Vaccine 27 (2009) 6974–6982

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Kinetics of antibody responses after primary immunization with meningococcal serogroup C conjugate vaccine or secondary immunization with either conjugate or polysaccharide vaccine in adults

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ARTICLE INFO

Article history:
Received 5 June 2009
Received in revised form 4 September 2009
Accepted 20 September 2009
Available online 1 October 2009

Keywords:
Meningococcal
Serogroup C
Conjugate
Kinetics
Antibodies

ABSTRACT

In the Netherlands the meningococcal serogroup C conjugate (MenCC) vaccine is administered as a single dose at 14 months. We evaluated the kinetics of isotype-specific antibodies in adults ($n = 21$) after primary immunization with MenCC or secondary immunization with MenCC or plain MenC polysaccharide vaccine. Blood samples were collected prior to immunization and at 6 additional time points, from 3 to 25 days post-immunization. Secondary immunization resulted in 5–10-fold higher IgG titers compared to the primary immunization group, 25 days post-immunization. Prior to the secondary immunization, but 5 years after the first immunization, protective bactericidal antibodies and levels of MenC-specific IgG and IgM were still present. Furthermore, IgG antibodies present before secondary immunization were of higher avidity compared to antibodies produced one month after primary immunization. In addition, secondary immunization with nonconjugated MenC polysaccharide seemed to induce a higher IgG2 response compared to MenCC immunization. The kinetics of the observed secondary immune responses were not really faster than the observed primary responses. However, the rate of increase in antibodies seemed faster than the primary responses, representing a booster response. As the course of infection by *Neisseria meningitidis* can be very rapid, these data support the idea that sustainment of high antibody levels induced by MenCC are important for immediate protection.

1. Introduction

Neisseria meningitidis is one of the major causes of septicaemia and meningitis. Meningococcal serogroups A, B, C, W-135 and Y account for the majority of disease, with serogroups B and C mainly causing endemic disease in Europe. Historically, serogroup B caused most of the clinical cases of meningococcal disease in the Netherlands. At the end of the year 2000, beginning of 2001 a sudden rise in the incidence of *N. meningitidis* serogroup C (MenC) in the Netherlands occurred. Due to this rise and the availability of MenC conjugate (MenCC) vaccines, it was decided to include MenCC into the national immunization programme (NIP) as a single dose vaccination at 14 months of age in 2002. In addition, in a catch-up campaign all persons between the age of 1 and 18 years were offered one dose of MenCC vaccine (94% coverage). The decision for a 1-dose schedule was based on epidemiologic, programmatic and economic reasons [1]. Following introduction, no cases of MenC disease occurred in vaccinated persons, and a sharp decline was observed in unvaccinated age groups in the Netherlands [2], with currently only a few cases occurring in unvaccinated persons.

The MenCC vaccine has been proven to be immunogenic in toddlers and adults after a single dose [3,4], and is able to prime for memory [5,6]. The vaccine induces antibodies specific for the MenC polysaccharide (PS) capsule with bactericidal activity that showed a good correlation with clinical protection [7]. However, it has also been shown that in children immunized at a very early age, before or around 1 year of age, protective antibody levels wane rapidly within 2 years following immunization [8,9]. In contrast, bactericidal antibodies can persist in older children and adolescents (6–15 years of age) up to at least 5 years following a single

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doi:10.1016/j.vaccine.2009.09.082

MenCC immunization [10]. Analysis of the kinetics of bactericidal responses following boosterimmunization show an immediate increase in response within 2–5 days, however these responses do not correlate well with a rise in IgG antibodies [11,12]. This indicates that next to the quantitative IgG antibody levels, also other factors are important for achieving and sustaining immune protection, such as antibody isotype (IgM, IgA or IgG subclasses) and affinity maturation.

Infection with *N. meningitidis* can develop rapidly with the interval between first symptoms and full blown disease as short as 12 h. It is therefore relevant to determine if previously induced memory immunity is able to respond quickly enough upon (re)stimulation. Currently, little is known about the characteristics and development of antibody levels shortly after primary or secondary immunization with MenCC or boostervaccination with MenC PS. Here we describe the kinetics of antibody responses of different (iso)types following the vaccination strategies described above in adults.

2. Materials and methods

2.1. Study

Healthy adult volunteers, 18–55 years of age, with or without a previous history of MenCC vaccination (Neisvac-C, Baxter, USA) were recruited for a comparison of primary immune responses to MenCC or memory immune responses to MenC conjugate or MenC PS (Mencevax A+C (MACPS), GSK, Belgium) vaccines. Persons were excluded if there was evidence of medical treatment that might interfere the study results, acute illness or fever (>38.5 °C) within 2 weeks before enrolment, allergies to any of the vaccine components (by medical history), immune deficiencies, history of any neurological disorder and previous administration of plasma products within the last 6 months and pregnancy. Adults ($n = 7$) with no history of MenCC received 0.5 ml dose of MenCC (containing 10 μg serogroup C polysaccharide conjugated to 10–20 μg of tetanus toxoid); 14 adults who were previously vaccinated with a single dose of MenCC were randomly assigned to receive either a 0.5 ml dose of MenCC or a 0.5 ml dose of MACPS (containing 50 μg of serogroups A and C polysaccharide each). All volunteers gave written informed consent before entering the study. Randomization of both secondary immunization groups was concealed to the point of enrolment. The study was non-blinded for clinical trial staff and participants, however blinded for laboratory personnel. Ethical approval for this study was obtained from the "Central committee on research involving human subjects" (CCMO, The Hague, The Netherlands). The trial is registered at the Dutch Trial register, identifier NTR1419.

In total, 21 subjects were enrolled and 7 persons were included in each group. Blood was withdrawn pre-vaccination and at days 3, 5, 7, 10, 17 and 25 after immunization.

2.2. Serological assays

N. meningitidis serogroup A, C, W-135 and Y specific IgG, IgG1 and IgG2 were detected by a fluorescent-bead-based multiplex immunoassay (MIA) as previously described [13,14]. *N. meningitidis* serogroup A, C, W-135 and Y specific IgA and IgM were also detected by a MIA as described previously [13,14], with the modification of using isotype specific R-phycoerythrin (RPE) conjugated antibodies to detect IgA and IgM antibodies. For the detection of serogroup specific IgA, a goat anti-human IgA RPE conjugate was used (α-chain specific, Southern Biotechnologies, USA) and serogroup specific IgM was detected by a donkey anti-human IgM RPE conjugate (Fc5μ-specific, Jackson ImmunoResearch, West Grove, PA). Standardized reference serum CDC 1992 was used in these assays (NIBSC, Potters Bar, UK). Tetanus and diphtheria IgG specific antibodies were determined by a MIA as described previous by van Gageldonk et al. [15].

The level of MenC-specific functional antibodies was determined by a serum bactericidal antibody (SBA) assay using baby rabbit complement (Pel-Freeze Incorporated, Rodgerson, AZ) as an exogenous source of complement [16]. The target strain for the assay was C11. SBA titers were expressed as the reciprocal of the final serum dilution yielding ≥50% killing at 60 min. SBA titers of <4 were assigned a value of 2 for statistical purposes.

Avidity of MenC-specific IgG antibodies was assessed by using a modification of the MIA for determining MenC-specific IgG [13,14]. Serum samples with an IgG concentration of ≥0.25 μg/ml were adapted to an antibody concentration of 25 ng/ml. Ammonium thiocyanate (NH_4SCN; Sigma–Aldrich, St. Louis, MO) was used to dissociate low-avidity antigen–antibody binding [17]. After the initial 20 min incubation period of polysaccharide-conjugated-beads with serum, 0.5 M NH_4SCN in phosphate buffered saline (PBS) pH 7.2 or PBS only was added for 10 min exactly, followed by a wash with PBS. Residual bound IgG antibodies were detected using a goat anti-human IgG RPE conjugate. Samples were analyzed using a Bio-Plex 200 system in combination with the Bio-Plex Manager software, version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). The avidity index (AI) was the percentage of antibodies that remained bound to the MenC PS-conjugated beads after treatment with NH_4SCN and was calculated as follows: AI = (amount of IgG with NH_4SCN)/(amount of IgG with PBS) × 100.

2.3. PBMC preparation

Blood for peripheral blood mononuclear cell (PBMC) isolation was collected in vacutainer cell preparation tubes (BD, Franklin Lakes, NJ) containing sodium citrate, polyester gel and Ficoll Hypaque solution. PBMCs were separated via density-gradient centrifugation within 2 h. Cells were washed twice in PBS containing 2% fetal calf serum (FCS; Hyclone, South Logan, UT), and finally suspended in Aim-V medium containing 10% FCS and 50 μM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO) and subsequently used for the detection of antibody secreting cells (ASC).

2.4. Ex vivo ELISPOT assay

96-Well Multiscreen-IP plates (PVDF membrane; Millipore, Billerica, MA), were coated with either 5 μg/ml MenC polysaccharide (NIBSC, Hertfordshire, UK) conjugated to methylated human serum albumin (NIBSC), 7 Lf/ml tetanus or diphtheria toxoid (Statens Serum Institut, Copenhagen, Denmark) or 10 μg/ml goat anti-human immunoglobulin G (Cappel, Illkirch, France) in sterile PBS pH 7.2. Plates were stored at 4 °C and used within a month. Isolated PBMCs were suspended to a final concentration of 2×10^6 PBMCs/ml. Plates were blocked with Aim-V medium and a total of 100 μl of the PBMC suspension was added and four serial 2-fold dilutions were made. Plates were incubated overnight at 37 °C in 5% CO_2 and 95% relative humidity. Cells were removed by washing plates with PBS-Tween 20; remaining cells were lysed with pyrogen-free water (B.Braun, Oss, The Netherlands) and bound IgG was detected using a 1/5000 dilution of goat anti-human IgG Fc-specific alkaline phosphatase conjugate (Sigma–Aldrich, St. Louis, MO) in PBS-Tween 20 with 1% goat serum. Spots were visualized by staining with 5-bromo-4-chloro-3-indolyl phosphate in nitro blue tetrazolium (Sigma–Aldrich, St. Louis, MO) dissolved in pyrogen-free water. Spot-forming cells were counted and con-

firmed by visual inspection and expressed as number of spots/10^6 PBMCs.

2.5. Statistical analysis

Specific antibody concentrations in serum samples were calculated as geometric mean concentrations (GMCs), with 95% confidence intervals (CI). Geometric mean SBA titers (GMTs) with corresponding CIs were determined and percentages of SBA titers $\geq$8 and $\geq$128 were calculated at each time point. Within group and between group comparisons of antibody concentrations and ASC numbers at various time points were made using the Wilcoxon signed rank test or the Mann–Whitney test, respectively. No calculations of study power were conducted prior to the start of the study.

The primary outcome measure of this study was the kinetics of the different Ig isotypes in the different immunization groups in order to compare these responses between the three groups. Antibody kinetics among groups were compared with a non-linear mixed effect model as described by Pinheiro and Bates [18]. In this model, the ^{10}log of the antibody concentrations are modeled by non-linear S-shaped function of the number days after immunization, described by four parameters. Each parameter is allowed to vary between persons around a mean group value. These mean group values can be subsequently used to determine differences between the three groups based on the onset of the rise in antibodies and inflection point of the responses. The onset time of rise in antibodies is defined as the point at which the antibody level is at 10% between the lower asymptote and the upper asymptote of the curve. The inflection time is defined as the point where the curve has its steepest slope. Groups were mutually compared for the response of IgG, IgA, IgM and the functional SBA response by calculating the discrepancies between the immunization groups, with 95% confidence intervals, and testing whether these significantly differed from zero ($\alpha = 0.05$).

3. Results

3.1. Inclusion

Between May and December 2007, all participants completed the study. Median age of the primary MenCC immunization group was 32.7 years (SD 9.6). Within the MenCC and MACPS boosterimmunization groups the median age was 23.3 (SD 7.5) and 23.3 (SD 12.1) years, respectively. The mean time since the primary immunization was, on average, 5.1 years (SD 0.3) in the secondary immunization groups. While all participants completed the study, not all blood withdrawals yielded sufficient material. The exact number of samples used for each assay is presented in Table 1.

3.2. Kinetics of meningococcal serogroup C specific IgG, IgA and IgM antibodies

Within the primary MenCC immunization group, pre-immunization GMC for MenC PS-specific IgG was 0.15 μg/ml, whereas a significantly higher IgG concentration was observed pre-immunization in both boostergroups. Six and five out of seven persons revealed levels of IgG $\geq$2 μg/ml in the MenCC and MACPS groups, respectively (Table 1 and Fig. 1a–c). An average 5-fold increase in IgG concentrations was observed between days 5 and 7 within all groups (Table 1 and Fig. 1a–c). In the MACPS boostergroup a 2.6-fold rise in IgG was already seen between days 3 and 5 (Table 1 and Fig. 1c). At day 25 after primary- or secondary immunization a significant rise in IgG was observed for all groups compared to day 0 (Table 1). Re-vaccination with either nonconjugated or conjugated MenC vaccines induces a clear-cut booster response, with a 4.6- and 10.8-fold higher IgG antibody levels at day 25, in the MenCC and MACPS groups, respectively as compared to maximum IgG titers obtained in the primary MenCC vaccination group.

Table 1
MenC-specific antibody responses pre-immunization and at various days after primary or secondary immunization with MenCC vaccine or secondary immunization with MACPS vaccine.

Antibody response to MenC immunization					
Day	*n*	GMC[a] (95% CI)			
		SBA	IgG	IgA	IgM
Primary MenCC immunization					
0	7	2 (na)	0.15 (0.04–0.63)	0.06 (0.02–0.21)	0.09 (0.02–0.44)
3	7	2 (2–2)	0.14 (0.03–0.59)	0.05 (0.01–0.21)	0.09 (0.02–0.43)
5	6	6 (1–26)	0.17 (0.03–0.94)	0.06 (0.01–0.39)	0.19 (0.04–0.83)
7	7	290 (34–2464)	0.95 (0.16–5.78)	1.52 (0.36–6.46)	1.64 (0.54–4.97)
10	6	1699 (563–5127)	10.40 (1.99–54.26)	10.20 (1.57–66.22)	7.05 (2.43–20.44)
17	7	1425 (483–4209)	12.53 (4.10–38.33)	7.30 (1.48–36.07)	6.32 (2.62–15.24)
25	7	943 (328–2709)	11.42 (4.28–30.49)	5.26 (1.05–26.33)	5.38 (2.24–12.92)
Booster MenCC immunization					
0	7	362 (155–845)	6.22 (1.54–25.24)	0.56 (0.20–1.58)	0.78 (0.28–2.19)
3	5	320 (87–1180)	9.88 (1.31–74.47)	0.74 (0.25–2.17)	0.56 (0.13–2.38)
5	6	722 (375–1393)	7.90 (1.79–34.88)	0.62 (0.26–1.48)	0.68 (0.23–2.02)
7	6	1861 (646–5359)	39.42 (15.01–103.50)	2.88 (1.98–4.18)	1.13 (0.50–2.58)
10	7	4308 (2639–7033)	64.42 (29.89–138.80)	4.30 (2.78–6.65)	1.64 (0.88–3.06)
17	7	3621 (2061–6361)	56.09 (28.19–111.60)	2.53 (1.47–4.37)	1.35 (0.71–2.55)
25	7	3475 (1978–6105)	52.33 (26.02–105.30)	1.96 (0.98–3.89)	1.42 (0.63–3.22)
Booster MenC PS immunization					
0	7	280 (163–481)	5.13 (1.39–18.98)	0.34 (0.16–0.71)	0.40 (0.20–0.83)
3	6	278 (141–550)	4.58 (0.90–23.25)	0.40 (0.18–0.87)	0.51 (0.24–1.11)
5	7	612 (412–907)	11.75 (5.30–26.07)	1.04 (0.48–2.25)	1.11 (0.27–4.52)
7	6	1825 (779–4271)	52.46 (20.75–132.70)	4.72 (1.43–15.63)	2.94 (0.65–13.19)
10	7	4873 (2351–10101)	118.70 (62.66–224.90)	10.71 (4.21–27.24)	2.92 (0.82–10.45)
17	7	5472 (2936–10198)	109.10 (50.70–234.70)	9.43 (3.28–27.07)	2.84 (0.83–9.73)
25	7	5164 (2532–10532)	123.50 (56.57–269.70)	8.13 (3.08–21.49)	2.19 (0.71–6.72)

na: not applicable.
[a] SBA titers expressed as geometric mean titers (GMT). IgG, IgA and IgM expressed in μg/ml.

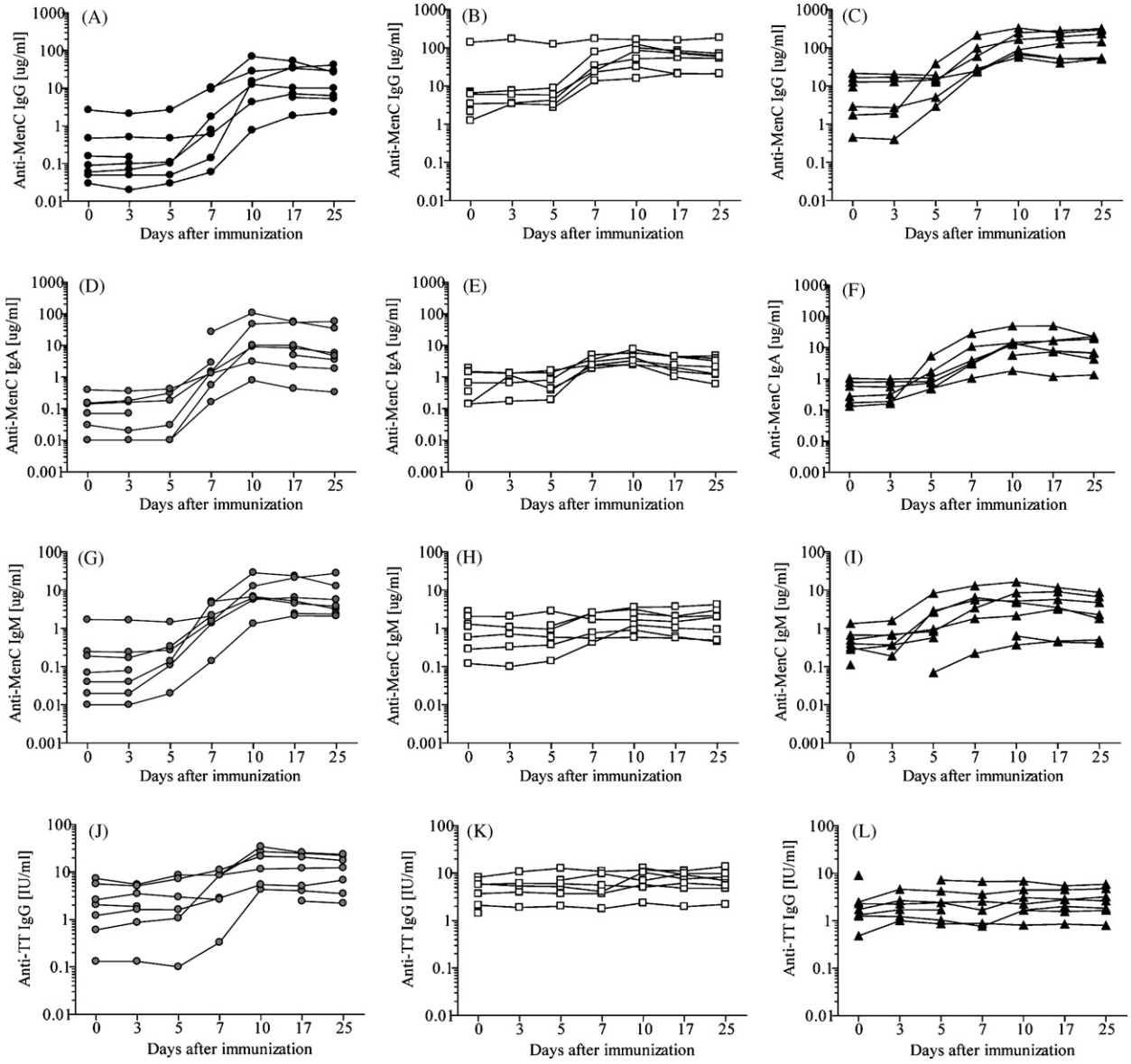

Fig. 1. MenC-specific IgG, IgA and IgM antibodies measured at each time point following primary (A, D, and G) or secondary (B, E, and H) immunization with MenCC vaccine or a secondary immunization with MACPS vaccine (C, F, and I). Furthermore, tetanus-specific IgG antibodies were determined following primary MenCC immunization (J), secondary MenCC immunization (K) and secondary MACPS immunization (L).

With respect to other isotypes, pre-immunization MenC PS specific IgA (GMC 0.06 μg/ml) and IgM (GMC 0.09 μg/ml) concentrations were significantly lower in persons receiving a primary MenCC vaccination, compared to the boostervaccinees (on average, GMC IgA = 0.45 μg/ml and GMC IgM = 0.59 μg/ml, both groups $p < 0.02$), indicating that primary MenCC vaccination induces a long lasting IgA and IgM response after 5 years. At day 25 following immunization, a 87- and 24-fold rise in IgA was observed in the primary MenCC vaccines and the MACPS boostergroup, respectively, to a GMC of 5.26 and 8.13 μg/ml. In contrast, the rise in IgA in the MenCC secondary vaccinees between day 0 and 25 was significantly 4-fold lower ($p < 0.05$), compared to the MACPS group (Table 1 and Fig. 1d–f). A rise in IgM titers was observed between days 3 and 10 in the MenCC primary and MACPS secondary vaccinees, IgM concentrations were significantly higher at day 25 after immunization ($p < 0.05$) in these groups. The level of IgM on day 25 was, respectively, 3.8- and 2.5-fold higher in the primary immunization MenCC group, compared to the MenCC and MACPS boostergroups (Table 1 and Fig. 1g–i) and therefore indicates a true primary response in this group.

To ascertain that the increase in MenC antibodies was due to specific activation of the immune system and not due to vaccine components causing polyclonal activation of B-lymphocytes, antibodies to non-C meningococcal serogroups were determined. No significant change in IgG, IgA or IgM antibodies against meningococcal serogroups W-135 and Y was observed in either one of the vaccine groups (data not shown). Meningococcal serogroup A (MenA) IgG, IgA and IgM antibodies, showed a significant rise ($p < 0.001$) in vaccinees receiving the MACPS vaccine, which is expected because this vaccine contains also MenA PS (Supplementary Figure 1).

3.3. Kinetics of tetanus toxin specific IgG antibodies

Levels of Ttx specific IgG increased significantly 6-fold ($p < 0.05$) within the primary immunization group between days 0 (GMC 1.55 IU/ml) and 25 (GMC 9.25 IU/ml). Remarkably, secondary immunization with MenCC did not lead to an (further) increase in Ttx specific IgG (GMC on day 0, 4.15 and 6.44 IU/ml on day 25). As expected, no significant increase in Ttx specific IgG was observed

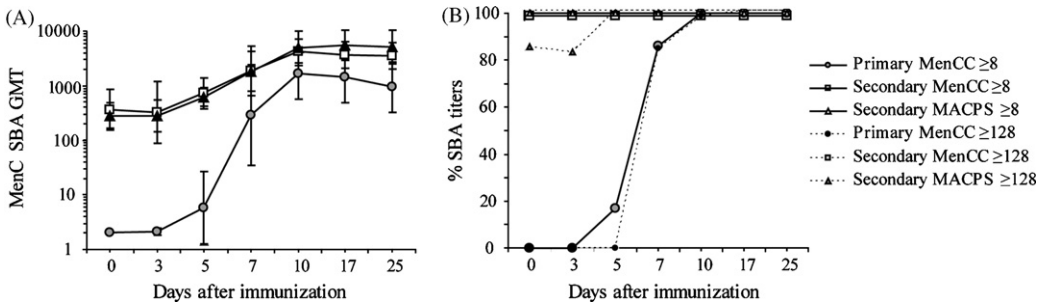

Fig. 2. Serum bactericidal activity using baby rabbit complement (SBA) was measured at each time point following primary or secondary immunization with MenCC or a secondary immunization with MACPS. (A) Grey circles indicate SBA kinetics following primary immunization with MenCC. White squares show SBA kinetics after secondary immunization with MenCC. Dark grey triangles illustrate SBA kinetics following a secondary immunization with MACPS. (B) Percentage of individuals at each time point in the different immunization groups obtaining a SBA titer ≥8 or ≥128.

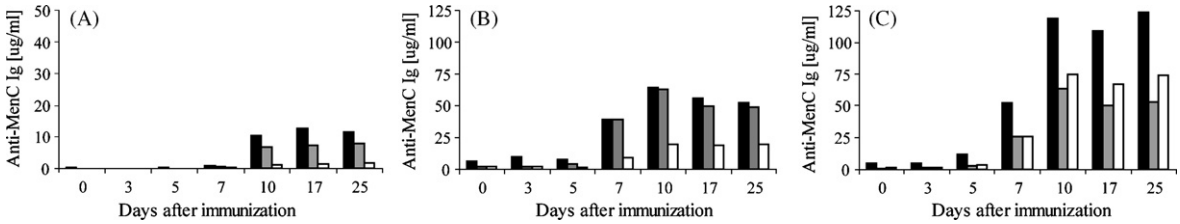

Fig. 3. MenC-specific IgG (black bars) and two IgG isotypes, IgG1 (grey bars) and IgG2 (white bars), measured at each time point following primary (A) or secondary (B) immunization with MenCC vaccine or a secondary immunization with MACPS vaccine (C).

in the MACPS secondary immunization group (Fig. 1j–l). Again, as a control for polyclonal activation of B-cells by the vaccines, Dtx specific antibodies were determined. In none of the 3 groups a significant rise in IgG specific for Dtx was observed (data not shown).

3.4. Kinetics of bactericidal antibodies

None of the persons included in the primary immunization group had a protective SBA titer pre-vaccination (GMT 2). A sharp increase in SBA titers was found between days 3 (GMT 2) and 7 (GMT 290) after immunization, but it took 10 days until all persons achieved a protective SBA titer of ≥8 (GMT 1699) (Table 1 and Fig. 2). In both boostergroups all included persons already had a protective SBA titer (GMT 321) prior to re-vaccination and within 5 days all persons in both boostergroups obtained a SBA titer ≥128 (Table 1 and Fig. 2). Secondary immunization with either MenCC (GMT 3475) or MACPS (GMT 5164) resulted in higher SBA titers on day 25 than primary immunization (GMT 943; $p < 0.05$).

3.5. Meningococcal serogroup C specific IgG1 and IgG2 antibody responses

The most abundant subclass induced after MenCC vaccination in the primary immunization group was IgG1 ($p < 0.05$). At day 25 post-immunization the average IgG1/IgG2 ratio was 4.8 (Fig. 3a), but great variation in this ratio was found between individuals. Within the MenCC boostergroup the subclass distribution was comparable to the primary immunization group (Fig. 3b). At day 25 post-immunization the average IgG1/IgG2 ratio was 2.5. The average ratio of IgG1/IgG2 on day 25 post-immunization in the MACPS boostergroup was 0.72, indicating a slightly better induction of IgG2 after a secondary immunization with plain polysaccharide, although the differences were not significant (Fig. 3c).

3.6. Avidity of meningococcal serogroup C specific IgG

Avidity of MenC-specific IgG antibodies was examined in all samples containing ≥0.25 μg/ml. Therefore, the avidity of samples in the primary immunization group could only be determined from day 7 onwards. Antibodies formed during a primary response are mainly of low-avidity: on average 8% of MenC-specific antibodies remained detectable after 0.5 M NH_4SCN treatment at day 25 (Fig. 4). Antibodies that were induced by previous immunization with MenCC in both boostergroups were of higher avidity: on average 31% of the MenC-specific antibodies remained detectable after 0.5 M NH_4SCN treatment. The avidity increased between days 5 and 10 after boosterimmunization, independently whether the nonconjugated or conjugated vaccine was used. Approximately 50% and 60% of the antibodies remained detectable after NH_4SCN treatment, in the MenCC and MACPS boostergroups, respectively at day 25. We were unable to detect any significant difference in avidity between the two boostergroups (Fig. 4).

3.7. Kinetics of meningococcal serogroup C and tetanus toxin specific IgG antibody secreting cells (ASC) in blood

No MenC- or tetanus-specific ASC were detected at day 0 in the blood in either of the three immunization groups. The first MenC

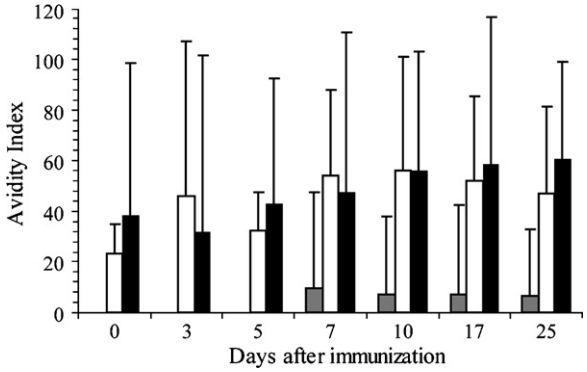

Fig. 4. Average avidity indices measured at each time point following primary (grey bars) or secondary (white bars) immunization with MenCC vaccine or a secondary (black bars) immunization with MACPS vaccine. Error bars indicate 2SD.

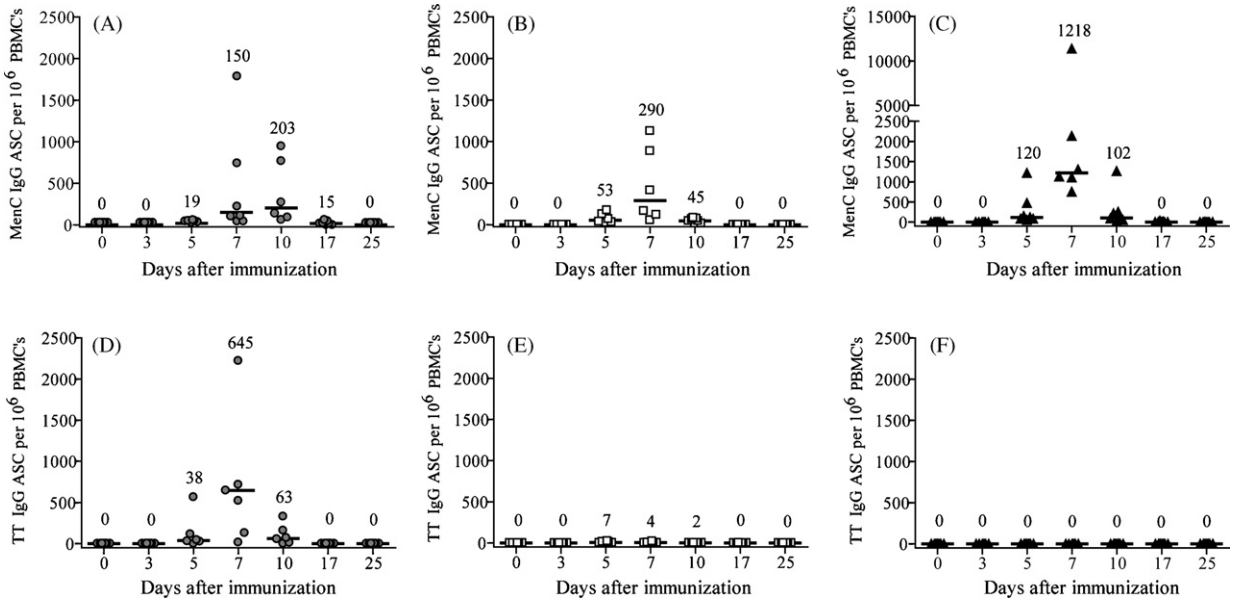

Fig. 5. MenC- and tetanus toxoid-specific plasma cell responses at each time point following primary (A and D) or secondary immunization (B and E) with MenCC vaccine or a secondary immunization with MACPS vaccine (C and F). Persons were immunized at day 0 and the frequency of MenC- and tetanus toxoid specific ASCs was measured at various days after immunization. The numbers above each time point and horizontal bars indicate the median number of specific ASCs at each time point.

(median of 19 ASC/10^6 PBMCs) and tetanus-specific (median of 38 ASC/10^6 PBMCs) ASC in the primary immunization group were detected at day 5, and their numbers gradually increased until day 10 for MenC (median of 203 ASC/106 PBMCs), while there was a clear peak in tetanus-specific ASC response at day 7 (median of 645 ASC/10^6 PBMCs), indicating a booster response for tetanus. Following MenCC and MACPS vaccination, the first MenC specific ASC appeared after 5 days in the blood, a peak in ASC response was detected at day 7 and was over 4-fold higher in the MACPS group ($p < 0.05$, median of 1218 ASC/10^6 PBMCs) compared to the MenCC group (median of 290 ASC/10^6 PBMCs). No significant increase in tetanus-specific ASC was observed within the MenCC secondary immunization group and no tetanus-specific ASC were detected in the MACPS group (Fig. 5).

3.8. Course of antibody kinetics: comparisons between primary and secondary immunizations

With a non-linear mixed effect model we were able to determine possible differences between the immunization groups based on the onset of rise, and the speed of the antibody responses. The onset time from which antibody titers increased was not significantly different between groups. However, the speed of the IgG antibody rise was significantly faster ($p = 0.045$) in the secondary MenCC immunization group compared to the primary immunization group. Secondary immune responses elicited by the MACPS vaccine were not significantly faster compared to secondary responses after MenCC immunization (Supplementary Figure 2).

4. Discussion

In this study we examined in detail the kinetics of different antibody isotypes following primary or secondary immunization with MenCC or a secondary immunization with MenC polysaccharide vaccine in adults. We found that MenC-specific IgG and SBA titers, 5 years after primary immunization, were still well above protective cut-off values and that a long lasting IgM response is present. Furthermore, although the onset time in rise of antibodies between the different groups is similar, a booster response in terms of the rate of increase in IgG antibodies is observed following secondary immunization. We found that it takes up to 7 days before a significant increase in antibody levels is observed. In addition, the type of booster vaccine, clearly affects the antibody (iso)types induced, and the course of the booster immune response.

4.1. MenC-specific IgG responses, persistence and avidity

Our observations concerning the antibody kinetics after primary conjugate immunization in adults are in concordance with previously published data [19,20]. A rise in IgG concentration in these studies was observed between days 4 and 10. Although we did not observe a rise in antibodies after primary immunization before day 7, MenC-specific ASC could already be detected at day 5. Furthermore, the rise in SBA titers before day 7 presumably reflects the small rise in IgM already present in some individuals. IgG and functional antibody titers increased further from day 10 to 17, supported by the number of IgG-specific ASCs still detectable in the blood at day 10 [21]. Antibody persistence following MenCC immunization was previously shown to be age dependent. Several studies have shown that antibodies rapidly wane after infant immunization, while in adolescents and adults antibodies persist for a longer period [10,22]. We also found that IgG antibodies and SBA titers were still present above protective levels in adults 5 years after a single immunization. While persisting antibody levels are probably due to the activity of long-lived plasma cells, it is unclear where, how and to what extend these plasma cells are generated following immunization [23]. Murine models have shown that in early life the homing of long-lived plasma cell in the bone marrow is limited, and this might explain why vaccine-induced antibody levels rapidly decline in infants and to a lesser extend in adults [24,25]. Also, the potential role of memory B-cells in continuously replenishing the plasma cells pool is unclear [26,27].

In this study, the kinetics and magnitude of the IgG response following a secondary immunization indicate that a single MenCC immunization induces immunological memory, and that this memory is at least (re)callable up to 5 years, as was shown for adolescents by Snape et al. [28]. Immunization with the MACPS vaccine could to a certain level be viewed as a model for the response that would occur after an exposure to *N. meningitidis* [28]. In this context it is important to note that the secondary response to the

MenC polysaccharide vaccine is not really faster than a primary response and takes at least 5–7 days, the rate of increase seems on the other hand faster than the observed primary response. The first MenC IgG-specific ASC also did not appear in peripheral blood until 5 days after secondary immunization. Therefore, if antibodies titers have fallen below protective levels, the secondary immune response might not be fast enough to enable protection in case of exposure to *N. meningitidis* serogroup C.

The process of affinity maturation takes place in germinal centers, and results in antibody titers with increased avidity. An increase in avidity has been a proposed marker of successful induction of immunological memory [29]. Because avidity maturation is an ongoing process, this may explain why IgG antibodies present 5 years after initial MenCC vaccination are of higher avidity than one month after primary immunization [29,30]. This might also explain why no clear differences are seen in avidity one month following secondary immunization with MenCC or plain polysaccharide [17].

4.2. MenC-specific IgA and IgM responses

Meningococcal conjugate immunization results in a reduced nasopharyngeal carriage of *N. meningitidis* [31]. MenCC as well as polysaccharide vaccines have been shown to induce IgG and IgA in serum but also in saliva [32,33]. We observed a rise in serum IgA after primary or secondary immunization up to day 10. Hereafter titers decreased in all groups, likely due to the short half-life of this isotype. Furthermore, our data emphasize that secondary immunization with MenCC seems to result in immunoglobulin class switching more towards IgG than IgA, as in the MACPS group an equal rate in increase of IgG and IgA is seen, while in the MenCC group a higher rate in increase of IgG compared to the increase of IgA is observed [21,33].

Surprisingly, we observed a significantly higher level of IgM in individuals immunized 5 years earlier with MenCC compared to MenCC naïve persons and this might contribute to the persistence of functional SBA titers in adults [20]. The reason for this higher level is unclear, since it is rather unlikely that this is caused by exposure to the MenC bacterium, due to a very low incidence rate and virtually no carriage of MenC currently in The Netherlands [2,34]. Furthermore, the early rise in SBA titers in some primary MenCC and secondary MACPS vaccinees, as was also seen in other studies [11,28], seemed to be associated with an early appearance of IgM (Figs. 1 and 2).

4.3. Tetanus-specific IgG responses

Carrier-induced epitope suppression is the phenomenon which, in our model, would imply that high levels of anti-tetanus antibodies would suppress the response to the MenC polysaccharide. The anti-tetanus antibodies result from childhood vaccination and, in the booster groups, from the primary immunization with the MenCC conjugate vaccine, in which tetanus is used as carrier protein [35–38]. In this study we were unable to observe impaired tetanus- or MenC-specific antibody responses following primary MenCC immunization. Following a secondary immunization with MenCC no further increase in tetanus antibodies is found. These data do not allow to conclude about potential mechanisms such as carrier suppression. On the other hand, despite the high tetanus antibody levels, a clear-cut booster response was found for the MenC polysaccharide [39,35,40]. Carrier-induced epitope suppression therefore probably does not play an overriding role in the response to MenCC vaccines. Burrage et al. showed that MenCC immunization rapidly followed by a diphtheria–tetanus vaccination did not result in an increase in diphtheria or tetanus titers [35]. Studies in infants with MenCC vaccines in contrast showed that after boosterimmunizations antibody levels specific for the carrier protein were able to increase [12]. However, a study by Nanan et al. suggested that the potency of specific B-cells to expand decreases with every antigenic re-challenge [41], possibly explaining why in adults booster immunizations do not result in an increase in tetanus-specific ASC and IgG antibodies.

4.4. Comparison between secondary immunizations

Small differences in the secondary immune response elicited by the MenCC or plain polysaccharide vaccine were found. The secondary IgG, IgA and IgM response was slightly faster in the MACPS group compared to the MenCC group. However, the magnitude of the response was not significantly different for IgG and IgA and only significant for IgM in the MACPS group. The MACPS vaccine was used at the recommended dosage of 50 μg of MenC PS, while the recommended dose of MenCC contains only 10 μg of MenC PS. Therefore this slightly faster response might be related to the vaccine dosage. Furthermore, the administration of plain polysaccharide may not only stimulate the previously induced MenC-specific memory B-cells, but might also activate cells that are involved in T-cell independent responses, such as marginal zone B-cells in the spleen [42–44], reflecting a primary response in which IgM is produced.

Snape et al. [28] described that the MenCC vaccine induces a significant better immune response than the MACPS vaccine, when an adjusted dose of 10 μg MenC PS for this vaccine was used. We did not detect differences in the IgG and SBA responses after a secondary immunization with either one of the two vaccines despite the different PS-contents. Moreover, the MenCC vaccine has the advantage of inducing a long persisting antibody response in adolescents and adults, and induces memory B-cells [6,12].

4.5. MenC-specific IgG1 and IgG2 response

A high IgG1/IgG2 antibody ratio following conjugate vaccination is considered to reflect activation of cellular mechanisms of a T-cell-dependent immune response in infants [30,45]. However, in adults the antibody response to conjugate vaccines is heterogeneous but seems to be more dominated by IgG2 [45–49]. As expected, also in our study the IgG1 and IgG2 isotype response was heterogeneous within each study group. Nonetheless, the major IgG isotype induced following primary or secondary immunization with MenCC was IgG1. Secondary immunization with plain polysaccharide resulted on average in a higher level of IgG2 relative to IgG1, which reflects the more T-cell independent isotype response [49,50]. These trends in IgG1 and IgG2 isotype responses might be of influence on the overall functionality of the antibodies elicited by MenCC of MACPS secondary immunization. While the MACPS vaccine induces approximately 2.4 times more IgG at day 25 compared to the MenCC vaccine, the SBA titers are only 1.5 times higher. This is possibly a result of the presence of IgG2, induced by the MACPS vaccine, that binds complement less effectively than IgG1 [51].

4.6. Study limitations

The results described here are from a small study group and therefore biases may be introduced. However, despite the small sample size we were able to follow antibody kinetics within single individuals and were therefore able to reveal differences between the three vaccine groups based on swiftness and levels of antibody isotype responses.

5. Conclusion

Our data show that functional antibodies elicited by MenCC are able to persist in adults at least 5 years, and supports other studies. We observed clear-cut secondary antibody responses upon booster immunization, but these responses were relatively late, and may therefore perhaps not be in time to prevent infection if antibodies would have waned. Furthermore, the composition of antibody isotypes may be of influence for sustainment of protection as well as level of functionality. This study highlights the importance of sustained protective antibody levels in subpopulations in which antibody levels have waned and herd-immunity is low, such as infants and children [9,22], in which secondary immune responses may be even more impaired against a rapidly invasive organism as *N. meningitidis*.

Conflict of interest

Dr Sanders reports receiving unrestricted grants from Wyeth and Baxter for research, consulting fees from Wyeth and GlaxoSmithKline, lecturing fees from Wyeth and grant support from Wyeth and GlaxoSmithKline for vaccine studies. All other authors have no conflict of interest to declare.

Acknowledgements

We thank all persons who participated in the study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.09.082.

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