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B Cell Memory to a Serogroup C Meningococcal Conjugate Vaccine in Childhood and Response to Booster: Little Association with Serum IgG Antibody

Kirsten P. Perrett,*†‡ Celina Jin,*† Elizabeth Clutterbuck,*† Tessa M. John,*† Amy P. Winter,*† Elizabeth Kibwana,*† Ly-Mee Yu,§ Nigel Curtis,‡ and Andrew J. Pollard*†

The maintenance of adequate serum Ab levels following immunization has been identified as the most important mechanism for individual long-term protection against rapidly invading encapsulated bacteria. The mechanisms for maintaining adequate serum Ab levels and the relationship between Ag-specific memory B cells and Ab at steady state are poorly understood. We measured the frequency of circulating serogroup C meningococcal (MenC)-specific memory B cells in 250 healthy 6- to 12-y-old children 6 y following MenC conjugate vaccine priming, before a booster of a combined *Haemophilus influenzae* type b-MenC conjugate vaccine and then 1 wk, 1 mo, and 1 y after the booster. We investigated the relationship between circulating MenC-specific memory B cell frequencies and Ab at baseline and following the booster vaccine. We found very low frequencies of circulating MenC-specific memory B cells at steady state in primary school-aged children and little association with MenC IgG Ab levels. Following vaccination, there were robust memory B cell booster responses that, unlike Ab levels, were not dependent on age at priming with MenC. Measurement of B cell memory in peripheral blood does not predict steady state Ab levels nor the capacity to respond to a booster dose of MenC Ag. *The Journal of Immunology*, 2012, 189: 2673–2681.

For most countries, without access to timely specialized intensive care, invasive meningococcal disease remains a serious global public health problem, causing significant mortality and morbidity (1, 2). The introduction of serogroup C meningococcal (MenC) polysaccharide–protein conjugate vaccine in the United Kingdom and other developed countries had an immediate and profound effect on the incidence of invasive MenC disease in the targeted age groups (3–5). However, postlicensure studies demonstrated that, despite excellent short-term immunogenicity, substantial reduction in carriage (6), and a resultant increase in herd immunity (7), vaccine effectiveness and Ab titres wane rapidly in the youngest children (8).

Long-term protection against encapsulated bacteria following immunization with conjugate vaccines depends on the persistence of serum anti-capsular Ab, immunological memory, and herd immunity (9). The in vitro functional assay, the serum bactericidal activity (SBA), which measures bactericidal killing by anti-capsular Ab, is the only validated measure of protection against MenC disease. Experience with *Haemophilus influenzae* type b (Hib) conjugate vaccine has demonstrated immunological memory despite low or undetectable Ab levels (10–12). Furthermore, children who do not make a detectable primary Ab response mount immunological memory responses following a booster MenC vaccine (13). These findings suggest that, theoretically, individuals with low or undetectable serum Ab titres may still be protected from disease caused by encapsulated bacteria through the presence of Ag-specific B cell memory cells that can be activated in response to encapsulated bacterial invasion.

However, the presence of immunological memory has been demonstrated in some cases of Hib and MenC vaccine failures (significantly higher convalescent Ab titres were found in cases than in vaccine-naive controls) (14, 15). Furthermore, Ab kinetic studies in primed adolescents showed that it takes 5 d to increase serum functional Ab following MenC polysaccharide antigenic challenge (16). In contrast, an invasive strain of meningococcus may invade in hours. These data suggest that for rapidly invading encapsulated bacteria, the presence of immunological memory alone, without adequate levels of functional Ab, cannot guarantee protection (9, 17). Accordingly, the most important determinant identified for individual long-term protection against encapsulated bacteria is the maintenance of adequate serum Ab levels following immunization. Understanding the mechanisms for maintaining serum Ab is therefore critical for individual protection and tailoring vaccine design against encapsulated bacteria.

Memory B cells are thought to be responsible for the secondary (anamnestic) immune response to an Ag (18–20) and, indirectly, Ab persistence (18), by replenishing the pool of long-lived Ag-specific plasma cells [which are responsible for the continuous maintenance of serum Ab levels (21–23)]. It is thought that at steady state (>1 y following vaccine Ag encounter), memory
B cells, plasma cells, and Abs are part of a robust homeostatic system (24). The exact nature of the long-term relationship between memory B cells, plasma cells, and Ab levels following MenC immunization during childhood has not been previously described.

We measured the circulating MenC-specific memory B cells in 250 children, aged 6–12 y of age who had been immunized 6 y previously between 2 mo and 6 y of age in the United Kingdom MenC mass immunization campaign (25). A Hib-MenC conjugate booster vaccine was then given to all children. We assessed the frequency of MenC-specific memory B cells at 1 wk, 1 mo, and 1 y and MenC-specific plasma cells at 1 wk following the booster. MenC SBA titers and IgG concentrations were measured at all time points (data previously published) (25). The relationship between circulating MenC-specific memory B cell frequencies or Ab at baseline and B cells and Ab following the booster was investigated.

Materials and Methods

Study design and participants

An open-label phase IV study was done in Oxfordshire, United Kingdom, from September 2006 to July 2008. Details of the study design, recruitment, inclusion, and exclusion criteria have been previously described (25). Briefly, participants were healthy, aged 6–12 y of age, had received all United Kingdom scheduled immunizations, and had received primary MenC vaccine during the 1999–2001 United Kingdom MenC mass immunization campaign. Participant’s individual MenC immunization history was verified from medical or centralized immunization records. Children were stratified into seven groups on the basis of their MenC immunization history (age of child at receipt of MenC vaccine) and age at enrollment. Ethical approval was obtained from the Oxfordshire Research Ethics Committee (06/Q1605/100). The study was registered at http://www.controlled-trials.com (ISRCTN77285898).

Vaccines and blood sampling

At study enrollment, a maximum of 10 ml blood was obtained by venepuncture and placed in tubes containing heparin. All participants subsequently received a single booster dose of a Hib-MenC conjugate vaccine (Menitorix; GlaxoSmithKline Vaccines) administered i.m. into the deltoid with a 23-gauge, 25-mm needle. Additional blood samples were collected at 1 wk (in 75 participants; 25 participants each from groups I, III, and VII), 1 mo, and 1 y after administration of the Hib-MenC booster vaccine (Fig. 1).

Preparation of PBMCs

PBMCs were isolated by density-gradient centrifugation, as previously described (26). Briefly, a maximum of 7 ml heparinized blood was available for the separation of PBMCs. Whole blood was diluted with an equal volume of RPMI 1640 medium (Sigma-Aldrich) to which penicillin-streptomycin solution (Sigma-Aldrich) and 200 mM L-glutamine had been previously added at a dilution of 1/100 (complete medium). The PBMCs were separated by density-gradient centrifugation over Lymphoprep (Axis Shield). The PBMCs were then washed with complete medium and resuspended in complete medium to which inactivated newborn bovine serum (Sigma-Aldrich) had been previously added at a dilution of 1/10. The final concentration prior to ELISPOT assay or cell culture was 2 × 10^6 cells/ml.

Preparation of ELISPOT plates

Multiscreen 96-well plates with a polystyrene nitrocellulose membrane (Millipore) were coated with 5 µg/ml MenC polysaccharide (National Institute of Biological Standards and Controls, U.K.), conjugated to methylated human serum albumin (National Institute of Biological Standards and Controls) or 10 µg/ml goat anti-human Ig (Caltag Laboratories). Sterile PBS was added to the Ag blank wells. The coated plates were sealed and stored at 4°C.

Detection of MenC-specific memory B cells

PBMCs were stimulated for 5 d with polyclonal stimuli to induce development of memory B cells to Ab-secreting cells (ASC). The B cell stimulation culture was prepared by adding 100 µl PBMCs/well to a 96-well round-bottom cell culture plate (Greiner OneBio) to give a concentration of 2 × 10^5 PBMCs/well. Then 100 µl optimized polyclonal stimulant mix was added to each well. The stimulated medium final well concentrations were as follows: 1/5000 Staphylococcus aureus Cowan (Calbiochem), 83 ng/ml PWM (Sigma-Aldrich), and 2.5 µg/ml CpG (InvivoGen). A minimum of 20 wells was cultured for each participant at each time point. The PBMCs were stimulated in vitro for 6 d at 37°C, 5% CO₂, and 95% humidity. On day 6, the cultured cells were harvested, pooled, washed three times, resuspended to yield a concentration of 2 × 10^6 cultured PBMCs/ml, and plated onto precoated ELISPOT plates, as described for plasma cells below.

Detection of MenC-specific plasma cells

MenC-specific IgG ASC were detected by adding 100 µl in vitro stimulated or fresh PBMCs per well to the precoated ELISPOT plates (giving a total of 2 × 10^5 PBMCs/well) and incubated overnight at 37°C, 5% CO₂, and 95% humidity. After 16–20 h, unbound cells and supernatant were discarded and wells were washed. A 1/5000 dilution of goat anti-human IgG–alkaline phosphatase conjugate (Calbiochem) was added to bound Ab secreted by the cells. Bound conjugate was revealed with the addition of a substrate buffer (alkaline phosphatase substrate kit made according to the manufacturer’s instructions; Bio-Rad Laboratories).

ELISPOT counting

Spots were counted using an automated ELISPOT reader (ELR02) and ELISPOT software, version 3.2.3 (Cadam Medical, Stourbridge, U.K.). Automated Ag-counting settings (intensity, size, gradient, and emphasis) were identical for each plate and manually checked by an operator blinded to the sample.

Serogroup C meningococcal polysaccharide serum bactericidal activity assay and ELISA

Serum was separated from a maximum of 3 ml blood and stored in up to 5 cryovials at or below −20°C. MenC SBA assays and MenC polysaccharide-specific IgG Ab concentration were determined using standard SBA and ELISA methods, as previously described (25). The MenC SBA assays used baby rabbit complement, reference strain C11 (C:16:P1.7-1,1), and were done by the Vaccine Evaluation Unit (Manchester, U.K.).

Statistical analysis

ELISPOT assays that contained <1000 total IgG spots/million PBMCs were excluded from the analysis (representing assays with failed in vitro stimulation). The mean number of spots counted in the two Ag blank wells was subtracted from the mean spot count for MenC from the replicates. B cell frequencies of Ag-specific plasma (fresh) cells or Ag-specific memory B cells (cultured lymphocytes) were expressed per million PBMCs. All ELISPOT data were analyzed using Excel 2007 software (Microsoft Office), Stata software (version 10; StataCorp), and SAS software (version 9.1).

Results

Participants

Full demographic results have been published previously (25). In brief, of 250 enrolled participants, 249 received the Hib-MenC booster, 244 (98%) were included in the analysis, and 230 (92%) completed all study visits. The mean age of enrollment was 9.1 y; age range 6.4 (group I)–12.6 (group VII). The mean age at primary MenC immunization ranged from 0.2 (group I) to 5.8 (group VII) y, and the mean interval since MenC immunization was 6.75 y (SD, 0.36 y).

Frequency of circulating MenC-specific memory B cells at baseline

The frequency of circulating MenC-specific memory B cells at baseline, 6 y following priming MenC immunization in 6–12-y-old children (study enrollment), was calculated by age groups (Table I, Fig. 1A). Circulating MenC-specific memory B cells were found infrequently in all age groups. MenC-specific memory B cell geometric mean frequency (GMF) ranged from 0.57 (95% CI: 0.27–1.20) in group I (mean age 7.0 y, mean age at priming MenC vaccine 0.2 y) to 2.18 (95% CI: 1.01–4.67) in group VI (mean age...
12.1 y, mean age at priming MenC 5.8 y). Overall, 84 of 244 (34%) participants had ≥5 MenC-specific memory B cells per million PMBCs. There was no statistically significant difference across the age groups (p value test for trend = 0.14).

Frequency of plasma and memory B cells 1 wk post–Hib-MenC booster vaccine in 6-, 7-, and 12-y-old children

Seven days after the Hib-MenC booster vaccine, a very high frequency of circulating MenC-specific IgG plasma cells was detected. The geometric mean frequency of MenC-specific plasma cells per million ASC ranged from 551.6 (95% CI: 218.86–1390.02) to 704.8 (95% CI: 390.1–1273.5). There was no statistically significant difference across the age groups (Table II, Fig. 2).

MenC-specific memory B cells were seen at significantly higher frequencies 7 d after the Hib-MenC booster than at baseline. The geometric mean frequency of MenC-specific memory B cells ranged from 83.3 (95% CI: 51.8–134) in group I to 146 (95% CI: 95.9–222) in group III. Overall, 60 of 61 (98%) participants had ≥5 MenC-specific memory cells per million PMBCs (range 1.25–1000). There was no statistically significant difference across the age groups (Table I, Fig. 3B). One week postbooster, the median frequency of circulating MenC-specific IgG memory B cells was significantly higher than at steady state (prebooster): 0.29% of total circulating IgG memory B cells.

Frequency of MenC-specific memory B cells 1 mo post–Hib-MenC booster vaccine in 6- to 12-y-old children

At 1 mo post–Hib-MenC booster, the frequency of circulating MenC-specific IgG memory B cell GMF ranged from 23.9 (95% CI: 13.1–43.8) in group I to 110 (95% CI: 82.8–147) in group IV. There were ≥5 MenC-specific memory B cells per million cultured PBMCs seen in 97% of participants at 1 mo. Again the p value test for trend across the age groups did not reach significance (Table I, Fig. 3B). One month postbooster, the median frequency of circulating MenC-specific IgG memory B cells was significantly higher than at steady state (prebooster): 0.17% of total circulating IgG memory B cells.

Frequency of MenC-specific memory B cells 1 y post–Hib-MenC booster vaccine in 6- to 12-y-old children

One year postbooster, circulating MenC-specific memory B cells had declined from the 1-mo frequencies, and no significant test for trend was seen between the groups (p = 0.72). However, frequencies of circulating MenC-specific memory cells remained higher than at baseline (63% of participants had ≥5 MenC-specific memory cells per million cultured PBMCs) (Table I, Fig. 3B).

Correlation of immunogenicity measures

Spearman correlation, adjusting for age at baseline, was applied to MenC-specific memory B cells and MenC-specific IgG at baseline or steady state and memory B cells, IgG, SBA, and plasma cells at other relevant time points (Table III). MenC IgG geometric mean concentration (GMC) and MenC SBA GMT data for baseline and 1 mo and 1 y postbooster were previously published (25).

There was a weak correlation between the frequency of circulating MenC-specific memory B cells and MenC-specific IgG at baseline (r = 0.19, p = 0.003) at steady state (study enrollment or baseline) 6 y following priming MenC immunization.

There was also a weak correlation between the frequency of circulating MenC-specific memory B cells at baseline and MenC-specific memory B cell frequencies at 1 mo (n = 225, r = 0.26, p < 0.001) and 1 y postbooster (n = 213, r = 0.25, p < 0.001). The frequency of circulating memory B cells 1 wk postbooster was
also related (although not statistically significant at \( p < 0.05 \) cutoff) to the circulating frequency at steady state (\( n = 61, r = 0.24, p = 0.06 \)).

In addition, there were correlations between Ab measures: a weak correlation between MenC-specific IgG at baseline and MenC IgG at 1 mo (\( n = 231, r = 0.15, p = 0.03 \)) and a somewhat higher correlation between MenC-specific IgG and MenC IgG at 1 y (\( n = 225, r = 0.38, p < 0.0001 \)). Furthermore, MenC IgG at baseline correlated with SBA at steady state (\( n = 243, r = 0.31, p < 0.001 \)) and weakly at both 1 mo (\( n = 231, r = 0.13, p < 0.04 \)) and 1 y (\( n = 225, r = 0.13, p < 0.049 \)).

**Discussion**

**Frequency of MenC-specific memory B cells at steady state**

To our knowledge, this is the first study to describe the frequency of circulating memory B cells at steady state, some years following priming immunization to a polysaccharide–protein conjugate vaccine in early childhood. This study showed that the frequency of circulating MenC-specific memory B cells in primary school-aged children at steady state is low. Specifically, only one-third (84 of 244 or 34%) of 6- to 12-y-old children had ≥5 circulating MenC-specific memory B cells per million cultured PBMCs, 6 y following MenC priming. The frequency of circulating MenC-specific memory B cells in primary school-aged children was not dependent on age at priming and showed only a weak association with MenC-specific IgG at steady state. Furthermore, to our knowledge, this was also the first study to assess the B cell response to a MenC booster given >5 y following priming in young children.

Previous studies on the frequency of circulating memory B cells at steady state following priming immunization are few. Age-specific memory B cells have been detected in peripheral blood many years following immunization of adults with protein vaccines. In one study there was a frequency of 0.01–1% of total IgG-secreting memory B cells for diphtheria and 0.1–1% for tetanus (27). Another study detected smallpox-specific memory B cells >50 y following immunization at a frequency of 0.1% of total IgG-secreting memory cells (23).

In this study, the median frequency of MenC-specific memory B cells was 0.005% of total circulating IgG memory B cells; GMF ranged from 0.57 in group I children (mean age 7.0 y, mean age at priming MenC 0.2 y) to 2.18 in group VI (mean age 12.1 y, mean age at priming MenC 5.8 y). A previous study of the frequency of MenC-specific memory B cells in toddlers following a three-dose priming schedule found that 7 of 23 (30%) had ≥5 MenC-specific memory B cells per million cultured PBMCs; the median frequency of MenC-specific memory B cells was 0.02% of total circulating IgG memory B cells or 3.5 MenC-specific memory B cells per million cultured PBMCs (28). Whereas a similar percentage of toddlers had ≥5 MenC-specific memory B cells per million cultured PBMCs as the children in our study (~30%), there was a higher median frequency of MenC-specific memory B cells in the toddler study. This is likely to be related to the more recent time since priming, 8 mo in the toddlers compared with 6 y in this study.

Table II. GMF of MenC-specific plasma cells per 10^6 PBMC, concentration of MenC IgG and titres of MenC SBA, 1 wk post–Hib-MenC booster

<table>
<thead>
<tr>
<th>Group (Age at Baseline, Years)</th>
<th>I (7.0)</th>
<th>III (8.3)</th>
<th>VII (12.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MenC plasma cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>GMF (95% CI)</td>
<td>574 (428; 768)</td>
<td>567 (402; 798)</td>
<td>722 (581; 898)</td>
</tr>
<tr>
<td>MenC IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>GMC (95% CI)</td>
<td>15.6 (9.8; 25.0)</td>
<td>21.1 (12.7; 35.1)</td>
<td>22.1 (15.6; 31.3)</td>
</tr>
<tr>
<td>MenC SBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>GMT (95% CI)</td>
<td>12,417 (8,328; 18,512)</td>
<td>20,427 (12,007; 34,750)</td>
<td>20,245 (13,067; 31,367)</td>
</tr>
</tbody>
</table>

MenC IgG GMC and MenC SBA GMT results for baseline, 1 mo, and 1 y time points have been previously published (25). GMT, Geometric mean titer.
This study also measured the frequency of Hib, tetanus, and diphtheria memory B cells at steady state in 6- to 12-y-old children. These were enumerated at a frequency of 0.001, 0.04, and 0.04% of total circulating IgG memory B cells, respectively (data not shown). These data support the suggestion that memory B cells specific for protein Ags (e.g., tetanus and diphtheria) may persist better than polysaccharide-specific (e.g., MenC and Hib) memory B cells in children (28). Alternatively, the ELISPOT assay may have greater sensitivity for protein than polysaccharide Ags.

Another study measured the frequency of MenC-specific memory B cells in adolescents who had been primed with MenC conjugate vaccine 3–4 y previously (29) and found that only 8% (3 of 37) had measurable MenC-specific memory B cells compared with 34% (84 of 244) of children 6 y following MenC priming in our study. However, it is possible that other adolescents had memory B cells that were not detected in the former study for two reasons. First, the assay sensitivity in the adolescent study was low (0.05%) due to limited blood volume (29). Second, the in vitro stimulants used for polyclonal activation were different. In the former study, these were SAC and IL-2 compared with the combination of SAC, PWM, and unmethylated CpG used in the toddler study (28) and this study. SAC is known to cross-link the BCR; CpG acts through TLR9 (30); and IL-2 and PWM act as costimulatory molecules to stimulate the proliferation of activated B cells and T cells present in the PBMC culture. Crotty et al. (20) studied a number of different mitogens in a variety of combinations for maximal polyclonal proliferation of circulating human IgG memory B cells and differentiation into ASC, and identified that the combination of PWM, SAC, and CpG was the best combination to culture with PBMCs for maximal detection of IgG ASC.

**FIGURE 2.** Geometric mean frequency of MenC-specific plasma B cells 1 wk post–Hib-MenC booster in 7-, 8-, and 12-y-old children ≥6 y following priming with a MenC conjugate vaccine (error bars depict 95% CI).

This study also measured the frequency of Hib, tetanus, and diphtheria memory B cells at steady state in 6- to 12-y-old children. These were enumerated at a frequency of 0.001, 0.04, and 0.04% of total circulating IgG memory B cells, respectively (data not shown). These data support the suggestion that memory B cells specific for protein Ags (e.g., tetanus and diphtheria) may persist better than polysaccharide-specific (e.g., MenC and Hib) memory B cells in children (28). Alternatively, the ELISPOT assay may have greater sensitivity for protein than polysaccharide Ags.

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**FIGURE 3.** Geometric mean frequency of MenC-specific IgG memory B cells per 10⁶ cultured PBMC at: (A) baseline and (B) baseline, 1 wk, 1 mo, and 1 y post–Hib-MenC booster, by age at baseline (error bars depict 95% CI).
MenC-specific memory B cells at steady state are not dependent on age at priming

This study showed that the frequency of MenC-specific memory B cells at steady state was low in all enrolled primary school-aged children, regardless of the age at MenC priming. This finding was not unexpected. At steady state the majority of memory B cells are thought to reside in the peripheral lymphoid tissue (31, 32). Therefore, the number of circulating Ag-specific memory B cells at steady state may not accurately reflect the total number of Ag-specific memory B cells available to be activated (to proliferate, differentiate into Ag-specific plasma cells, and secrete Ab) on re-encounter with Ag.

The finding of low numbers of circulating MenC-specific memory B cells in all age groups contrasts to the strongly significant age-dependent trend for increasing persistence of functional MenC Ab with age at priming (25). It has been postulated that the poor ability of infants to generate persistent Ab to polysaccharide–protein conjugate vaccines is due to a reduced ability to retain and support long-lived plasma cells in the bone marrow (33, 34). Additional factors include immaturity of the follicular dendritic cell network and therefore limited germinal center responses (35), limited costimulatory signals (e.g., CD21), decreased and/or delayed affinity maturation, and limited plasma cell supporting factors. For example, decreased expression of CD40 and TNFR family member ligands (BAFF and a proliferation-inducing ligand [APRIL]) results in decreased responses to their corresponding ligands (CD40L, expressed by T cells) and receptors (TACI, BCMA, and BAFF-R).

Recently, studies in mice have discovered that both the protein and carbohydrate components of the glycoconjugate are processed by the B cell-generating glycan peptides. MHCII binds the peptide portion, allowing the carbohydrate (glycan) epitope to be presented to the CD4+ T cell (36). It is now thought that the variable immunogenicity of certain glycoconjugates may be actually related to the efficiency of carbohydrate presentation. Critical to this interaction are the costimulatory molecules (e.g., CD40/CD40L) and T cell production of cytokines (e.g., IL-4 and IL-2), which induce maturation of the B cell. It is likely that all of these and other as yet unknown mechanisms that govern glycoconjugate processing and presentation by B cells to CD4+ T cells are immature in infants and result in defects in isotype switching, Ag presentation, germinal center development, and memory responses by B cells (35, 37, 38).

Consequently, it might be expected that children (primed with MenC at 6 y of age) would have higher postbooster B cell responses than infants, as they would have produced more long-lived plasma cells and memory B cells following priming immunization. Therefore, it might be expected that the frequency of MenC-specific plasma or memory B cells enumerated at day 7 post-booster more accurately reflects the total Ag-specific B cell pool and may demonstrate age-dependent differences.

MenC-specific plasma cell immune responses postbooster

Plasma cells are not detectable in peripheral blood at steady state (29, 39). They are thought to reside predominantly in the bone marrow (40, 41). Hence, the measurement of Ag-specific plasma cells is possible during a limited window following immunization. Plasma cells in primed individuals are seen as early as day 4, peak by day 6–7, and are not detectable by day 12 following immunization (29, 39, 42). This window represents the period of migration of newly generated plasma cells (derived from Ag-specific activation of naive B cells that have proliferated and differentiated into Ag-specific ASC) from the germinal centers of lymphoid tissues to the bone marrow and mature plasma cells (derived from pre-existing Ag-specific memory B cells generated at priming that have differentiated and proliferated) and released into the circulation to secrete Ab.

In this study, MenC-specific plasma cells were detected at very high frequencies 7 d post–Hib-MenC booster in all three age groups measured, and perhaps surprisingly there was no statistically significant difference (test for trend) in frequency of MenC-specific plasma cells between the groups. This may be due to the limited number of children with blood drawn at this time point (n = 20, 22, and 23 for groups I, III, and VII at mean ages of 7.0, 8.3, and 12.1 y, respectively). It is also possible that 7 d postbooster, the Ag-specific plasma cells being measured are predominantly newly generated plasma cells, rather than mature plasma cells. The effectiveness of MenC polysaccharide–protein conjugate vaccine given to school-aged children is similar (3), so it follows that the plasma cell response (frequency of circulating newly generated MenC-specific plasma cells 7 d postbooster) in this study’s 6- to 12-y-old children might be similar. Future research possibly using cell sorting and fluorescent labeling techniques may be able to determine whether the Ag-specific plasma cells measured postbooster are newly generated or mature.

The frequency of MenC-specific plasma cells 7 d postbooster was not statistically associated with the frequency of MenC-specific memory B cells or IgG at steady state. This finding was not unexpected as all age groups had very low frequencies of MenC-specific memory B cells at steady state and the circulating

### Table III. Correlation between MenC memory B cells and Ab (IgG) at steady state (6 y following priming MenC immunization) in 6- to 12-y-old children with the Ab, plasma, and memory B cells measured at 1 wk, 1 mo, and 1 y post–Hib-MenC booster (Spearman correlation, adjusting for age at baseline)

<table>
<thead>
<tr>
<th></th>
<th>MenC-Specific Memory B Cells at Steady State</th>
<th>MenC-Specific IgG at Steady State</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>r</td>
</tr>
<tr>
<td>Memory B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>61</td>
<td>0.24</td>
</tr>
<tr>
<td>1 mo</td>
<td>225</td>
<td>0.26</td>
</tr>
<tr>
<td>1 y</td>
<td>213</td>
<td>0.25</td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>242</td>
<td>0.19</td>
</tr>
<tr>
<td>1 mo</td>
<td>230</td>
<td>0.02</td>
</tr>
<tr>
<td>1 y</td>
<td>224</td>
<td>0.15</td>
</tr>
<tr>
<td>SBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>65</td>
<td>0.12</td>
</tr>
<tr>
<td>1 mo</td>
<td>230</td>
<td>0.009</td>
</tr>
<tr>
<td>1 y</td>
<td>224</td>
<td>0.12</td>
</tr>
<tr>
<td>Plasma cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>65</td>
<td>−0.15</td>
</tr>
</tbody>
</table>
memory B cell frequency may not be relied upon to be proportional to the total Ag-specific memory B cell pool. Determining whether the high plasma cell frequencies at day 7 postbooster reflect mostly newly generated or mature plasma cells requires further study.

**MenC-specific memory B cell immune responses postbooster**

The accurate measurement of human memory B cells is complicated as the majority of these cells are thought to reside in peripheral lymphoid tissue (31, 32). Memory B cells have been consistently shown to increase in frequency in the circulation following immunization (23, 27, 29, 39), when newly generated Ag-specific memory B cells may be transiting through the circulation to other lymphoid tissues. Following a booster, Ag-specific memory B cells are detected at the end of the first week and are persistent for at least 1 mo (28, 29, 39).

Following the Hib-MenC booster immunization, almost all children showed robust memory B cell responses, evidence of immune memory to priming MenC conjugate vaccine. As is the case for Ag-specific plasma cells, human phenotypic markers distinguishing newly generated memory B cells from recirculating memory B cells are poorly understood. It is assumed that the IgG ASC detected are of memory B cell origin [as they have CD27⁺ memory B cell surface expression (43), and mature plasma cells are unable to survive more than 2–3 d in cell culture (33, 44)]; however, there may be different populations of somatically mutated memory B cells originating from the germinal center compartments or preplasma cells (plasmablasts) involved. Further research is required to delineate the subpopulation of B cells stimulated to proliferate in the ELISPOT assay. This might involve phenotyping and cell sorting to determine whether removing the B cell subset results in the removal of the IgG ASC. Further research in this area will help to determine what proportion of MenC-specific memory B cells at 7 d and 1 mo postbooster is newly generated and whether this proportion is dependent on age at priming in childhood.

**Correlation between circulating MenC-specific memory B cells and Ab levels at steady state and postbooster**

This study found little association between the frequency of circulating MenC-specific memory B cells and persistence of Ab in steady state in primary school children (6 y following MenC priming).

Previous studies in adults have found strong correlations between circulating memory B cells and Ab levels at steady state for smallpox (23), measles and tetanus (45), and plasmodium falciparum Ags (46). These studies suggest that even in the absence of re-exposure to an Ag, memory B cells continuously divide and differentiate into plasma cells either spontaneously or via intermitting Ag-independent mechanisms such as microbial products (polyclonal stimulation of B cells via TLRs), cytokines (secreted by T cells activated by another Ag, also known as bystander T cell help), or possibly other as yet unknown stimuli (24). Consequently, this theory suggests that memory B cells continuously recirculate through the blood and secondary lymphoid organs (27), and therefore circulating levels of Ab are in proportion to the total Ag-specific memory B cell pool that is reflected by the frequency of circulating Ag-specific B cells. This theory is supported by a B cell kinetic study in humans (47). Using in vivo labeling of dividing cells, cell sorting, and gas chromatography–mass spectrometry, the authors showed that naive B cells divide slowly (0.46% per day), whereas memory B cells proliferate more rapidly (2.66% per day). This supports the view that B cell memory may be maintained independently of Ag by clones of spontaneously proliferating B cells (47).

The authors of another study that did not find a correlation between circulating memory B cells and Ab suggest that long-term persistence of Ab is provided by long-lived plasma cells rather than the differentiation of memory B cells into short-lived Ab-secreting plasma cells (48). This hypothesis suggests that long-lived plasma cells are predominantly responsible for the maintenance of long-term persistence of Ab, independent of memory B cells (31, 41, 48, 49). It is thought that Ag-specific long-lived plasma cells may live for decades, possibly in bone marrow niches (18, 41).

In our study, there was neither a strong correlation between memory B cells and Ab at steady state or postbooster nor were there age-dependent differences in frequencies of circulating MenC-specific memory B cells at steady state or any time postbooster. These findings are in contrast to strongly significant changes in concentration of MenC-specific Ab with age at priming immunization with MenC vaccine (25). Siegrist and Aspinall (35) suggest that the large load of environmental Ags in early life causes competition between plasma cells for access to a limited set of survival niches in bone marrow. Therefore, infants and young children have a suboptimal bone marrow plasma-cell pool and poor maintenance of persistent Ab. In contrast, primary immunization of older children and adults, who have less exposure to novel environmental Ags, lay down more long-lived plasma cells for the maintenance of persistent Ab. It is also suggested that early-life limitations of B cell responses result in a skewed response toward the production of memory B cells rather than long-lived plasma cells in infancy and young childhood. Findings from studies of neonatal immunization lend support to this theory. The administration of a vaccine at birth (e.g., hepatitis B) may fail to elicit Ag-specific serum Abs, but may still be able to prime for a secondary response (35). In addition, infants respond poorly to immunization with pure polysaccharide vaccines (T-independent Ags). Vaccination may induce tolerance or hyporesponsiveness to further doses rather than prime for a secondary response (50).

Our findings found similar levels of circulating memory B cells at steady state in children who received MenC priming in infancy up to 6 y of age, yet significantly different levels of Ab persistence, consistent with a skewed response to memory B cells rather than long-lived plasma cells in the younger children. We speculate that younger children in our study produced less long-lived plasma cells and accordingly had less persistent Ab measured at steady state (6 y postpriming). The postbooster data found that expansion of memory B cell responses occurred in almost all children regardless of age at priming (2 mo to 6 y of age). However, serum Ab was only maintained (continuously produced independently of persisting immunizing Ag) in older children (we speculate that this was because older children had sufficient long-lived plasma cells laid down in survival niches in the bone marrow at priming).

In a previous study, we reported that the magnitude of the IgG Ab and memory B cells 1 mo postpriming with MenC vaccine in infants was associated with the intensity of the booster response at 12 mo of age (28). In our current study that found no such association between Ab and memory B cells, immune responses were measured at very different time points. Our current study measured steady state immune measures (>6 y postpriming vaccinations) compared with the former study that measured immune responses 1 mo postpriming when Ab responses are at their peak and reflect the magnitude of the priming response. These differences highlight the importance of the time point at which Ab and cellular immune responses are measured following immunization with polysaccharide–protein conjugate vaccines for accurate interpretation of the findings.

In summary, this study found very low frequencies of circulating MenC-specific memory B cells at steady state in primary school-
aged children, 6 y following priming immunization with MenC conjugate vaccine, and showed little association with MenC IgG Ab levels. Children between 6 and 12 y of age showed evidence of immunological memory and expansion of memory B cell responses. MenC-specific memory B cell frequencies at steady state and postbooster, in contrast to serum Ab, were not dependent on age at priming immunization with MenC vaccine. These data lend support to the hypothesis that humoral and B cell memory immunity represent independently controlled forms of immuno-
logic memory. Measurement of B cell memory at steady state in peripheral blood of children does not predict steady state Ab levels or the capacity to respond to a booster dose of MenC Ag. Further research into the understanding of this phenomenon will assist in optimizing vaccines and immunization schedules for the mainte-
nance of protective serum functional Ab against rapidly invading encapsulated bacteria in childhood.

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