the single-dose regimen employed for 1–2 year olds children, vaccine effectiveness had fallen to 61% and for all other age groups immunised effectiveness remained above 80%.

Timing of vaccination	Time since vaccination	MenC (combined) effectiveness (95% CI) [§]	MenC-CRM ₃₀		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 [§]		
	10 months		13.5 (7.3–25) [†]	53 [‡]	21.5 (15.8–29.2) [‡]	99 [§]
	9–12 months		10.3 (5.2–20.6) [†]	46 [§]		
	> 1 year					
1–3 years (single dose)	4 years	88% (65–96)	3.2 (1.9–5.5) [†]	12 [§]		
	< 1 year					
	6 months		51 (30–85) [†]	75 [§]	166 (99–281) [‡]	86 [§]
	> 1 year		19 (11–32) [‡]	57 [§]		
	2 years		8.7 (5.8–13.2) [†]	27 [§]		
Adolescence (single dose)	3 years	96% (89–99)	2.5 (2.0–3.1) [*]	25 [§]		
	< 1 year					
	1 year		81.3 (54.5–121.3) [*]	95 [§]		
	> 1 year	90% (77–96)				
	3 years			92 [§]		

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

Table 2: Vaccine effectiveness and persistence of serum bactericidal activity following immunisation with either MenC-CRM₃₀ or MenC-TT

Table 5: Effectiveness of MenC vaccine in UK (2)[6]

The decrease in efficacy is particularly alarming in infants and toddlers, the age groups at highest risk of MD and in whom peak titres of bactericidal antibodies in serum (1 month after vaccination) had waned to low or undetectable levels by 7 to 9 months after vaccination [43].

2.5.3.4 Potential negative effects of MenC introduction

Capsule switching, the change from one capsular polysaccharide to another is well known in *Neisseria meningitidis* [109]. There are major concerns that introduction of MenC could apply selective evolutionary pressure on the hyperinvasive ST11 clone to switch to another serogroup, potentially B [110]. Further, there is also the theory of serogroup replacement, i.e. that another meningococcal serogroup could fill the ecological niche left by the reduction in carriage of serogroup C meningococcus. To date, there was no increase in the incidence of serogroup B meningococci bearing the ST11, following vaccine introduction in the UK (see also chapter 2.1.4).

The other concern was that introduction of the MenC vaccine might induce a reduction of MenC carriage and lead to reduced natural immune boosting. Recent studies in the UK reported that only 10 to 30% of sera from unimmunised adults were positive for serogroup C bactericidal antibodies. Therefore, a decrease in levels of serum bactericidal antibodies in adults might explain the apparent increase in the rate of meningococcal disease, because of a decrease of acquisition of maternal antibodies transplacentally [42]. The risk of invasive disease in a vaccinated population is determined by the complex interactions between vaccine-induced and natural immunity, carriage and herd effects.

2.5.4 Optimal MenC schedule

2.5.4.1 Reduced two dose MenC schedule

All MenC vaccines in the UK are now licensed as a 2-dose schedule in infants from 2 months of age with an interval of at least 2 months between doses. Studies with MenC vaccine on a 3-dose schedule did not show a higher induction of antibody titres with the third dose compared to the second dose [111-113]. However, post vaccination antibody titres following one dose of MenC vaccine were significantly lower than 2 doses of MenC vaccine [114], suggesting that two doses of MenC vaccine in the first year of life induced higher protection than only one dose.

2.5.4.2 Why not only a single dose in the second year of life?

In Switzerland and in other countries with low MenC incidence, the recommended schedule is a single dose of MenC in the second year of life in contrast to the UK recommendation of primary immunisation in the first year of life with a booster in the second year. In fact, the two dose schedule provides no significant benefit compared to a single dose of MenC in the second year of life alone [105].

The higher effectiveness of MenC in infants immunised at age 5-11 months compared with those immunised at 2-4 months suggests that the age at which the final dose is given rather than a booster dose might be important. [5] As with Hib vaccine, the protection induced by MenC vaccination is age dependent, and infants vaccinated at older ages seem to acquire greater protection and for longer duration [5]. These findings suggest that accelerated schedules are not optimal for conjugate vaccines. Furthermore, even if immunisation before 1 year of life with MenC primes for memory 4 years after primary immunisation, it does not seem to be sufficient to provide long-term protection. Delaying the primary immunisation to 1 year of life will leave infants below this age unprotected, even if this is an age of relatively low incidence of MD. Further, immunisation in the first year of life has the advantage to prime for following booster doses.

2.5.4.3 Interaction between vaccines when administered in combinations or simultaneously during infant immunisation

It has been demonstrated that MenC vaccine co-administered with DTaP-Hib and PC vaccine elicited lower antibody levels than when MenC was given separately from PCV vaccine [114]. Interactions between co-administered vaccines have been found among vaccines conjugated to the same carrier proteins (MenC-CRM₁₉₇, PCV-CRM₁₉₇) and also to different carrier proteins (PCV-CRM₁₉₇, Hib-TT).

2.5.4.4 Why adolescents should be included in the immunisation campaign ?

It has been shown that adolescents were the predominant carriers of serogroup C meningococci. Therefore, their inclusion in the catch up campaign seems essential to generate herd immunity. A possible schedule is a dose of MenC given at 12-18 months of age and another one at 12 years of age, as was recently introduced in Switzerland. These doses of MenC would provide an increase in bactericidal activity at 1 year of age and just before the period of adolescence, which are two periods of high incidence of MD. Without booster doses of MenC vaccine after early childhood immunisation, teenagers would be very susceptible to MD, with their bactericidal antibody titre being lower than protective threshold.

Chapter 3: Determination of memory response to MenC conjugate vaccine in children of one year of age, after priming at 2, 3 and 4 months

3.1 Rationale

Although the MenC conjugate vaccine has been shown to be effective, there are unanswered questions around its ability to provide protection against disease in the long term. The antibody level against MenC polysaccharide falls after vaccination and it has been proposed that any continuing protection is in part due to the presence of immunological memory, known to be induced by conjugate vaccines. However, recent vaccine failures for both MenC and Hib conjugate vaccines have raised questions about the ability of immunological memory to provide protection against disease. Due to these concerns, a booster dose of vaccine against both Hib and MenC at one year of age has been introduced to the routine immunisation schedule for children in the UK.

The generation and measurement of immunological memory is poorly understood. One way to assess immunological memory is to repeatedly measure antibody and the response to further vaccine doses at progressively longer intervals from vaccination. Quantifying the number of plasma and memory B cells produced by vaccination is also an important step towards understanding the development of long-term protection for a particular vaccine. Assessment of the characteristics of the B cell response will help to improve understanding of the generation of sustained immunity and B cell memory. The number of antigen-specific B cells prior to and following administration of MenC vaccine can be measured by the Enzyme-Linked-Immuno-Spot-Assay (ELISpot) technique

The objectives of this clinical study are thus :

- To establish whether MenC-specific memory B cells and plasma cells persist in the blood in children of one year of age, following primary immunisation with MenC vaccine at 2, 3 and 4 months of age.
- To establish the kinetics with which MenC-specific plasma cells and memory B cells increase in the blood of healthy children after a booster dose of MenC vaccine.
- To compare the results of MenC-specific B cells with B cells specific for other antigens, such as CRM₁₉₇ (a mutant of diphtheria toxoid used as carrier protein in MenC vaccine), diphtheria toxoid, Hib, and tetanus toxoid.

3.2 Study design

Subjects:

This was a phase IV, open-label study to investigate the B cell memory immune response to the C polysaccharide component of a MenC vaccine. A total of 33 healthy children of at least 12 months of age to which the vaccine had been administered after priming with meningococcal C conjugate vaccine at 2, 3 and 4 months of age were recruited. These children were recruited from a cohort of 72 subjects who participated in a previous study in Oxfordshire (study M14P5, Eudract number 2004-004962-33), assessing the B cell response to the C polysaccharide component of a MenC vaccine during routine infant immunization schedule (at 2,3 and 4 months of age).

Information and consent:

Informed consent was obtained from the parents and the protocol approved by the Research Ethics Committees of Oxfordshire (approval number CO6/Q1604/N41) and from the clinical trials regulations of Novartis vaccine (Eudract number 2006-000732-28).

Vaccine administration

After consent by the parents, participants received a dose of serogroup C meningococcal polysaccharide conjugate vaccine (0.5 ml) (Menjugate), by intramuscular injection into the antero-lateral thigh. The 0.5 ml dose contained 10 µg of the polysaccharide and 10 µg of the conjugate CRM₁₉₇ and was adsorbed on aluminium phosphate. At the same time, but in the other anterolateral thigh, a dose of Hib conjugate vaccine (Hiberix) was administered following the new routine immunization schedule of UK since 2006 [105]. Each 0.5 ml dose of the Hib conjugate vaccine contained 10 µg of purified Hib capsular polysaccharide covalently bound to approximately 30 µg tetanus toxoid.

Samples

A blood sample was obtained from each child prior to the vaccination and at day 30 after the vaccination. The 33 children were enrolled into 5 groups to allow investigation of the kinetics of the antibody, plasma cell and memory cell response between day 2 and day 9. Blood from children allocated into the different subgroups were obtained on either day 2, day 4, day 6, day 8 or day 9. There were a total of 3 blood draws for any one child (Table 6).

Group (subjects)	Visit 6	Visit 7	Visit 8
1 (5)	Day 0	Day 2	Day 30
2 (7)	Day 0	Day 4	Day 30
3 (6)	Day 0	Day 6	Day 30
4 (7)	Day 0	Day 8	Day 30
5 (6)	Day 0	Day 9	Day 30

Table 6: Schedule of visits for blood sampling

The total volume of blood sample was maximum 6 ml. From the 6 ml of blood, 5 ml was decanted to a heparinised container for B cell studies by ELISpot and 1 ml of blood (clotted sample) was decanted for antibody studies by ELISA and SBA. If the total volume of blood was less than 6 ml, the availability of samples for B cell studies was the priority for each visit, except visit 8 (30 days after immunisation). This was because the SBA and ELISA assay were deemed to be more important to assess whether the child was protected or not after the booster dose of MenC-conjugate vaccine.

3.3 Materials and Methods

3.3.1 Preparation of PBMCs for culture or for ex vivo ELISpot

Reagents

- RPMI-1640 Hepes modification 500 ml (Sigma-Aldrich, England)
- Penicillin (5000 units)/ Streptomycin (5 mg) in 100 ml (Sigma-Aldrich, England)

- L-Glutamine (200 mM) in 100 ml (Sigma-Aldrich, England)
- Lymphoprep (Axis-Shield Diagnostics, Norway)
- Foetal Calf Serum (**FCS**) (500 ml heat inactivated at 56°C for 30 minutes + frozen in 50 ml aliquots at -20°C) (Sigma Aldrich, England)
- **Complete medium** (RPMI (500 ml) + penicillin/streptomycin (5 ml) and L-Glutamine (5 ml)) + **10% FCS** (450 ml medium + 50 ml FCS.)

Process

A maximum volume of 5 ml of heparinised blood from children was available for the separation of PBMCs. The blood was diluted 1:2 in Complete medium. The PBMCs were then separated by density gradient centrifugation over Lymphoprep, which was added into a new universal tube at half the volume of the diluted whole blood. The diluted whole blood was then slowly layered on top of the lymphoprep without mixing the layers. The layered blood was then centrifuged at 2200 rpm for 30 minutes with the brake off. 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 10% FCS.

After the final wash, cells were resuspended into 1 ml of 10% FCS for counting by adding 50 µl of cells to 50 µl trypan blue + 50 µl PBS. The cells in a 10 µl volume of this mix were counted using a haemocytometer with a central 5x5 RBC grid and counting 5 squares within this grid. The number of cells obtained was then multiplied by 5 (allowing for number in total RBC grid), then by 3 (allowing for the cell dilution), and then by 10^4 (allowing for the depth of the haemocytometer). This gave the total number of cells/ml. The result was divided by the number of cells required / ml, which for cell culture and *ex vivo* ELISpot was 2×10^6 , which gave the final volume in ml of cell suspension.

3.3.2 Activation of memory B cells during 5 days for memory B cell ELISpot

Description of the assay

The ELISpot assay allows quantitation of antigen-specific memory B cells in human blood. This assay requires a 6-day polyclonal stimulation of PBMC to drive memory B cells to differentiate into antibody secreting cells (ASC) *in vitro*. A number of mitogens can be used to activate memory B cells *in vitro*. For example CpG-ODN induces IL-2 production, IL-2 receptor expression and thus activation and proliferation of immune cells via TLR9 and TLR4. IL2 also allows non-cognate T cell help. Pokeweed mitogen (PWM) and *Staphylococcus aureus* cowan strain (SAC) are polyclonal activators and activate all lymphocytes. SAC acts via B cell receptor (BCR) and T cell receptor (TCR) triggering. In a study by Bernasconi *et al.* it was observed that activation of naïve B cells was dependent on BCR signalling by anti-IgG and that they were unresponsive to T cell help and CpG. In addition, human memory B cells proliferated with bystander T cell help, when stimulated with CpG or IL2 and polyclonal activators without need for BCR triggering by addition of anti-Ig. In conclusion, in the absence of specific Ag only memory B cells proliferate and differentiate to ASCs in response to polyclonal stimuli derived from microbes or activated T cells [13]. Further it was found by Crotty *et al.* that PWM combined with SAC and a CpG oligonucleotide was the optimal combination identified. [115].

Reagents

- 10%FCS
- PWM (10mg)) reconstituted to 1mg/ml (Sigma-Aldrich, England)
- CpG-2006 TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT (200µg)) re-constituted in 1ml of distilled water (Invitrogen, England)
- SAC (Calbiochem, England)
- 96 well, round bottom, cell culture treated, sterile cell culture plates (Fisher, England)

Process

PBMCs prepared from peripheral blood were re-suspended in 10% FCS at a final concentration of 2×10^6 PBMC/ml and added to 96-well round bottomed culture plates in 100 µl/well. Stimulated medium, containing 1/5000 SAC, 1/6000 or 83 ng/ml PWM and 1/40 or 2.5 µg/ml CpG-2006 was added to the cells in 100 µl/well. The cells were incubated at 37°C in 5% CO₂ for 5.5 days.

3.3.3 Determination of antigen-specific-plasma cells and memory B-cells by ELISpot

Description of the assay

ELISpot assay permit to quantify antigen-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 6-day polyclonal stimulation of the PBMCs to assess Ag-specific memory B cells. The 6 day culture allows the detection of memory B cells that have differentiated into ASCs *in vitro* and allows detection of specific B-cells at a single-cell level.

Materials

- 10%FCS
- Purified tetanus toxoid (Statens seruminstitut, Denmark)
- Purified diphtheria toxoid (Statens seruminstitut, Denmark)
- Purified CRM₁₉₇ toxoid (Novartis vaccine, Italy)
- Methylated human serum albumin (mHSA) (NIBSC, England)
- Serogroup C meningococcal polysaccharide (NIBSC, England)
- Goat-anti-human immunoglobulins (Caltag laboratories, Burlingame, USA)
- ELISpot plates (96-well PVDF membrane) (Millipore, England)
- Alkaline phosphatase conjugates: Goat-anti-human IgG (Calbiochem, England)
- Alkaline phosphatase substrate kit (Bio-Rad, England)
- Tween20 (VWR International, England)
- EDTA (Ethylenediaminetetraacetic acid di-sodium salt dehydrate) (Sigma-Aldrich, England)
- PBS tablets (Sigma-Aldrich, england)
- NaCl (sodium chloride) (Sigma-Aldrich, England)
- KH₂PO₄ (potassium dihydrogen orthophosphate) (Sigma-Aldrich, England)

- Na₂HPO₄·7H₂O (di-sodium hydrogen phosphate-heptahydrate) (Sigma-Aldrich, England)
- KCl (potassium chloride) (Sigma-Aldrich, England)
- **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 before the solution was autoclaved. Following sterilization, the solution was stored at room temperature.
- **1xPBS (phosphate buffered saline)** : 10xPBS (100 ml) was added to distilled water (900 ml), and then the pH was corrected to 7.2.
- **Wash buffer (PBS-Tween 0.25)**: Tween20 (2.5 ml) was added to 1X PBS (1L), at pH corrected to 7.2.
- **Cell wash buffer (PBS-EDTA+0.05% FCS)** : A total of 5 PBS tablets and EDTA (0.744 g) were dissolved into distilled water (1L). The pH was then adjusted to 7.2 before the solution was autoclaved. Following sterilization, FCS (5 ml) was added to give a final concentration of this reagent of 0.05%. This solution was stored at 4°C.

Process

3.3.3.1 Preparation of ELISpot plates

MultiScreen™-IP 96 well filter plates were coated with either 5 µg/ml serogroup C meningococcal polysaccharide conjugated to methylated human albumin (5 µg/ml), CRM₁₉₇ (10 µg/ml), diphtheria toxoid (10 µg/ml), tetanus toxoid (5 µg/ml), Hib polysaccharide (2 µg/ml), or goat anti-human Ig (10 µg/ml) in sterile PBS (100 µl/well). PBS alone was added to the antigen-blank wells. The ELISpot plates were then stored at 4°C until use.

Just before the cell suspension was added, pre-coated ELISpot plates were washed and blocked with 10% FCS (200 µl/well) for at least 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 7: ELISpot plate layout (1)

Subject				Subject				Subject			
1	2	3	4	5	6	7	8	9	10	11	12
menc	Hib	Diph	Ig 1:10	menc	Hib	Diph	Ig 1:10	menc	Hib	Diph	Ig 1:10
menc	Hib	Diph		menc	Hib	Diph		menc	Hib	Diph	
menc	Hib	Diph		menc	Hib	Diph		menc	Hib	Diph	
menc	Hib	Diph	Ig 1:100	menc	Hib	Diph	Ig 1:100	menc	Hib	Diph	Ig 1:100
menc	crm	Tet		menc	crm	Tet		menc	crm	Tet	
menc	crm	Tet		menc	crm	Tet		menc	crm	Tet	
menc	crm	Tet	PBS	menc	crm	Tet	PBS	menc	crm	Tet	PBS
menc	crm	Tet	PBS	menc	crm	Tet	PBS	menc	crm	Tet	PBS

Table 8: ELISpot plate layout (2)

3.3.3.2 Detection of plasma cells

PBMCs prepared from peripheral blood were washed 3 times with 10% FCS and re-suspended to a final concentration of 2×10^6 PBMCs/ml. 100 μ l/well of the suspension was added to ELISpot plates pre-coated, and incubated overnight at 37°C in 5% CO₂. Cells and supernatants were then discarded from the plate before washing four times with 200 μ l/well PBS-Tween 0.25, and once with a 5 minutes incubation with PBS. IgG-Alkaline phosphatase conjugate at a concentration of 1:5000 (100 μ l/well) was added to the plate and incubated for 4 hours at room temperature. Wells were then washed four times in PBS-Tween 0.25 and three times in dH₂O before 50 μ l/well of substrate solution (substrate buffer 1/25, in dH₂O, with 50 μ l of each of solutions A and B per 5 ml of dH₂O) was added. The plates were developed, allowing spots to be observed and the reaction was stopped using dH₂O (200 μ l/well) without allowing the background to darken. Finally, the plates were washed four times with dH₂O (200 μ l per well) before the plates were dried in drying oven.

3.3.3.3 Detection of memory B-cells

After 5.5 days of culture, the cells were re-suspended and washed three times in PBS-EDTA/FCS (0.05%). Then the cultured cells were plated into pre-coated ELISpot plates at 2×10^5 cells /well and then incubated and developed as for the ex-vivo ELISpot (see above).

3.3.3.4 Calculation of memory B cell frequencies

Following development, plates were read on 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 and antigens and the operator was blinded to which sample was being counted.

The number of replicates depended on PBMC yield, which varied, so varying numbers of cultures were analyzed per donor, and donors with the most cells had more replicates of B-cell assays for each antigens tested. The mean number of spots was calculated for each antigen from the replicates. Between 4 and 20 replicates were obtained for MenC. Between 2 and 8 replicates were obtained for diphtheria toxoid, CRM197, tetanus toxoid and Hib. Anti-immunoglobulin assays were undertaken in 2 dilutions (1:10 and 1:100) with between 2 and 6 replicates per dilution. There were 1-6 replicates of the PBS control. For the culture B cell ELISpot samples were excluded from the analysis when less than 700 IgG secreting cells were detected per 2×10^5 cultured lymphocytes. This was to exclude assays with failure of memory B cell activation in culture.

3.3.3.5 Statistical analysis

Data was analysed using SPSS version 14.0 and represented as box plots, with median values, 25th-75th-percentile values, error bars (including all values within 1.5 times the interquartile range of the box), and values falling outside of the error bars indicating by a dot. Within-group comparisons of B cell numbers at varying time points were made using the Wilcoxon signed ranks test for unpaired data. A value of $P \leq 0.05$ was considered statistically significant and was indicated on the output graphics by an ** for $P \leq 0.05$ and *** for $P \leq 0.01$.

3.4 Results

Between May 10, 2006 and August 10, 2006, all 33 children completed the phase IV study. Samples from 27 children were obtained at visit 6, 31 at visit 7, and 29 at visit 8. For memory B cell assessment, samples were excluded because less than 700 IgG secreting cells were observed or due to culture contamination and therefore at V6 21 samples were available for analysis and at V7 and V8 23 samples were available for analysis.

The V7 sample was prioritized for use in the *ex vivo* ELISpot assay, whereas the V6 and V8 samples were prioritised for assessment of memory B cells by the cultured B cell ELISpot method. *Ex vivo* ELISpot was performed on these samples only if there were sufficient cells available after cultured ELISpot.

3.4.1 Persistence of IgG-specific memory B cells and plasma cells specific for MenC and for other antigens prior to vaccination

At one year of age, antigen-specific memory B cells for MenC, CRM₁₉₇, Hib and tetanus toxoid were infrequently detected in peripheral blood of infants previously primed with these antigens (Figure 7). Diphtheria toxoid-memory B cells were detectable with a median frequency of 3 ASCs per 2×10^5 cultured lymphocytes.

Prior to vaccination, only 9/21 children had detectable MenC-specific memory B-cells. Of these, 8 had 1 ASC per 2×10^5 cultured lymphocytes and 1 had 2 ASCs per 2×10^5 cultured lymphocytes. The median of the persistence of MenC-specific memory B cells was 0. By comparing the persistence of memory B cells specific for other antigens : 16/20 children had detectable CRM₁₉₇-specific memory B cells with a median of 1 ASC per 2×10^5 cultured lymphocytes; all children had detectable diphtheria toxoid-specific memory B cells with a median of 3 ASCs per 2×10^5 cultured lymphocytes; 3/11 children had detectable Hib-specific memory B cells with a median of 0 ASC per 2×10^5 cultured lymphocytes; and 6/11 children had detectable tetanus toxoid-specific memory B cells with a median of 1 ASC per 2×10^5 cultured PBMCs.

Prior to vaccination, there were no antigen-specific plasma cells detected in peripheral blood. Frequency was between 0 and 1 ASC per 2×10^5 PBMCs for all antigens and in all children (Figure 8).

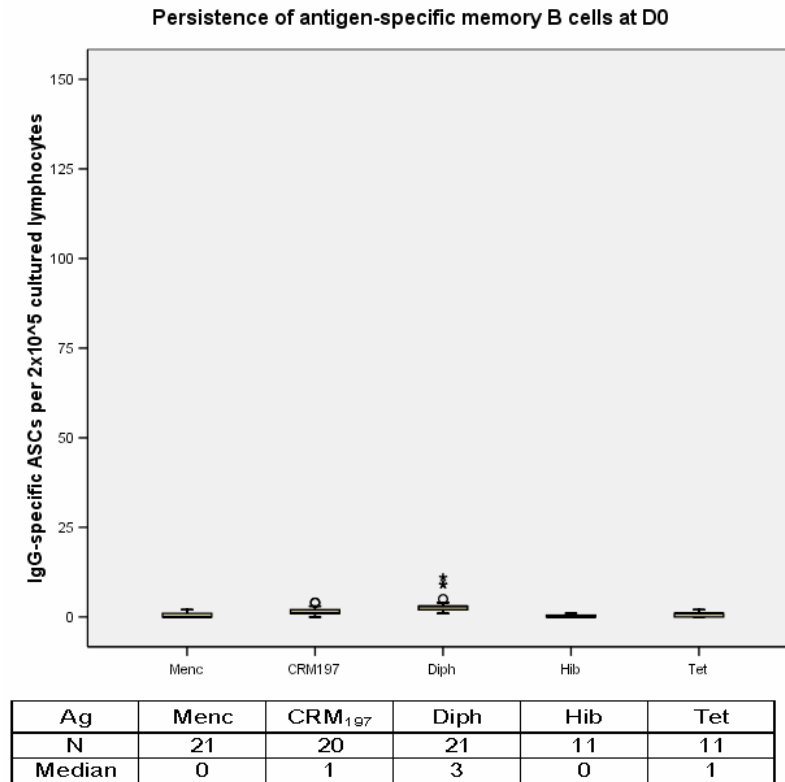


Figure 7: Persistence of Ag-specific IgG memory B cells prior to vaccination

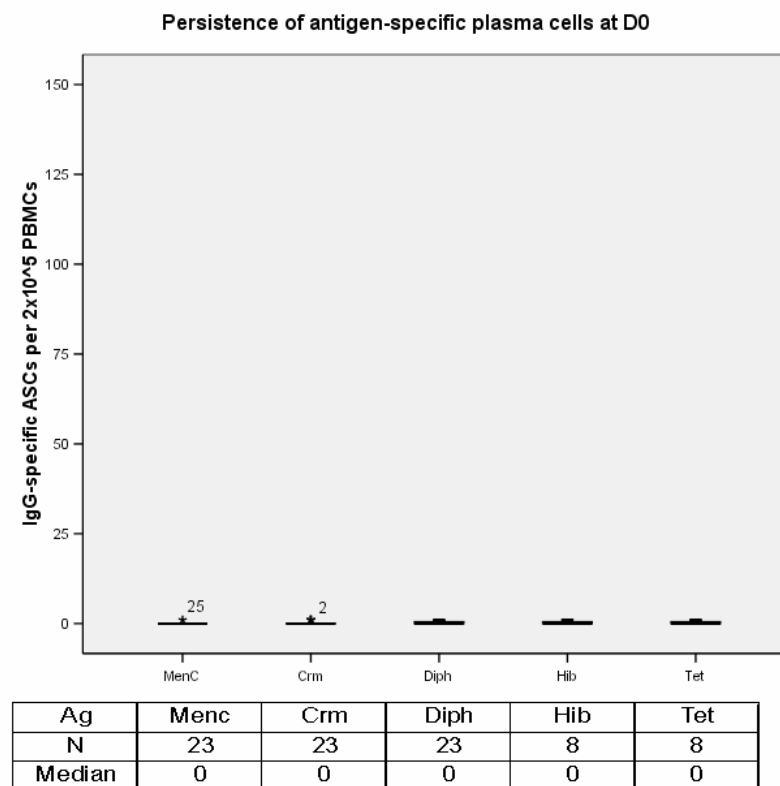


Figure 8: Persistence of Ag-specific IgG plasma cells prior to vaccination.

3.4.2 Kinetics of the response of MenC-specific plasma cells after a booster dose of MenC- and Hib- conjugate vaccines, and in comparison to other antigens.

Before immunization, there were no MenC-plasma cells detected in peripheral blood in the 22 children tested, except for one child who had 1 MenC-ASC per 2×10^5 PBMCs (Figure 8 and 9). Following immunisation, the first response in relation to baseline was seen by day 4, with a median of 1 MenC-ASC per 2×10^5 PBMCs (Figure 9). Peak plasma cell responses were seen at day 6, with a median of 22.5 MenC-ASCs per 2×10^5 PBMCs ($p \leq 0.01$ between D0-D6), followed by a rapid decline in the frequency of MenC-plasma cells already by day 8 and day 9 (median of 4 and 2 ASCs per 2×10^5 PBMCs). There were no more MenC-plasma cells at day 30.

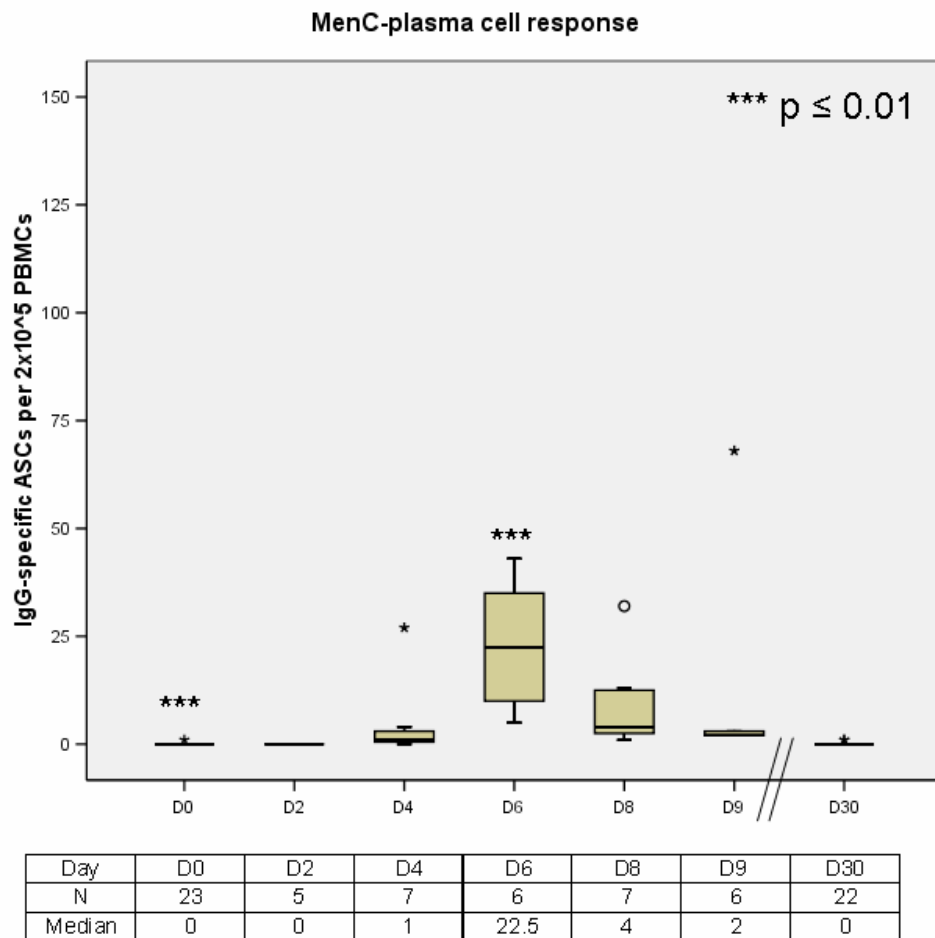


Figure 9: Kinetics of the response of MenC-specific IgG plasma cells after MenC- and Hib-conjugate vaccines.

Plasma cell responses specific for other antigens were also assessed for comparison with the MenC-plasma cell response. However, all other antigens tested were also contained or related to the vaccines administered at day 0; CRM₁₉₇ was used as carrier antigen in the MenCV. Tetanus toxoid was used as carrier antigen in the Hib-conjugate vaccine. Diphtheria toxoid-specific immune cells were also stimulated, as CRM₁₉₇ is a mutant protein derived from diphtheria toxoid. The magnitude and rate of decline in frequency was variable between individuals and for each antigen, while the peak response occurred consistently on day 6, (except for Hib-ASCs, where the peak was seen by

day 8-9, but the samples number were very low for day 6 and day 9) (Figure 10). The difference between baseline frequency and peak frequency at day 6 was statistically significant for CRM₁₉₇-, diphtheria toxoid-, tetanus toxoid- specific ASCs ($p \leq 0.05$ between D0-D6). For all the antigens tested, there were almost no detectable specific plasma cells at day 30 post vaccination (median between 0 and 1 ASC per 2×10^5 PBMCs for all antigens).

The magnitude of response was greater for diphtheria toxoid- specific plasma cells, compared to CRM₁₉₇- specific plasma cells, with a median at day 6 of 20.5 diphtheria toxoid-ASCs per 2×10^5 PBMCs and 9 CRM₁₉₇-specific ASCs per 2×10^5 PBMCs (Figure 10).

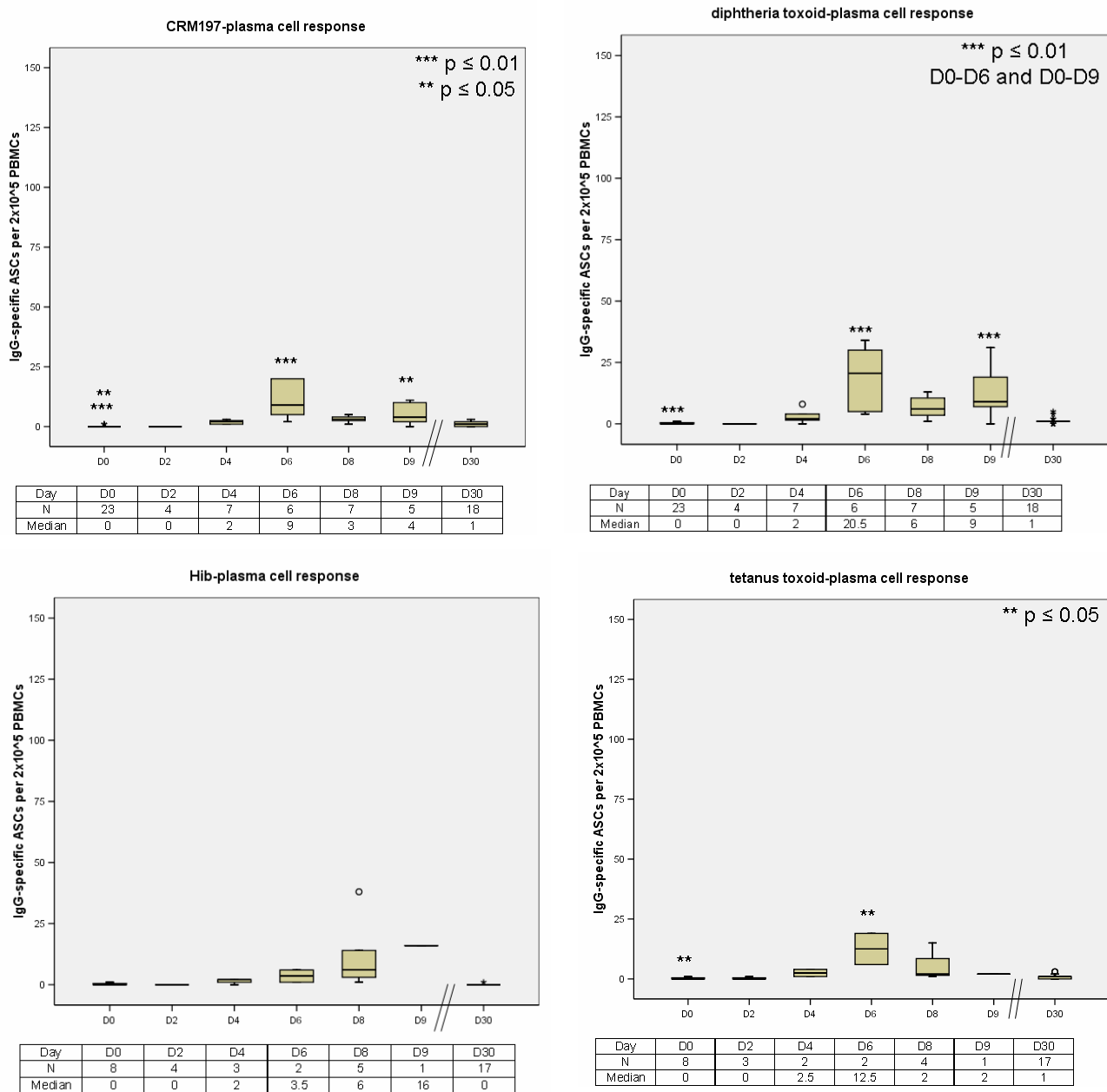


Figure 10: Kinetics of the response of CRM₁₉₇-, diphtheria toxoid-, Hib-, tetanus toxoid-specific IgG plasma cells after booster dose of MenC- and Hib- conjugate vaccines.

NB: There was only one subject at D9 for Hib and tetanus toxoid, and this subject was the same subject represented by the * at Day 9 on the Figure 11. This individual, therefore, responded particularly well to the booster dose of MenC- and Hib- conjugate vaccines.

There were no detectable change in the frequency of total-IgG plasma cell after immunization with MenC- and Hib- conjugate vaccines (Figure 11).

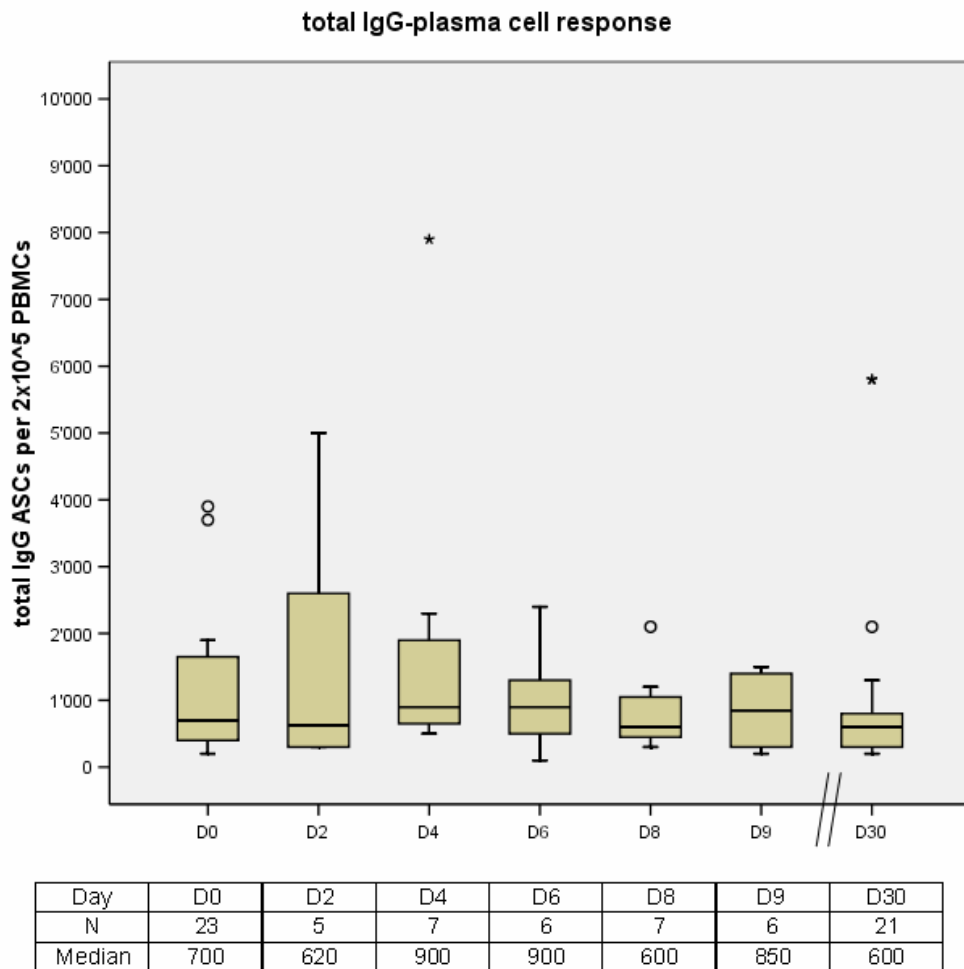


Figure 11: Kinetics of the response of total IgG-plasma cells after booster dose of MenC- and Hib- conjugate vaccines.

3.4.3 Kinetic of the response of Menc-specific memory B cells after booster dose of MenC- and Hib- conjugate vaccines, and in comparison to other antigens

Prior to vaccination, 9/21 children had detectable MenC-memory B cells (1/21 had 2 memory cells and the 8 others had 1 memory cell per 2×10^5 cultured lymphocytes), but the median frequency before vaccination was 0 MenC-memory cells per 2×10^5 cultured lymphocytes (Figure 7 and 12).

The first response above baseline was seen by day 8 for MenC-memory B cells, varying from 1 to 154 MenC-memory B cells and with a median of 8 MenC-memory B cells per 2×10^5 cultured lymphocytes and by day 9 between 1 and 80 MenC-memory B cells with a median of 3 MenC-memory B cells per 2×10^5 cultured lymphocytes (Figure 12). The difference of MenC-specific memory B cells per 2×10^5 cultured lymphocytes between D0 and D8 was statistically significant ($p \leq 0.05$ between D0-D8).

At one month after the booster dose of MenCV, MenC-memory B cells were still detected in blood. There were between 0 and 74 cells with a median of 4 MenC-memory B cells per 2×10^5 cultured lymphocytes. The level at D30 after immunisation was statistically different from baseline level ($p \leq 0.01$ between D0-D30) (Figure 12).

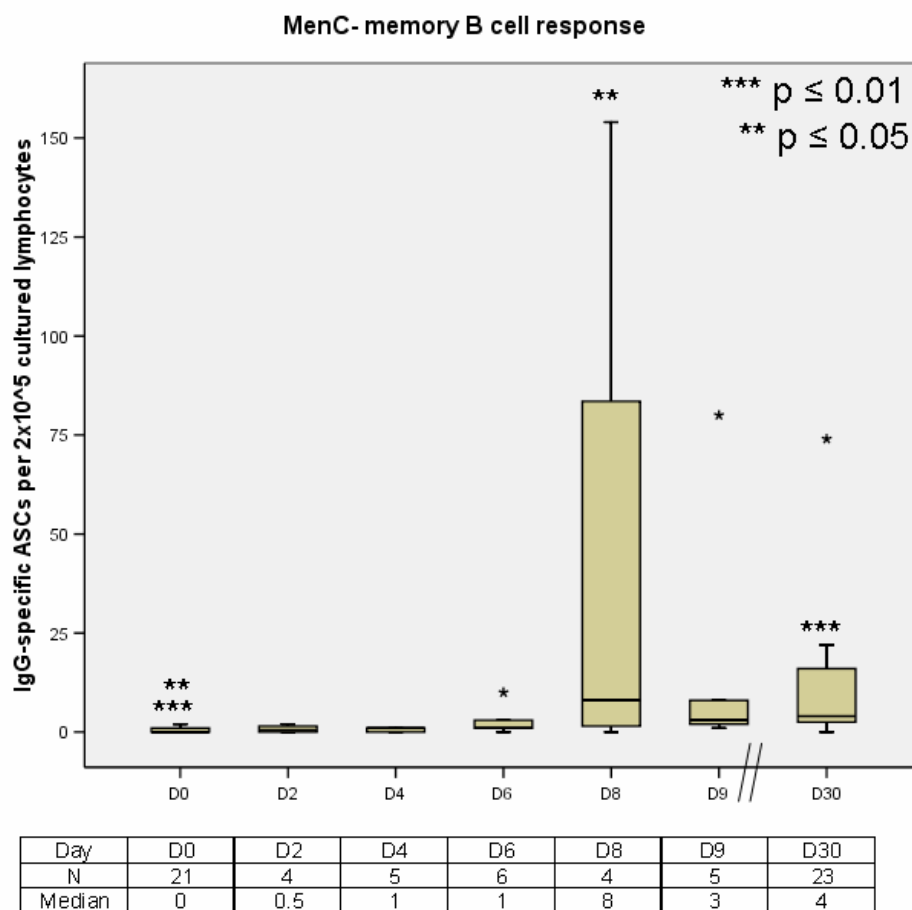


Figure 12: Kinetics of the response of MenC-specific IgG memory B cell response after booster dose of MenC- and Hib- conjugate vaccines

Memory-B cell responses specific for other antigens were also assessed for comparison (Figure 13). For Hib and tetanus toxoid, the number of subjects for the kinetics analysis were very low (in particular D4, D6 and D9) and for this reason, only baseline frequency at D0 and D30 and frequency at peak (D8), were used for comparison with other Ag-specific memory B cell frequency. For each antigen, the peak response of memory B cells appeared consistently on day 8, but the difference between D0 and D8 was not significantly different, except for Hib ($p = 0.2$ for CRM₁₉₇, $p = 0.068$ for diphtheria toxoid, $p = 0.001$ for Hib and $p = 0.17$ for tetanus toxoid) (Figure 13).

Post-day 8, antigen-specific memory cells declined variably, but for all antigens memory-B cells at day 30 post immunization were still significantly higher than baseline ($p \leq 0.001$ for CRM₁₉₇, diphtheria toxoid and Hib, ≤ 0.05 for tetanus toxoid) (Figure 13).

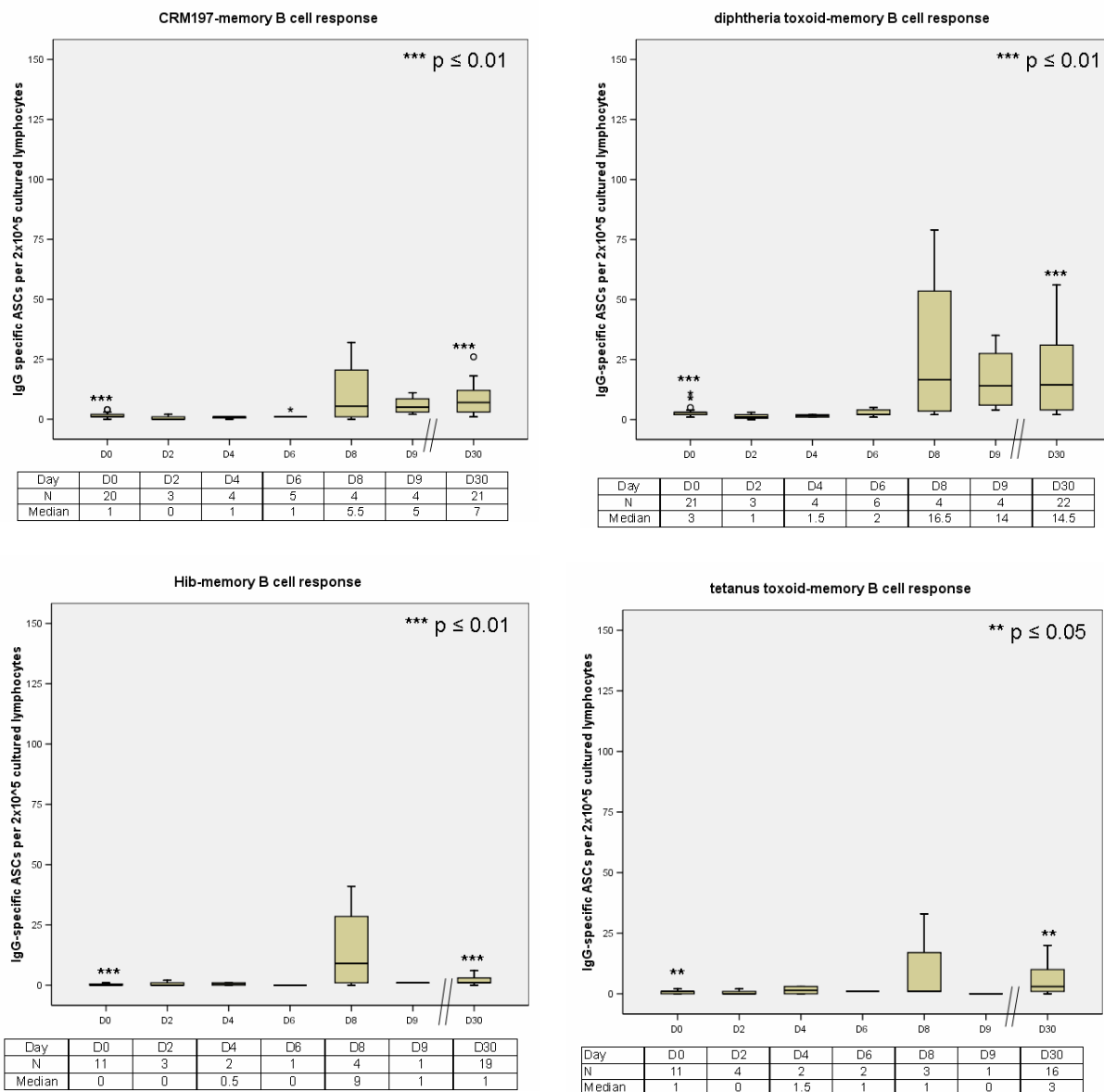


Figure 13: Kinetics of the response of CRM₁₉₇-, diphtheria toxoid-, Hib-, tetanus toxoid-specific IgG memory B cells after booster dose of MenC- and Hib- conjugate vaccines

When comparing memory B cell frequency at day 8 and at day 30 post-immunisation, there appeared to be a difference between polysaccharide antigens and protein antigens. MenC and Hib-specific memory B cells seemed to decline after the peak, and were seen at lower levels at day 30 than at day 8 whereas diphtheria toxoid-, CRM₁₉₇-, and tetanus toxoid- memory B cells appeared to remain at the same level at day 30 as at day 8. However for all antigen-specific memory B cells, the frequencies between D8 and D30 were not statistically different ($p = 0.92$ for MenC, $p = 0.86$ for CRM₁₉₇, $p = 0.97$ for diphtheria toxoid, $p = 0.29$ for Hib and $p = 0.92$ for tetanus toxoid)

At 1 month after immunisation, the number of children with detectable MenC-specific memory B cells was 20/22 with a median frequency of 4 ASCs per 2×10^5 cultured lymphocytes. The median for the other antigen-specific memory B cells were in the same range. The median of diphtheria toxoid-memory B cells was higher whereas the median of Hib-memory B cells was particularly low with 1 memory B cell per 2×10^5 cultured lymphocytes. Memory B cells specific to all antigens however, were still significantly higher compared to the baseline level ($p \leq 0.01$ between D0-D30 for MenC-, CRM₁₉₇, diphtheria toxoid and Hib and $p = 0.015$ for tetanus toxoid) (Figure 13 and 14).

As for plasma cells response, diphtheria toxoid-specific memory cells were much higher than CRM₁₉₇-specific memory B cells for all the time points (Figure 13).

There was a high variation in the frequency of total-IgG memory B cells after immunization, but total IgG-memory B cell frequencies were not statistically different between day 0 and day 9 ($p = 0.621$) and between day 0 and day 30 ($p = 0.224$) (Figure 14).

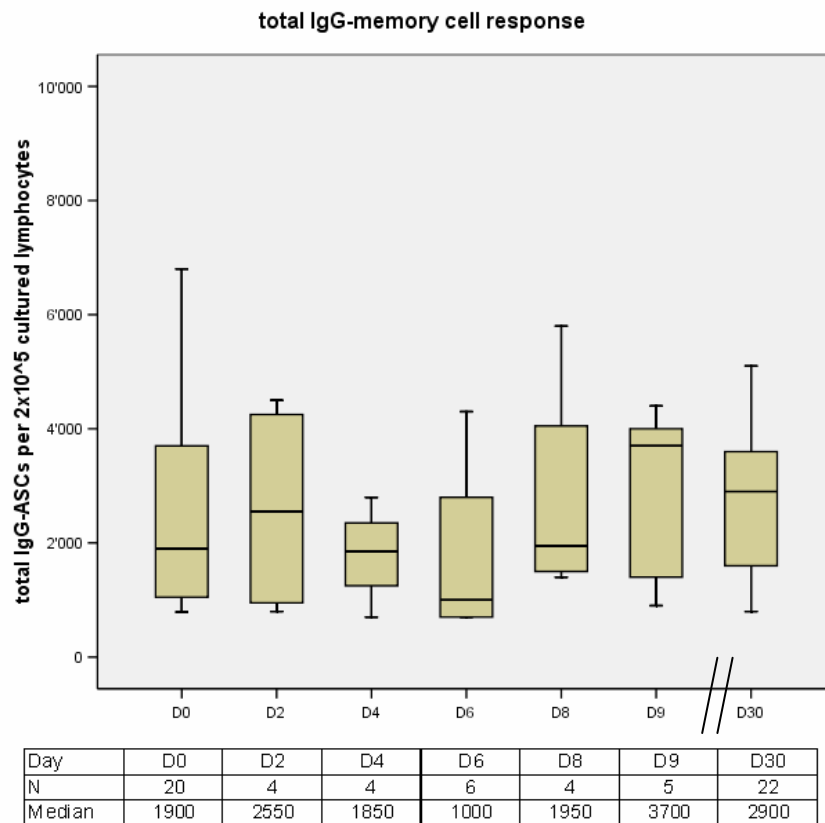


Figure 14: Kinetics of the response of total IgG memory B cells after booster dose of MenC- and Hib- conjugate vaccines

3.5 Discussion

3.5.1 **Persistence of IgG-specific memory B cells for MenC and for other antigens prior to immunisation and kinetics of memory B cell response following immunisation**

In this study of children previously primed with MenCV at 2, 3 and 4 months of age, MenC-memory B cells could not be detected in the blood by one year of age, as reflected by a median of 0 memory cells per 2×10^5 cultured lymphocytes (Figure 7). Persistence of memory B cells specific for other protein or polysaccharide antigens before vaccination was also low, with a median of between 0 and 1 memory B cells per 2×10^5 cultured lymphocytes for all antigens except diphtheria toxoid (3 memory cells) (Figure 7). This detectable frequency of diphtheria toxoid- and tetanus toxoid-specific memory B cells in children prior to immunisation was, in general, lower than their frequencies in primed adults studies in either our laboratory or in other studies. However in our laboratory, in some adults who had their last dose of diphtheria toxoid and tetanus toxoid immunisation more than 10 years previously, the frequency of memory B cells specific for these antigens were comparable to the values observed in children during the present study. Further, when memory B cell frequencies before immunisation were expressed as a percentage of total IgG-memory B cells, there were a median frequency of 0.06% of CRM₁₉₇-, 0.13% of diphtheria toxoid-, 0.01% of tetanus toxoid-specific memory B cells per total IgG-memory B cells (data not shown). These frequencies of memory B cells specific for diphtheria toxoid and tetanus toxoid in primed children were comparable although among the lower values of diphtheria toxoid- and tetanus toxoid- specific memory B cell frequency previously described in adults (between 0.01% and 1% of total IgG memory B cells) [11, 12].

Following immunisation, memory B cells appeared rapidly in blood, were detected from day 8 and persisted by 1 month. At 1 month following immunisation with MenC- and Hib-conjugate vaccines, frequencies of memory B cells expressed as a percentage of total IgG-memory B cells were: 0.29% for MenC, 0.24% for CRM₁₉₇, 0.67% for diphtheria toxoid, 0.05% for Hib and 0.16% for tetanus toxoid. This memory B cell kinetics was the same for all antigens assessed and was comparable to previous studies using a variety of vaccine antigens in primed individuals;

Nanan *et al.* [11] assessed frequencies of specific IgG-memory B cells after booster immunisation with diphtheria and tetanus toxoid in already primed persons (Figures 15, 16). They demonstrated that the total number of diphtheria-specific-B cells increased from day 0 to 12 and then gradually declined on day 90. Further, from day 0 to 90 after diphtheria immunisation, frequencies of tetanus-specific memory B cells remained unchanged.

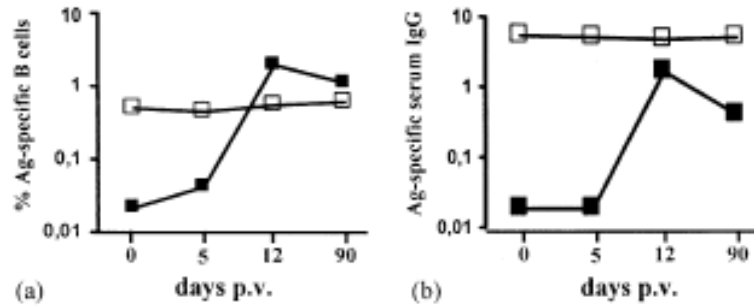


Figure 15: Secondary immune response after a booster dose of diphtheria vaccine: diphtheria- (filled squares) and TT-specific (open squares) memory B-cells expressed in percent of total-IgG-secreting cells (1 donor) [11]

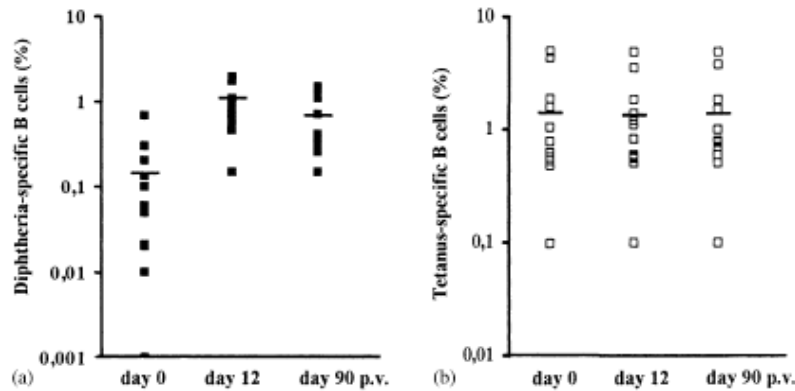


Figure 16: Diphtheria- and TT- specific memory B-cells during the first 90 days after booster dose of diphtheria toxoid vaccine (expressed as % of total IgG-secreting cells) (13 donors) [11]

Crotty *et al.* [12] described kinetics of memory B cells after smallpox vaccination as follows: specific memory B cells initially declined post-immunisation, but then reached a plateau ~10-fold lower than the peak and were maintained for >50years after vaccination at a frequency of ~0.1% of total circulating IgG+ B cells (Figure 17).

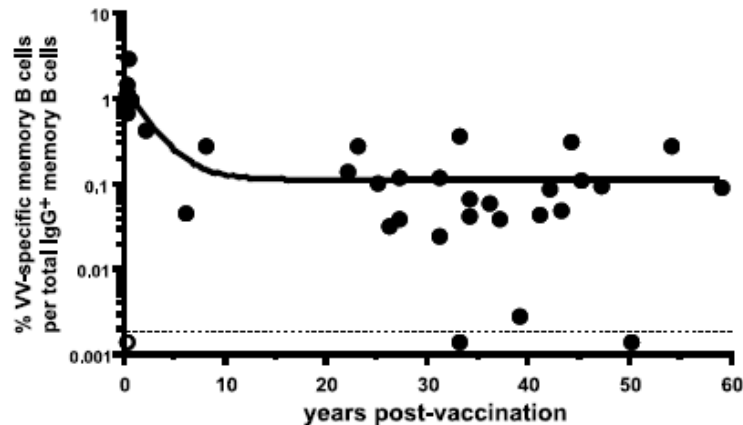


Figure 17: Longevity of smallpox vaccine-specific B cell memory [12]

These findings support the idea that following immunisation, memory B cells migrate out of circulation and home in secondary lymphoid organs, ready to mediate secondary immune response upon rechallenge. The time period over which memory B cells disappeared from peripheral blood varied in comparison to published studies with regards to the antigen under investigation and the age of the subjects.

It appears that memory cells are kept in circulation for a short period following antigen exposure, perhaps with the function of protecting against endemic disease [12] or because of cellular competition (or programmed cell death) making space for new memory B cells in secondary lymphoid organs [12].

These observations suggest that although MenC-specific memory B cells were not detectable in the blood of one year old children primed with MenCV at 2, 3, and 4 months of age, MenC-memory B cells may persist after priming and home to lymph nodes ready to rapidly differentiate and proliferate upon re-challenge with the antigen.

Memory cells, which appeared in the first week following immunisation in primed subjects, might be re-circulating memory B cells from previous priming rather than newly formed memory cells. The newly formed memory B cells might appear much later after newly stimulated naïve B cells have proliferated in germinal centres and differentiated into memory B cells.

In another study by Kelly *et al.* involving subjects primed with the MenCV and then vaccinated with either a plain polysaccharide or another dose of MenCV, it was found that only the conjugate vaccine was able to induce a significant increase of the frequency of MenC-memory B cells after immunisation, and these cells already appeared in blood by day 6-7 after the immunisation with a booster dose of MenCV [10]. 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 germinal centre 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 antigen.

It has also been proposed that memory B cells found in the circulation should be representative of the entire B cell pool, because memory B cells continuously re-circulate through the blood and secondary lymphoid organs for years after immunisation, allowing them to encounter and react with antigens at these sites [11]. However in our study, and other studies of previously primed subjects, the frequency of memory B cells specific for the vaccine antigen consistently increased 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. Whether this is the case remains unclear.

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 follicular DCs, or can be constantly generated from proliferating pathogen or periodic re-exposure to the pathogen can also stimulates the immune system [16]. In the Ag-independent model, several examples have shown that immune memory can persist for years following immunisation in the absence of antigen [12]. The Ag-independent mechanism postulates that memory B cells proliferate and differentiate to plasma cells *in vitro* in response to polyclonal stimuli of two types [13]. One type of stimulus is derived from microbial products, such as lipopolysaccharide or unmethylated single-stranded DNA motifs (CpG oligonucleotides), which

stimulate B cells via TLR4 and TLR9. The other type of stimulus is due to T cell activation by third party antigens, which stimulates B cells in a noncognate fashion via CD40 ligand and cytokine production (IL15), referred as bystander T cell help. Therefore during an antigen-specific response, the increased availability of activated T cells results in an increased production of all plasma cells [13]. However in several studies [11] there were no observed changes in the frequency of memory B cells specific for recall antigens following immunisation with an antigen.

3.5.2 Persistence of plasma cells specific for MenC and for other antigens prior to vaccination and kinetics of plasma cell response following immunisation

In this study, there were no antigen-specific plasma cells detectable in blood before immunisation for all antigens. The median frequency of total IgG-plasma cells was 700 per 2×10^5 PBMCs, suggesting that the frequency of antigen-specific plasma cells was too low to be detected in blood (i.e lower than 1/700 or 0.14% of total IgG-plasma cells). The first appearance of plasma cells 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. The plasma cell kinetics was the same for all antigen (all of which were included in the vaccines) and similar to previous studies using a variety of vaccine antigens in primed individuals [13, 68].

The kinetics of the plasma cell response after different antigen exposures have been considered previously: Traggiai *et al.* [116] analyzed the kinetics of specific plasma cells and serum IgG after a booster immunisation with TT, and found that following boosting, large numbers of specific plasma cells were detectable from day 5 to 10 and returned to baseline by day 15. Serum IgG increased from days 5 to 8, and reached a plateau level, which persisted for 1 month before declining with a half life of 40 days and after 250 days had reached pre-boost levels. Traggia *et al.* found that total IgG-secreting plasma cells increased with the same kinetics, and in all cases, their number was higher than that of TT-specific plasma cells, supporting the hypothesis of an ongoing activation. The number of plasma cells secreting antibodies to unrelated antigens to which the donor was immune (*Toxoplasma gondii* and measles virus) also increased by a factor of 10 with similar kinetics.

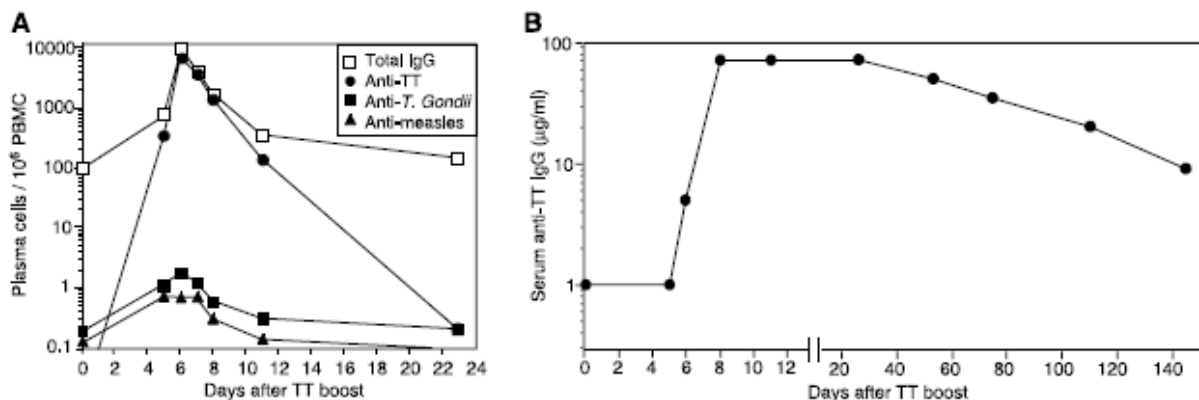


Figure 18: Kinetics of the response of plasma cells specific for tetanus toxoid and for unrelated antigens after a booster dose of tetanus toxoid vaccine (The donor was immune to TT, *T. gondii* and measles and was boosted with TT) [116]

Given the rapidity of plasma cell response following immunisation, these are likely to represent

plasma cells derived from pre-existing memory B cells. The transient appearance of the plasma cells is thought to represent their translocation from secondary lymphoid tissue, where they are formed following immunisation, to bone marrow via the blood-stream [63]. It is also possible that cells appearing at day 6 come from long-lived plasma cells residing within the bone marrow. These may then be subsequently activated to escape bone marrow by an unknown signal after new exposure to the antigen.

The kinetics of the tetanus toxoid-plasma cell response in adults observed by Traggia *et al.* were comparable to the kinetics of the tetanus toxoid-specific plasma cell response observed in the infants in this study. However, the peak frequency of tetanus toxoid-plasma cells was much lower in infants ($20/2 \times 10^5$ PBMCs) compared to adults ($10000/10^6$ PBMCs). This could be explained by a larger pool of B cells in adults, because of an increased number of booster doses of vaccine or due to natural exposure with cross-reactive antigens or the antigen itself. Another possible explanation could be that the long lived plasma cell reservoir might less well be maintained in children, because of a lower number of available niches in secondary lymphoid organs [63] or because plasma cells in adults might also have a better capacity for recirculation possibly due to higher chemokines responsiveness.

In our study, no significant variation in total IgG-plasma cell levels following immunisation was observed (Figure 11). The theory of a polyclonal activation of all B cells following immunisation with a specific antigen is a possible explanation for the findings of Traggia *et al.*, that following immunisation with an antigen, plasma cells specific for all recall antigens appeared in the blood at very low frequency. Another explanation might be that of a competition for bone marrow survival niches between newly generated plasma cells and old plasma cells. This suggests that these cells might be long-lived plasma cells of the bone marrow, dislocated from their niches by the newly formed antigen-specific plasma cells [63]. Perhaps newly formed plasma cells have an advantage over older plasma cells in terms of their capacity to respond to chemokines secreted by dendritic cells in bone marrow niches.

The polyclonal activation model suggests that under steady state conditions, Ag-specific plasma cells are continuously generated and should be detectable in peripheral blood on their way to the bone marrow and frequency of Ag-specific plasma cells and serum Abs should reflect the frequency of Ag-specific memory B cells activated by polyclonal mitogens [13]. In this study this theory does not appear applicable, because there were no plasma cells and no memory cells detected in peripheral blood at steady state, although memory B cells appeared rapidly in blood following immunisation.

Conventional models postulate that plasma cells are short-lived and are continuously generated de novo from long-lived memory B cells by stimulation with persisting Ag [16]. Recent studies by Manz *et al.* [63] and Slifka *et al.* [117] demonstrated however, that a fraction of plasma cells in the murine BM are long lived and can secrete antigen for extended periods of time in the absence of memory B cells, however, the existence of long-lived plasma cells in humans remains controversial.

It has been postulated that the B cell response generates short-lived plasma cells first, followed by long-lived plasma cells and memory B cells (Figure 19). In an infection, it is important to have antibodies produced as rapidly as possible. This is achieved by the antigen-specific B cells that differentiate into plasma cells early in extra-follicular foci. Generation of high affinity antibodies that are much more efficient at controlling and eliminating the pathogen is also important and is achieved by the Ag-specific B cells that initiate germinal centres [16].

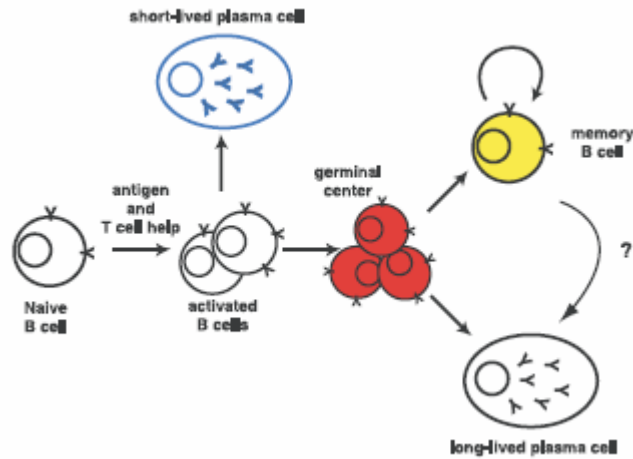


Figure 19: Memory B cell and plasma cell differentiation [16].

Considering these observations, mechanisms that contribute to sustaining serum antibody levels after infection or vaccination might be long-lived plasma cells that survive in appropriate bone marrow niches and persisting activation of memory B cells [116].

Traggiai *et al.* tried to explain the kinetic of antibody responses as follows:

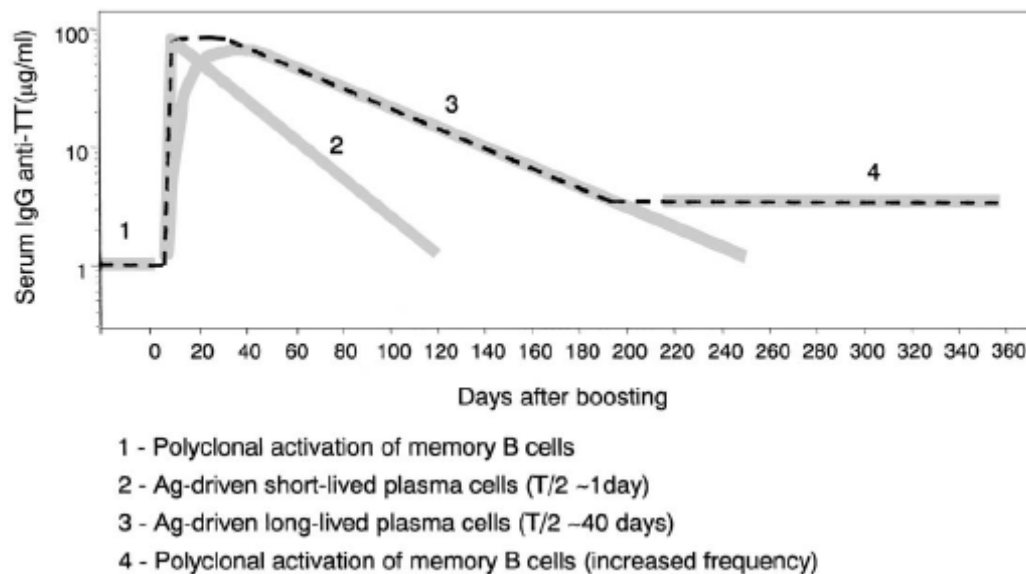


Figure 20: Mechanisms that sustain serum antibody levels following boosting [116].

In the Ag-dependent model, memory B cells proliferate and differentiate massively to short-lived plasma cells. This response is transient because of the negative feedback of the high level of antibodies present. Some plasma cells generated in this way become long-lived, survive in bone marrow niches and continue to produce antibodies for few months. In the polyclonal model, all memory B cells respond to environmental antigens and continuously proliferate and differentiate into plasma cells, maintaining a constant level of serum antibodies and plasma cells throughout life. In conclusion, there is a “short term serological memory” which is Ag dependent and lasts for a few

months, and a “long-term serological memory” resulting from Ag-independent polyclonal activation of memory B cells [13].

The kinetics of the antibody response have not been considered yet in this study, but it has been observed in several other investigations that antibodies appear in the blood by day 4 post immunisation in primed subjects. This suggests that following immunisation, plasma cells appear in the blood later in comparison to the appearance of antibodies. A possible explanation might be that antigen-specific plasma cells formed during previous exposure to the antigen and homing in bone marrow niches could rapidly increase their rate of antibody secretion following re-challenge with the antigen. Later, plasma cells differentiated from circulating memory B cells might contribute to further increases in antibody level in the blood. Another explanation for the later appearance of plasma cells compared to antibodies might be a difference in sensitivity between the two assays used to measure antibody titer in blood (ELISA) and to detect ASCs (ELISpot). It is assumed that ELISA has a lower detection threshold compared to ELISpot which directly detects B cells secreting antibodies and which requires the survival of these cells during the assay.

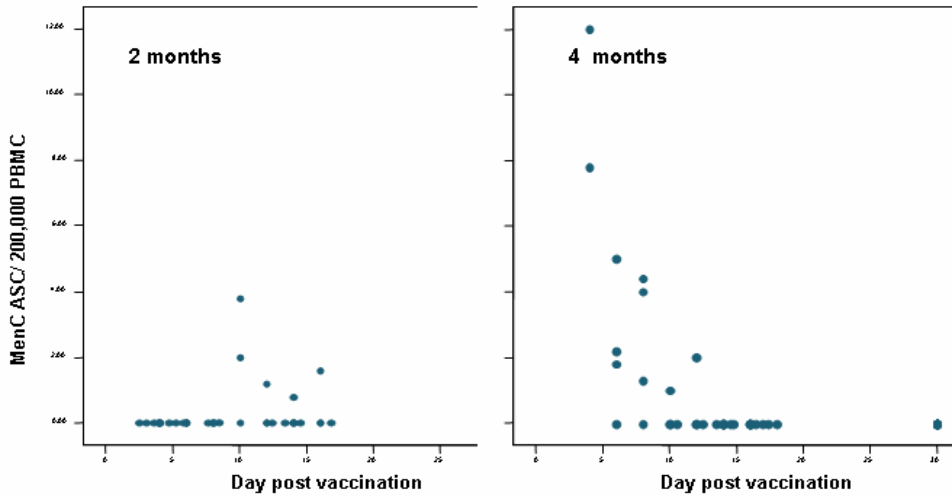
3.5.3 Comparison of the results of the present study with the one performed on the same infants, during priming at 2, 3 and 4 months of age

Comparing MenC-specific plasma cell and memory B cell response after primary immunisation with 3 doses of MenCV at 2, 3 and 4 months in the same infants (Figure 21), the principal difference was the highest frequency of plasma cells and memory B cells at one year of age: at 4 months, the peak median frequency of plasma cells was 9.9 ASCs per 2×10^5 PBMCs and at one year it was 22.5 ASCs per 2×10^5 PBMCs. The memory B cell peak frequency was at 4 months 2.5 memory B cells per 2×10^5 cultured lymphocytes and at one year of age 8 memory B cells per 2×10^5 cultured lymphocytes.

When comparing the plasma cell response at 4 months and one year of age with the one at 2 months of age following the first dose of MenCV, plasma cells appeared in blood much later, by day 10-15 compared to day 4-6 by 4 months and one year of age. These findings suggest two possible explanations: the maturation of the immune system between 2 months and 4 months/one year of age, or the difference in the characteristics of a primary immune response compared to a secondary immune response.

To distinguish between these two possibilities, it might be interesting to assess the immune response to MenCV in non-primed infants of the same age (respectively 4 months and one year of age). If the immune response observed at 4 months or at one year is comparable to the primary immune response that was observed in these children at 2 months of age, it would suggest that the delay might be characteristic of a primary immune response rather than immaturity of the immune system. The higher frequency of B cells at one year of age compared to 4 months of age might be due to the expansion of the B cell pool by one year of age because of increased exposure to MenC antigens or to cross-reactive antigens. The other possible explanation might be the maturation of the immune system with age. In a study by Kelly *et al.* [10] assessing a secondary immune response in teenagers previously primed with MenCV and receiving a booster dose of MenCV, kinetics of plasma cell and memory B cell response (Figure 22) were comparable to that observed at one year of age, but the frequency of B cells was also much higher than in infants with a peak of 125 ASCs per 2×10^5 PBMCs and 15 memory B cells per 2×10^5 cultured lymphocytes respectively.

Plasma cell responses to MenC conjugate vaccine at 2 months of age and 4 months of age – infant vaccination schedule



Memory B-cell responses to MenC conjugate vaccine at 2 months of age and 4 months of age – infant vaccination schedule

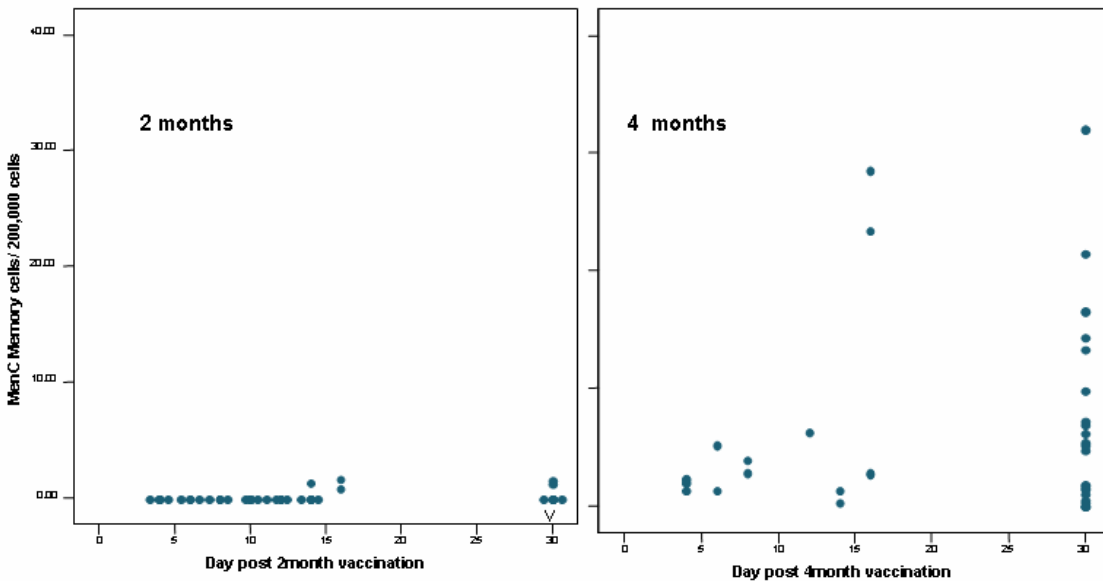


Figure 21: Plasma cell response (top) and memory B cell response (bottom) at different days after immunisation with 3 doses of MenCV at 2, 3, 4 months of age (Kelly *et al.* unpublished).

It would be interesting to assess a primary immune response in adults and in children with the same novel antigen, as this would help to determine whether increased B cell frequency in adults is due to the existence of a larger pool of these cells or to maturity of the immune system. Further, this type of experience would also aid in answering the question of whether the delay in the response of

plasma cells at 2 months of age is due to a primary exposure or to an immaturity of the immune system. This would only be possible, using a novel antigen to which both adults and infants have not been exposed. Rabies antigen would be a good candidate for these studies.

An increase of the B cell pool with age may result from natural or artificial boosting, through natural exposure to the antigen, or to cross-reacting antigens or by vaccination. Immaturity of the immune system may be manifested as a lower number of available niches in secondary lymphoid organs to home plasma cells and memory B cells, or different chemokines able to keep cells in life or to guide them in available sites.

Fig 1b

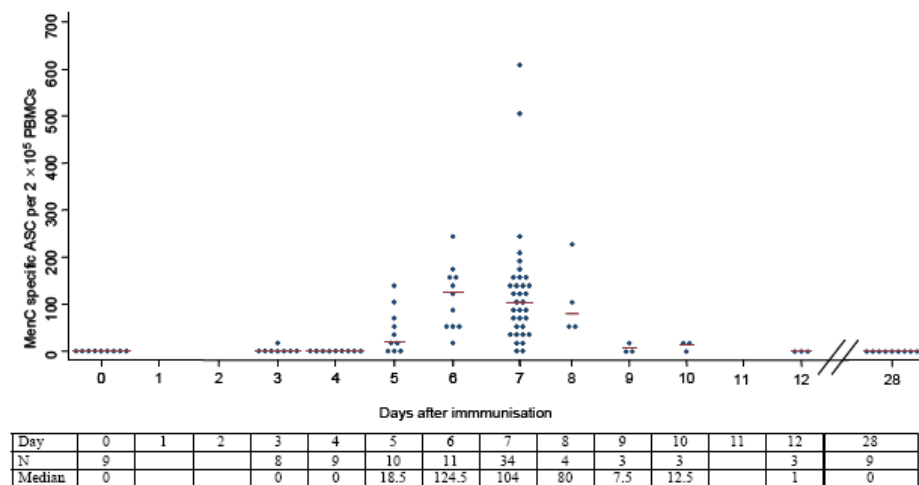


Fig 3b

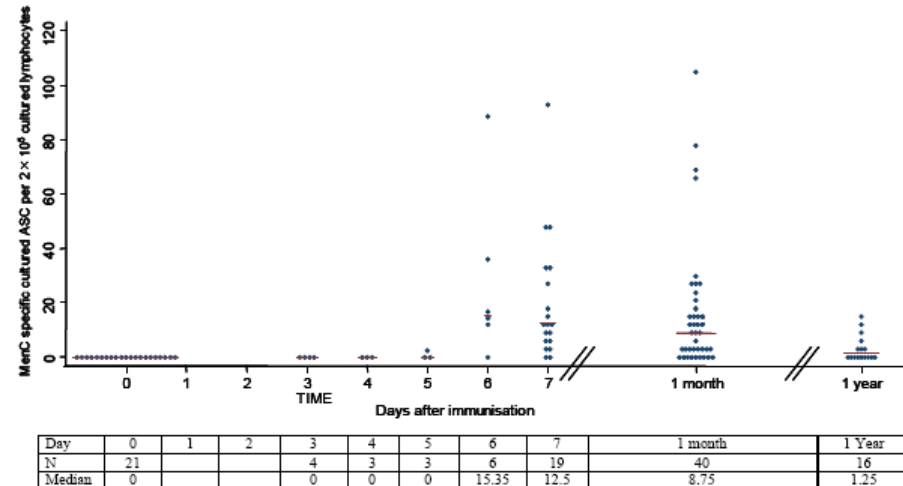


Figure 22: MenC-plasma cell (top-1b) and memory B cell response (bottom-3b) following immunisation with MenCV in teenagers previously primed with MenCV.

3.5.4 Why CRM197-specific B cell frequency was lower than diphtheria toxoid-specific B cell frequency?

In this study, frequencies of diphtheria toxoid-specific plasma cells or memory B cells were always greater than the frequencies of CRM₁₉₇-plasma cells or memory B cells.

Non-toxic variants of diphtheria toxoid known as the cross-reacting materials (CRMs) are produced by different bacteriophages. In comparison to diphtheria toxoid, CRM₁₉₇ contains a mutation where a glycine residue has been replaced by a glutamic acid residue in the catalytic domain of the toxin molecule. This small structural difference to the wild type toxoid makes CRM₁₉₇ more sensitive to proteolytic degradation and therefore less immunogenic in comparison. However, it has been shown that CRM₁₉₇ could be stabilised and protected from proteolysis by treatment with formaldehyde [118, 119]. It has been demonstrated in several studies that diphtheria toxoid and CRM₁₉₇ were equally effective immunogens if the latter had been treated with a certain concentration of formalin [118]. However, it has been found in a study by McNeela *et al.* that although the effects of formaldehyde treatment on antibody responses are beneficial, T cell responses to CRM₁₉₇ were progressively reduced with increasing concentration of formaldehyde [120]. This suggests that formaldehyde treatment might result in the alteration or loss of T cell epitopes, interfering with TCR recognition of the antigen processed and presented by MHCII molecules. These observations might explain the differences between CRM₁₉₇-specific and diphtheria toxoid-specific B cell frequencies found in this study.

3.5.5 Expression of the memory B cell frequency as a percentage of total IgG ASCs

Different research groups have expressed frequencies of memory B cells per total PBMCs, such as Bernasconi *et al.* [13] and Dorfman *et al.* [121]. Others groups, such as Nanan *et al.* [11] or Crotty *et al.* [12, 115], expressed frequency of memory B cells as a percentage of total IgG-secreting B cells. It has been suggested that it is more accurate to express memory B cell frequency as a percentage of total IgG-secreting B cells. This is because the number of memory cells observed in the ELISpot assay after 5 days culture does not directly reflect the number of specific memory cells seeded into a culture well, but despite *in vitro* expansion the ratio of memory B cells per total IgG memory B cells does not change. However, in this study and other studies of adults from the same laboratory, it has been found that the total number of IgG-memory cells varied in one individual over time and that the frequency of total IgG-spots detected by the ELISpot assay was too high and therefore less reliable. It was therefore decided in the present study to express the frequency of memory B cells per the total frequency of PBMCs. Results were expressed per 2×10^5 PBMCs (for plasma cells) or 2×10^5 cultured lymphocytes (for memory B cells), rather than as percentage of total IgG-B cells. Although the totals of IgG plasma and memory B cells were highly variable in one individual over time, the same bias was applied for all individuals, all antigens and all time points.

It is also important to mention that assessment of plasma cells and memory B cells was performed using the ELISpot assay and the only difference was the fact that memory B cells assessment was performed after polyclonal stimulation during 5 days of culture.

Chapter 4: Conclusions

This study has shown that MenC-specific memory B cells were infrequently detected in the peripheral blood of infants of one year of age who had been primed previously with three doses of MenCV at 2,3 and 4 months of age. Furthermore, this study has shown that MenC-specific plasma cells and memory B cells appeared rapidly following immunisation with MenCV. Given the rapidity of this B cell response following immunisation, these findings suggest that although memory B cells were not detectable in peripheral blood before immunisation they might persist after priming, and home in secondary lymphoid organs ready to rapidly differentiate and proliferate upon re-challenge. These findings are important since it is known that MenC-specific antibody levels are not sustained after a primary immunisation with MenCV in infancy [5, 6].

However it has been suggested that in case of low baseline level of anti-capsular antibodies, mechanisms of memory immunity (inducing rapid production of high quality specific antibodies) might be too slow to protect an individual in the immediate hours or days following exposure to invasive meningococcus [67].

It has been shown that antibody responses following re-challenge with the antigen are not detectable earlier than 4 days following antigen exposure, even in primed individuals. This suggests that the only way to protect children from invasive meningococcal disease might be the induction of sustained anti-capsular antibodies. It is hoped that a booster dose of MenCV at one year of age, when the immune system is more mature, will induce a better persistence of plasma cells and memory B cells to sustain serum antibody levels.

Further studies are now essential to better understand the development of long-term protection against protein-polysaccharide conjugate vaccines given in infancy, in particular the relationship between plasma cells/memory B cells and long term humoral immunity. Such data may permit adaptation of the infant vaccine schedule to provide sustained protection against encapsulated bacteria and reduce infant mortality.

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