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Cross-Reactivity of Antibodies against PorA after Vaccination with a Meningococcal B Outer Membrane Vesicle Vaccine

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The cross-reactivity of PorA-specific antibodies induced by a monovalent P1.7-2,4 (MonoMen) and/or a hexavalent (HexaMen) meningococcal B outer membrane vesicle vaccine (OMV) in toddlers and school children was studied by serum bactericidal assays (SBA). First, isogenic vaccine strains and PorA-identical patient isolates were compared as a target in SBA, to ensure that the vaccine strains are representative for patient isolates. Geometric mean titers (GMTs) in SBA against patient isolates with subtypes P1.5-2,10 and P1.5-1,2-2 after vaccination with HexaMen were generally lower than those against vaccine strains with the same subtype, although the percentage of vaccine responders (≥4-fold increase in SBA after vaccination) was not affected. Using various P1.7-2,4 patient isolates, GMTs as well as the number of vaccine responders were higher than for the P1.7-2,4 vaccine strain, indicating that the use of the P1.7-2,4 vaccine strain may have underestimated the immunogenicity of this subtype in HexaMen. Secondly, the cross-reactivity of antibodies induced by MonoMen and HexaMen was studied using several patient isolates that differed from the vaccine subtypes by having minor antigenic variants of one variable region (VR), by having a completely different VR or by having a different combination of VRs. MonoMen induced P1.4-specific antibodies that were cross-reactive with P1.4 variants P1.4-1 and P1.4-3. HexaMen induced a broader cross-reactive antibody response against various patient isolates with one VR identical to a vaccine subtype or a combination of VRs included in HexaMen. Cross-reactivity, measured by a fourfold increase in SBA after vaccination, against these strains ranged from 23 to 92% depending on the subtype of the tested strain and was directed against both VR1 and VR2. The extended cross-reactivity of vaccinee sera induced by HexaMen against antigenic variants has important favorable implications for meningococcal B OMV vaccine coverage.

Neisseria meningitidis remains an important cause of bacterial meningitis and septicemia in humans, with peak occurrences in toddlers and adolescents. Despite optimal treatment, mortality rates are relatively high. Therefore, prevention of this disease is of great importance. Nowadays, effective vaccines are commercially available against serogroups A, C, W135, and Y of *N. meningitidis*, but not against serogroup B. As the group B capsular polysaccharide is not a suitable vaccine component, attention is focused on outer membrane proteins. The National Institute of Public Health and the Environment (RIVM) of The Netherlands has developed two vaccines based on outer membrane vesicles (OMV) of *N. meningitidis* serogroup B. Table 1 shows all phase II trials that have been performed with these vaccines, in which the most important antigen is the porin A (PorA). This transmembrane protein consists of eight loops, of which the surface-exposed loops 1 and 4 contain one variable region each (VR1 and VR2, respectively). The variation in these two loops determines the subtype. First, a recombinant, hexavalent vaccine consisting of two OMV, each containing three different PorA subtypes, was constructed, including the subtypes P1.5-2,10; P1.12-1,13; P1.7-2,4; P1.19,15-1; P1.7,16; and P1.5-1,2-2 (HexaMen). In clinical phase II trials in infants, toddlers, and school children, subtype antigens P1.5-2,10 and P1.5-1,2-2 were most immunogenic, whereas the most-prevalent subtype in The Netherlands, P1.7-2,4, induced the lowest bactericidal activity assessed by the corresponding vaccine strains in the serum bactericidal assay (SBA). The geometric mean titers (GMTs) measured by SBA against the P1.5-2,10 and the P1.5-1,2-2 vaccine strains were at least four to six times higher than the GMT against the P1.7-2,4 strain (2, 3). A second OMV vaccine was constructed expressing only the P1.7-2,4 subtype (MonoMen). This vaccine was highly immunogenic in toddlers and induced a booster response in children previously vaccinated with HexaMen (4, 5). The booster response was not only observed for the P1.7-2,4 subtype but to a lesser extent also for other subtypes included in HexaMen. This suggests that cross-reactivity could contribute to the immune response and hence protection from disease.

The theoretically predicted coverage of MonoMen and HexaMen based on the exact match of vaccine subtypes is 39 and 50% of group B meningococci in The Netherlands, respectively (10). In 2000, up to 72 different subtypes of *N. meningitidis* were isolated in The Netherlands (10). It is impossible to include all of these variants in an outer membrane protein-based vaccine. Little is known about the cross-reactivity of antibodies induced against a specific PorA subtype present in

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TABLE 1. Phase II clinical trials in The Netherlands using RIVM meningococcal B OMV vaccines in children

Trial no. (reference)	Vaccine	Immunization schedule and adjuvant[a]	Population
I (3)	Hexavalent	2 + 1, $AlPO_4$	Toddlers (n = 172) and schoolchildren (n = 165)
II (4)	Monovalent	One booster, $AlPO_4$	Toddlers and schoolchildren from trial I, 2.5 yr after HexaMen (n = 177)
III (5)	Monovalent	2 + 1 vs. 3 + 1, $AlPO_4$ vs. $Al(OH)_3$	Toddlers (n = 134)

[a] Abbreviations: $Al(OH)_3$, aluminium hydroxide; $AlPO_4$, aluminium phosphate; 2 + 1, two primary vaccinations followed by a booster vaccination; 3 + 1, three primary vaccinations followed by a booster vaccination.

either a monovalent or a hexavalent OMV vaccine. Theoretically, four kinds of cross-reactivity could be found: (i) cross-reactivity against strains having minor antigenic variants of one VR, (ii) cross-reactivity with strains sharing only one VR1 or VR2 with a vaccine subtype, (iii) cross-reactivity against strains having a different combination of VRs compared to vaccine subtypes, and (iv) cross-reactivity against strains having VR combinations not included in the vaccine. Only data on cross-reactivity against minor VR variants of the P1.5-2,10 subtype in HexaMen are available, and these show a reduced killing of strains with minor sequence variants of the P1.10 epitope (8). The presence of cross-reactive antibodies of any kind would have great implications on vaccine efficacy.

We studied the specificity of antibodies in serum samples of toddlers or school children vaccinated with HexaMen, MonoMen, or both. First, to investigate whether vaccine strains used in SBA are representative for patient isolates of the same subtype, we collected a set of patient isolates with subtypes P1.5-2,10; P1.5-1,2-2; and P1.7-2,4. These strains were used as a target in SBA with sera collected in phase II trials, and titers were compared with titers obtained when the vaccine strains were used in SBA. Secondly, we collected several patient isolates which frequently cause meningococcal disease in The Netherlands; these isolates differ from vaccine subtypes either in minor VR variants, having a completely different VR or having a different combination of VRs to study the cross-reactivity of the antibodies induced by HexaMen and MonoMen in SBA.

MATERIALS AND METHODS

Vaccine and serum samples. Available sera from three different clinical phase II trials in children were used. These trials have been described earlier and are summarized in Table 1 (3–5). Only the prevaccination serum samples and the samples drawn postbooster were used for analysis.

Bacterial strains. The vaccine strains used in our experiments are all isogenic variants of strain H44/76 P1.7,16, each expressing one of the vaccine subtypes (TR52, TR10, TR1213, and TR4) (11). The *N. meningitidis* patient isolates used to study the cross-reactivity of the immune response induced by the two RIVM vaccines are listed in Table 2. These strains were obtained from The Netherlands Reference Laboratory for Bacterial Meningitis (AMC/RIVM) and from the RIVM collection. Serosubtyping of the strains was based on the sequence of PorA variable regions and classified according to the recently changed nomenclature (7; M. Maiden, I. Feavers, and K. Jolley, unpublished data [http://neisseria.org/nm/typing/pora/]).

Strain characterization. Expression of PorA, Opa, Opc, and lipopolysaccharide (LPS) phenotype was determined using semiquantitative whole-cell enzyme-linked immunosorbent assay (ELISA). The following monoclonal antibodies were used: MN 14C11.6 (anti-P1.7), B306 (anti-Opc), MN43F8.10 (anti-L8), 4A8B2 (anti-L3), MN42F12.32 (anti-L2), 17-1-L1 (anti-L1), MN5A10F (anti-P1.9), MN5C11G (anti-P1.16), and MN20B9.34 (anti-P1.4) and two monoclonal antibodies against Opa proteins: 15-1-P5.5 (anti-P5.5) and MN20E12.70 (anti-B128).

Colony blot for antigen expression. The strains used for vaccine production express a low amount of Opa and Opc proteins. When ELISA results of the patient isolates used in SBA were positive for Opa and/or Opc, a negative colony was selected using double-staining colony blotting to exclude possible cross-

TABLE 2. Characterization of patient isolates used in cross-reactivity experiments

Strain	Serologic classification[b]	PorA	Sequence		Reaction with MAb[a]	
			VR1	VR2	P1.4	P1.7
N405/94	B:19,7,1:nt	P1.18,4-1	PPSKGQTGNKVTKG	HVVVNNNVATHVP	−	−
200487	B:4:nt	P1.7-2,del1	AQAANGGASGQVKVTKA	HVVVNNKV	−	−
MI/97027	B:4:nt	P1.7-2,del2	AQAANGGASGQVKVTKA	HVV	−	−
200483	B:4:15	P1.7-2,del3	AQAANGGASGQVKVTKA	H	−	−
60/96	Y:1:4	P1.21-5,4-3	QPQVPNSVQGNQVKVTKA	HVVVNNKVTTHVP	++	−
MC 50	C:nt:16	P1.21,16	QPQVTNGVQGNQVKVTKA	YYTKDTNNNLTLVP	−	−
M1080	B:19,7,1:7,1	P1.7-1,1	AQAANGGAGASGQVKVTKVTKA	YVAVENGVAKKVA	−	++
200840	B:4:nt	P1.7-2,13-2	AQAANGGASGQVKVTKA	YWTTVNTGSATTTFVP	−	
2001642	B:4:nt	P1.7-2,13-2	AQAANGGASGQVKVTKA	YWTTVNTGSATTTFVP	−	−
2001825	B:1,4:nt	P1.7-2,13-1	AQAANGGASGQVKVTKA	YWTTVNTGSATTTTFVP	−	+/++
2001395	B:nt:nt	P1.7-2,13	AQAANGGASGQVKVTKA	YWTTVNTGSATTTTTFVP	−	−
200486	B:4:nt	P1.7,13-2	AQAANGGASGQVKVTKVTKA	YWTTVNTGSATTTFVP	−	−
2001624	B:1:16	P1.7-2,16	AQAANGGASGQVKVTKA	YYTKDTNNNLTLVP	−	−
200408	B:4:2	P1.7-2,2	AQAANGGASGQVKVTKA	HFVQQTPKSQPTLVP	−	−
200488	B:nt:nt	P1.7-2,9	AQAANGGASGQVKVTKA	YVDEQSKYHA	−	−
200041	B:4:4	P1.7-11,4	AQAANGGASGQVKVTKVTKVTKA	HVVVNNKVATHVP	++	−
2001738	B:4:7	P1.7-8,4-1	AQAANGGAGASGQVKVTKVTKVTKA	HVVVNNNVATHVP	−	++
2001795	B:nt:12,4	P1.12-1,4	KPSSTNAKTGNKVEVTKA	HVVVNNKVATHVP	++	−

[a] Reaction with monoclonal antibodies (MAb) in whole-cell ELISA against P1.4 (MN20B9.34) and/or P1.7 (MN14C11.6). Symbols: −, no reaction; +/++, weak to moderate reaction; ++, strong reaction.

[b] nt, nontypeable.

TABLE 3. GMTs and 95% CIs for SBA titers and the percentage of vaccine responders after vaccination against patient isolates of the same subtype as the P1.7-2,4 vaccine strain

Strain	Trial I post booster (n = 46)		Trial II post booster (n = 130)		Trial III post booster (n = 60)	
	GMT (95% CI)	% of children with 4-fold increase in titer	GMT (95% CI)	% of children with 4-fold increase in titer	GMT (95% CI)	% of children with 4-fold increase in titer
Isogenic H44/76 P1.7-2,4	2.9 (1.9–4.3)	37.0	4.0 (3.0–5.1)	48.5	57.0 (41.3–78.8)	100
91/40 B:4:P.1.7-2,4	2.5 (1.7–3.8)	26.0	3.8 (2.7–5.3)	31.2	27.4[a] (19.0–39.6)	100
92/53 B:4:P1.7-2,4	5.6[a] (3.5–8.8)	65.2	10.8[a] (7.3–16.0)	51.2	86.5[a] (54.5–137.3)	100
97/181 B:14:P1.7-2,4	11.3[a] (7.5–17.1)	84.8	11.0[a] (7.8–15.4)	61.9	123.5[a] (88.0–173.3)	100

[a] Significantly higher or lower than GMT against the isogenic strain in SBA (Wilcoxon signed rank test).

reactivity with class 5 proteins. This method has been described by Kuipers et al. (6). For the double staining the appropriate monoclonal against Opa or Opc together with a monoclonal of another immunoglobulin G isotype against a common antigen that should be present on all colonies (e.g., against the PorA subtype or LPS phenotype) were used. The colony blot procedure was repeated on the picked colonies as a control, and the selected strains were checked by ELISA for expression of several proteins.

Immunoblotting. The amount of PorA expressed by each strain was determined by semiquantitative immunoblotting (6), using a monoclonal antibody against loop 3 of PorA (MN23G2.38), which is a shared epitope of all PorA subtypes. The molecular weight marker was stained separately with AuroDye to visualize the protein bands.

Selection of a PorA-negative strain. Strain 2000488, subtype P1.7-2,9, was grown on GC agar plates overnight for 18 to 20 h. Four microliters of the plasmid pTZ19R, which contains a PorA gene with a kanamycin resistance box (G. Vidarsson, personal communication), was cut in the presence of 1 μl of the restriction enzyme *Eco*RI, 3 μl of buffer H 10x, and 22 μl of water for 1 h at 37°C. Bacteria were removed from the plate with a sterile swab and resuspended in 10 ml of Müller-Hinton Broth (MHB; Foundation for the Advancement of Public Health and Environmental Protection, Bilthoven, The Netherlands)–10 mM $CaCl_2$. A dilution of 1:5 was made in 5 ml of MHB–10 mM $CaCl_2$, and this was divided into two tubes each containing 2.5 ml of the suspension. Thirty microliters of restricted plasmid was added to one tube, whereas nothing was added to the other tube. After 2 h of incubation in a 37°C, 5% CO_2 incubator, 250 μl of each tube was plated on a GC plate containing kanamycin (100 μg/ml) in triplicate. These plates were incubated overnight. The next day, plates on which the suspension without any DNA was incubated showed no bacterial growth, whereas the other plates contained several colonies. These colonies were picked and checked for PorA expression using immunoblot.

SBA. SBA was measured as described earlier (15). The serum bactericidal titer was reported as the reciprocal of the lowest serum dilution yielding ≥90% killing. A vaccine responder was defined as having an increase in SBA titer of fourfold or more compared with the prevaccination titer. For the patient isolates of subtype P1.5-1,2-2 and P1.5-2,10, SBA was performed using threefold dilutions of sera, because of expected high titers against these subtypes.

Statistics. GMTs and 95% confidence intervals (CI) were calculated. Wilcoxon signed rank tests were used to evaluate the significance of differences between titers. A P of <0.05 was considered significant.

RESULTS

Vaccine strains versus patient isolates with identical subtypes. Three P1.7-2,4 isolates (strain 91/40 B:4:P1.7-2,4; strain 92/53 B:4:P1.7-2,4; and strain 97/181 B:14:P1.7-2,4) from New Zealand patients were used as target strains in SBA with 46 postbooster samples from trial I, all postbooster sera from trial II, and 60 postbooster serum samples with a measurable SBA titer from trial III. Results are shown in Table 3. In all tested sera higher GMTs and a significant increase in vaccine responders were observed for two out of three P1.7-2,4 patient isolates compared to the P1.7-2,4 vaccine strain. The other isolate gave a significantly lower GMT only in children vaccinated with MonoMen, although the percentage of vaccine responders was similar. These results indicate that the SBA titers obtained with the P1.7-2,4 vaccine strain may have underestimated the immunogenicity of P1.7-2,4 in HexaMen. To confirm this result, postbooster sera of a subgroup of 11 children from trial I with a high bactericidal GMT of 52.7 (95% CI, 30.8 to 90.1) were tested against six other P1.7-2,4 patient isolates. For only one out of the six isolates a significantly lower GMT was observed, whereas the percentage of responders did not vary significantly (range, 64 to 91%), and for the other five isolates GMTs were similar or even higher (data not shown).

Sera from children vaccinated in trial I with HexaMen were also tested using patient isolates with VR1-VR2 combinations P1.5-1,2-2 (two strains) and P1.5-2,10 (three strains). For one P1.5-1,2-2 patient isolate a significantly lower GMT was measured than for the P1.5-1,2-2 vaccine strain, whereas the GMT based on the other patient isolate did not differ significantly (Table 4). However, the percentage of vaccine responders in the SBA was very similar for each strain. For P1.5-2,10, three patient isolates showed significantly lower GMTs than the vaccine strain, but the percentage of children with a fourfold increase in SBA after vaccination was 100% for all three P1.5-2,10 strains.

Cross-reactivity against VR1 and VR2. Serum samples drawn before vaccination and after the booster vaccination of a subgroup of 50 toddlers and school children immunized with HexaMen (trial I) were used to study cross-reactivity. The focus was on patient isolates with the P1.7 and the P1.13 epitopes, since strains with these epitopes are highly prominent in The Netherlands. In addition, we included two patient iso-

TABLE 4. GMTs and 95% CIs for SBA titers and the percentage of vaccine responders after vaccination against patient isolates of the same subtype as the vaccine strains with subtype P1.5-1,2-2 and P1.5-2,10

Strain	GMT (95% CI)	% of children with 4-fold increase in titer
P1.5-1,2-2		
Isogenic H44/76	53.2 (35.8–79.1)	97.8
126E	37.0 (24.1–56.5)	95.6
5372	20.8[b] (13.7–31.7)	95.6
P1.5-2,10		
Isogenic H44/76	383.0 (288.3–508.6)	100
B40	29.7[b] (21.8–40.5)	100
21/51	70.3[b] (52.7–93.7)	100
870227	25.8[b] (19.4–34.2)	100

[a] All data are from trial I post booster (n = 46).
[b] Significantly higher or lower than GMT against the vaccinee strain in SBA (Wilcoxon signed rank test).

TABLE 5. GMTs and 95% CIs for SBA titers and the percentage of vaccine responders after vaccination with a hexavalent meningococcal OMV vaccine

Strain	Relation to vaccine VRs[b]	GMT (95% CI)	% of children with 4-fold increase in titer
P1.7-2,13-2 I	NC, VR1 = i, VR2 = v	8.0 (4.6–13.9)	56
P1.7-2,13-2 II	NC, VR1 = i, VR2 = v	2.6 (1.5–4.5)	23
P1.7-2,13-1	NC, VR1 = i, VR2 = v	59.6 (33.8–105.0)	92
P1.7-2,13-2	NC, VR1 = i, VR2 = v	3.2 (1.8–5.8)	28
P1.7-1,1	VR1 = v, VR2 = u	2.2 (1.4–3.5)	23
P1.21,16	VR1 = u, VR2 = i	6.7 (3.8–11.9)	58
P1.7-2,9	VR1 = i, VR2 = u	4.3 (2.6–7.2)	36
P1.7-2,16	NC, VR1 = i, VR2 = i	7.1 (4.4–11.4)	54
P1.7-2,2	NC, VR1 = i, VR2 = v	8.8 (5.2–15.0)	60
P1.7-2,13	NC, VR1 = i, VR2 = i	5.3 (3.6–7.7)	40
P1.7,16 H44/76	Vaccine strain	15.8 (10.1–24.5)	84
P1.7-2,4 TR4	Vaccine strain	5.7 (3.8–8.4)	64
P1.12-1,13 TR1213	Vaccine strain	15.6 (10.1–24.0)	88

[a] All data are from trial I post booster ($n = 50$).
[b] Abbreviations: NC, new combination; of vaccine VRs; i, identical to vaccine VR; v, variant of vaccine VR; u, unrelated to any of the vaccine VRs.

lates with P1.7-2,13-2 as a VR1-VR2 combination in this study, since this subtype of *N. meningitidis* was the second most important cause of meningococcal sepsis or meningitis in The Netherlands in 2000. Table 5 shows a great variation in GMT, ranging from 2.2 (95% CI, 1.4 to 3.5) against P1.7-1,1 to 59.6 (95% CI, 33.8 to 105.0) against P1.7-2,13-1. The percentage of children with at least a fourfold increase in SBA titers varied from 23% for P1.7-1,1 to 92% for P1.7-2, 13-1 (Table 5). Unexpectedly, the GMT and percentage of vaccine responders differed significantly between two strains with the identical subtype P1.7-2,13-2. Furthermore, the response against the patient isolate with subtype P1.7-2,9 was rather high, although P1.9 is not included in the vaccine and P1.7-2 is thought to be a hidden epitope in the vaccine. Nevertheless, 36% of all children had a significant increase in SBA titer against this strain. To ensure that the response against the P1.7-2,9 strain was directed against PorA, we created a PorA-negative mutant of this strain and used this mutant as a target in SBA. The GMT was considerably lower for the mutant than for the PorA-positive strain, and the percentage vaccine responders decreased from 36 to 5% (data not shown). The response against two patient isolates with P1.16 as VR2 was rather similar: 54 and 58% for P1.7-2,16 and P1.21,16, respectively. Since P1.21 is not present in the vaccine, this shows that the presence of the VR1-VR2 combination P1.7,16 in HexaMen induced bactericidal antibodies specific for VR2.

To determine the cross-reactivity induced by vaccination with MonoMen, a subgroup of sera from 20 toddlers vaccinated with MonoMen (trial III) with a high titer against P1.7-2,4 was also tested against patient isolates having a different VR2. None of the toddlers had a significant response against strains with VR1-VR2 combinations P1.7-2,16; P1.7-2,13; P1.7-2,2; or P1.7-2,13-2—and only 1 (5%) had a response against the P1.7-2,9 isolate. This indicated that the majority of antibodies induced by MonoMen were directed against VR2 P1.4. However, 100% of toddlers had a fourfold increase against a patient isolate with VR1-VR2 combination P1.7-11,4; and only 20% experienced a fourfold increase against a P1.12-1,4 patient isolate, indicating an effect of VR1 on the accessibility for bactericidal antibodies directed against VR2 (see also Table 6).

Cross-reactivity against P1.4 variants. As bactericidal antibodies induced by MonoMen were mainly directed against VR2, we tested cross-reactivity against P1.4 variants. We were unable to obtain patient isolates with VR1-VR2 combinations P1.7-2,4-1; P1.7-2,4-3; or any isolate with P1.4-2 as VR2 epitope. Results are shown in Table 6. In 40% of the children vaccinated with MonoMen a fourfold increase in SBA titer was found against a patient isolate with VR1-VR2 combination P1.7-8,4-1. Furthermore, 60% of the sera from trial III had a significant response to a P1.18,4-1 patient isolate. Against a serogroup Y strain with VR1-VR2 combination P1.21-5,4-3 a very high GMT of 512 and 95% vaccine responders in trial III were measured. This response was induced by MonoMen and not by any cross-reacting antibodies against serogroup Y capsular polysaccharide, since prevaccination titers were all low. Thus, vaccination with MonoMen induced bactericidal antibodies against P1.4 that can at least cross-react with P1.4-1 and P1.4-3. In sera from the trials I and II only 15 and 6%, respectively, of children had a significant response against the P1.7-8,4-1 patient isolate. To study the cross-reactive antibody response against VR2 more closely, we used deletion mutants obtained from patients. The response against the deletion mu-

TABLE 6. GMTs and 95% CIs for SBA titers and the percentage of vaccine responders after vaccination with a monovalent and/or hexavalent OMV vaccine

Strain	Trial I ($n = 20$)		Trial II ($n = 20$)		Trial III ($n = 20$)	
	GMT (95% CI)	% of children with 4-fold increase in titer	GMT (95% CI)	% of children with 4-fold increase in titer	GMT (95% CI)	% of children with 4-fold increase in titer
P1.7-2,del1	168.9 (105.9–269.3)	100	128.0 (69.2–236.9)	95	21.1 (9.0–49.1)	68
P1.7-2,del3	68.6 (36.8–127.8)	90	52.0 (25.0–108.0)	80	16.6 (7.1–38.5)	60
P1.7-2,del2	5.7 (3.1–10.3)	30	6.7 (3.9–11.5)	50	2.5 (2.2–3.0)	0
P1.7-11,4	19.0 (9.9–36.6)	80	107.6 (56.0–206.8)	100	163.1 (103.5–257.0)	100
P1.12-1,4	ND[a]	ND	ND	ND	3.2 (2.0–5.2)	20
P1.7-8,4-1	2.8 (1.9–4.1)	15	3.7 (2.0–6.8)	6	5.5 (3.1–9.7)	40
P1.18,4-1	ND	ND	ND	ND	8.0 (4.8–13.2)	60
P1.21-5,4-3	ND	ND	ND	ND	512	95
P1.7-2,4 vaccine strain	13.0 (7.9–21.3)	100	28.8 (16.2–51.4)	100	71.0 (43.7–115.4)	100

[a] ND, not done.

tant which still had most of its VR2 conserved (P1.7-2,del1) showed very high GMTs in children vaccinated in trials I and II. Against strain P1.7-2,del3, in which only one amino acid of the P1.4 epitope was present, higher GMTs than those observed against the vaccine strain were observed, but the percentage of vaccine responders was lower. Strain P1.7-2,del2 showed the lowest GMTs and the lowest number of responders. Children from trial III in general showed lower GMTs against all deletion strains, indicating that the specific anti-P1.4 antibodies were less able to kill these strains, compared to antibodies evoked by HexaMen.

DISCUSSION

The RIVM PorA-based OMV vaccines have been studied in clinical phase II trials in infants, toddlers, and school children. So far, only isogenic vaccine strains have been used as indicators to assess the immunogenicity of MonoMen and HexaMen in SBA against patient isolates of the same subtype. HexaMen induced a poor SBA response against subtype P1.7-2,4 as measured with the P1.7-2,4 vaccine strain (3). This was of great concern, since this is the most-prominent subtype of meningococcus B from patients in The Netherlands as well as in New Zealand. In this study we showed that GMTs as well as percentages of vaccine responders were higher when P1.7-2,4 patient isolates in stead of the P1.7-2,4 vaccine strain were used in SBA, indicating that the immunogenicity of P1.7-2,4, especially in the HexaMen vaccine, may have been underestimated. A reason for this finding might be a higher expression of capsular polysaccharide or LPS in the P1.7-2,4 vaccine strain which hampers the accessibility of the PorA epitopes. Alternatively, the level of PorA expression could be much lower in the vaccine strain. However, on immunoblots, levels of PorA expression were comparable for vaccine strains and patient isolates (data not shown). The number of vaccine responders when patient isolates with subtype P1.5-2,10 or P1.5-1,2-2 were used as target strains in SBA was consistently 100%. The bactericidal GMTs were significantly lower compared to the vaccine strains, but the significance of this finding is unclear since the level of SBA titers required for protection against meningococcal serogroup B disease is unknown.

To assess the probable effectiveness of a vaccine based on a protein as variable as PorA in *N. meningitidis*, the cross-reactivity of antibodies induced by such a vaccine is an important issue. There is some evidence that the monovalent Norwegian (P1.7,16) and Cuban (P1.19,15) meningococcal OMV vaccines induce cross-reactive bactericidal immune responses, since toddlers showed a cross-reactive immune response ranging from 24 to 41% of vaccine responders against heterologous strains (14). Antibodies induced by class 5 proteins, which are present in the Norwegian vaccine and can induce bactericidal antibodies, may have caused this cross-reactivity (13). However, infants did not show any cross-reactive antibodies after vaccination with the Norwegian or the Cuban vaccine in SBA (14). Using a whole-blood assay, Morley et al. found very limited cross-reactive antibodies against one out of four tested heterologous serogroup B strains after immunizing infants with the Cuban vaccine but SBA did not show any cross-reactive immune response either (9). Apparently, opsonophagocytosis measured by Western blot assay also contributes to cross-reactivity of serum antibodies. A clinical phase III study in young adults suggested that these monovalent vaccines were not efficacious against non-vaccine type strains. In order to obtain protection against more subtypes, multivalent vaccines are desirable (12).

We showed that HexaMen, containing six different combinations of VR1 and VR2 epitopes in one vaccine, induced a broad response against various patient isolates sharing one VR with a vaccine subtype and to minor VR variants of subtypes included in HexaMen. Cross-reactivity, measured by a fourfold increase in SBA after vaccination against a variety of patient isolates, ranged from 23 to 92%. Just like the difference in SBA when vaccine strains are compared with some of the patient isolates with the same VR1-VR2 combination, the difference in percentage of vaccine responders against the two patient isolates with the VR1-VR2 combination P1.7-2,13-2 partly depended on so far unknown strain characteristics. LPS phenotype, Opc and Opa expression, and the amount of PorA were all similar on immunoblots, yet twice as many vaccinees showed a significant antibody response against one strain compared to the other. In an earlier study, a subset of sera from the same vaccinees was tested against the PorA-deficient mutant vaccine strain H1.5. There was no increase in SBA against this strain (3). The vaccine did not contain any class 3 or 4 proteins. This indicated that the induced antibody response by HexaMen was mainly directed against PorA and that apparently other variable parts besides VR1 and VR2 of the PorA protein also contributed to a significant immune response.

After vaccination with MonoMen a strong bactericidal antibody response was observed against the P1.7-2,4 patient isolates. Patient isolates having P1.7-2 as VR1 together with a completely different VR2 like P1.9 or P1.16 were not killed by these sera, indicating that this monovalent vaccine induced in general VR2-specific antibodies. These antibodies were mainly directed against P1.4 but also showed bactericidal activity against P1.4-1 and P1.4-3. Thus, even though a monoclonal antibody against P1.4 does not cross-react with P1.4-1, sera from vaccinees show cross-reactivity with patient isolates having minor varieties in their VR2 epitopes. Children vaccinated with HexaMen and (older) children vaccinated with HexaMen and MonoMen did not respond to the P1.7-8,4-1 patient isolate. This indicates that they had fewer antibodies that were specifically directed against P1.4 and therefore were less able to kill minor P1.4 variants. Martin et al. also showed that antibodies against the most-immunogenic subtype of HexaMen, P1.5-2,10, showed significantly lower GMTs against variants P1.5-2,10-1 and P1.5-2,10-6, although the percentage of infants with an SBA titer of >4 after vaccination with HexaMen was still reasonably high (8).

Only 20% of the toddlers responded with a fourfold increase in SBA titer against a patient isolate with subtype P1.12-1,4. This indicates that the presence of another VR1 besides P1.7-2 in combination with P1.4 as VR2 probably results in differences in the conformation of PorA, changing the accessibility of the VR2 epitope for P1.4-specific antibodies. Furthermore, cross-reactivity against subtype P1.7-2,9 was induced by HexaMen, but not by MonoMen. We hypothesized that the response against this strain evoked by HexaMen is probably caused by cross-reactivity of antibodies directed against

P1.7,16. The short size of loop 4 encoding the P1.9 epitope (Table 2) could make VR1 more accessible for antibodies induced by the P1.7 epitope of P1.7,16 (1). The results in Table 6 using several patient isolates with second loops shortened by naturally occurring deletions indeed show that antibodies induced by HexaMen were better able to kill patient isolates in which most of the P1.4 epitope was deleted compared to antibodies induced by MonoMen. The phenomenon that some of the deletion mutants showed higher bactericidal titers than the vaccine strain has been shown earlier for mutants in which one VR was totally deleted. Other (unknown) epitopes may become more accessible. As a consequence, deletion mutants are not ideal tools in studying epitope specificity of serum antibodies (8, 15).

We conclude that sera from children vaccinated with a monovalent as well as a hexavalent vaccine created by the RIVM killed disease-causing strains of the same subtypes as present in the vaccine and that the immunogenicity of P1.7-2,4 in HexaMen was probably underestimated. When children were immunized with MonoMen, most antibodies were directed against VR2 of PorA. These antibodies were cross-reactive with minor variants of P1.4 but not with strains having a different VR2 together with P1.7-2 as a VR1. HexaMen induced cross-reactive antibodies against all tested patient isolates with minor VR variant strains and strains containing (combinations of) VRs not present in the vaccine, although the number of vaccine responders was rather unpredictable. Antibodies were directed against VR1 as well as VR2 of PorA, but the accessibility of VR epitopes for these bactericidal antibodies depended on the specific VR1-VR2 combination. As a consequence, protection as predicted from SBA induced by a multivalent PorA-based vaccine is probably not restricted to the vaccine VR1-VR2 combinations but is much broader. The extended cross-reactivity of vaccinee sera induced by a hexavalent vaccine against antigenic variants has important favorable implications for meningococcal B vaccine coverage, thereby limiting the number of VR combinations required in a multivalent PorA-based vaccine.

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