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Human B- and T-Cell Responses after Immunization with a Hexavalent PorA Meningococcal Outer Membrane Vesicle Vaccine

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The PorA protein from *Neisseria meningitidis*, a potential vaccine candidate, induces human bactericidal antibodies which are serosubtype specific. We developed a hexavalent PorA outer membrane vesicle vaccine based on reference strain H44/76. This vaccine contains the six most prevalent PorA serosubtypes as found in many countries. We previously reported on the immune responses of 20 adult volunteers after a single immunization with this vaccine. In this study, the B- and T-cell responses in three adult volunteers were studied after three consecutive immunizations (0, 2, and 11 months). The first immunization induced a strong B-cell response resulting in high immunoglobulin G levels in an outer membrane vesicle enzyme-linked immunosorbent assay. At least a fourfold increase in bactericidal activity was observed against the majority (four to six) of the vaccine antigens compared to prevaccination titers. Immunodominance was observed for one or two of the PorAs in the bactericidal assay with a set of six isogenic H44/76-derived PorA target strains. These strains carry the individual PorAs as present in the vaccine. The second and third immunizations did not induce a further increase in the immune responses. A decline in time with respect to PorA-specific antibodies was observed after each immunization. These observations were reflected by the T-cell proliferation responses. Two additional sets of isogenic H44/76-derived mutant strains were used to study the specificity and/or cross-reactivity of the induced bactericidal antibodies. These target strains differ only in expressing mutant family variants of either PorA P1.7,16 or P1.5,10, both present in the PorA vesicle vaccine. The bactericidal antibody responses found were directed predominantly against the P1.7 (loop 1 of P1.7,16) and the P1.10 (loop 4 of P1.5,10) epitopes. This indicates that different portions of PorA were involved in the induction of bactericidal antibodies depending upon the PorA serosubtype.

Infectious disease caused by *Neisseria meningitidis* is associated with high mortality and morbidity (13). Poor immune responses are induced against the B polysaccharide while serogroup B meningococci cause most disease in many countries (3–5, 27). Therefore, vaccines based on other surface antigens are currently under investigation. Clinical trials have demonstrated the induction of bactericidal antibodies by experimental outer membrane protein vaccines. The presence of bactericidal antibodies has been shown to correlate with protection against disease (11). It was found that the PorA protein, one of the major outer membrane proteins having a porin function, was critically important for the induction of bactericidal antibodies. However, such antibodies are serosubtype specific and recognize only the two variable regions of PorA, located in the surface-exposed loops 1 and 4 of the topology model (1, 8, 14, 16, 22, 24). Recently, a hexavalent PorA outer membrane vesicle (OMV) vaccine was developed, containing the most prevalent PorAs responsible for 60 to 80% of the cases of meningococcal disease in The Netherlands (2, 19, 25). Two vaccine strains were constructed by recombinant DNA technology to express three PorA proteins each (2, 25). The vaccine strains were derived from strain HIII5, a PorB-deficient mutant of the wild-type case isolate H44/76 (6, 21). This wild-type case isolate was also used for the production of a meningococcal vaccine in Norway (1, 16). In addition, the multivalent vaccine strains were made capsule (CPS) deficient. This also resulted in expression of a truncated GalE lipopolysaccharide (LPS) (25). In this way, the presence of host-identical saccharide structures was avoided (3, 25). Previously, a phase I clinical trial was carried out with 20 adult volunteers with 50 or 100 μg of the vaccine, implying the application of 7.5 or 15 μg each of the six PorAs (12). That study revealed that the bactericidal antibodies induced after one immunization were directed against PorA. A follow-up study showed that these antibodies recognized mainly the predicted loops 1 and/or 4 of the topology model. In the case of PorA P1.7,16, the antibodies showed a preference for the P1.7 (loop 1)-associated epitopes (17, 22). For several PorA serosubtypes, point mutation or loop deletion variants have been found to occur naturally (9, 15, 17, 20, 26). This antigenic variation seems to occur more frequently in loop 4 than in loop 1 and might serve the organism as a possible mechanism of escape from the human immune response. The altered epitopes are generally not recognized by the bactericidal subtyping murine monoclonal antibodies

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TABLE 1. Amino acid sequences of PorA P1.5,10 and P1.7,16 variants as present in an identical H44/76 meningococcal background

Gene donor strain (reference[s])[a]	Subtype	Deduced amino acid sequence[b]	
		VR1 (loop 1)	VR2 (loop 4)
870227 (this study)	P1.5^c,10	PLPNIQ-PQVTKR	HFVQNK------QNQRPTLVP
2400 (this study and 20)	P1.5^a,10^a	**Q***Q******	******------***P*****
2403 (this study and 20)	P1.5^a,10^f	**Q***Q******	******QNQQNQ***P*****
2208 (this study and 17)	P1.5^c,−	******-******	Deleted
PCR mutagenesis (this study and 17)	P1.−,10	Deleted	******------*********
H44/76 (17)	P1.7,16	GGASGQVKVTKVTK	PAYYTKDTNNNLTLVP
MC58 (17)	P1.7,16^b	**************	******N*********
41/90 (17)	P1.7,16^c	**************	***-**********
IHN7634 (17)	P1.7,16^d	**************	******H*********
2208 (this study and 17)	P1.7,−	**************	Deleted
PCR mutagenesis (17)	P1.−,16	Deleted	**********
PCR mutagenesis (17)	P1.−,−	Deleted	Deleted

[a] Origins of the strains from which the *porA* genes were derived.
[b] Only the differences from the canonical PorA (870227 and H44/76) subtype sequences are indicated. *, identical (compared to canonical sequence); −, absent (compared to canonical sequence).

(MAbs) and were characterized by PCR techniques (7). This report describes the analysis of the specificity and cross-reactivity against such PorA variants of the bactericidal immune response in adults induced after three consecutive immunizations with the hexavalent PorA vaccine. The immune responses were studied by enzyme-linked immunosorbent assay (ELISA), bactericidal assay, and T-cell proliferation assay. The trivalent vaccine vesicles were used as antigen in an OMV ELISA (12). The bactericidal activity was determined against six isogenic PorA strains derived from reference strain H44/76 expressing different PorAs (6, 12) and against H44/76-derived strains carrying loop 1 and/or 4 deletions as well as point mutations within loops 1 and/or 4 of PorAs P1.7,16 and P1.5,10 (reference 17 and this study). To study the human T-cell responses, peripheral blood lymphocytes (PBLs) of the vaccinees and nonimmunized donors were collected and tested for antigen-specific proliferation with OMV preparations from strain H44/76 (B:15:P1.7,16) and from strain HI5 (B:15:−, a PorA-deficient H44/76 variant [6, 11]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *N. meningitidis* strains were grown overnight at 37°C on gonococcal medium base (Difco) supplemented with IsoVitaleX in a humid atmosphere containing 5% CO_2. The antibiotic used for selection of meningococcal transformants was kanamycin (100 μg/ml). *Escherichia coli* NM522, used for the propagation of plasmids, was grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Recombinant DNA techniques. Plasmids were constructed by standard recombinant DNA techniques (18). Restriction fragments were purified from low-melting-point agarose gels (NuSieve GTG Agarose; FMC BioProducts). Sticky ends were made blunt by incubation with T4 DNA polymerase as described by the manufacturer (Boehringer Mannheim). PCR was performed for 30 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C on a Bio-Med Thermocycle 60. The PCR buffer (10×) contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin. *Taq* polymerase was purchased from Perkin-Elmer Cetus.

Construction of plasmids. The plasmid vector used for all constructs was pTZ19R (10). Complete *porA* genes were obtained by PCR with primers 8 (5′-GGAAGCTTGGTTGCGGCATTTATCAGATATTTGTTCTG-3′) plus 7 (5′-CGTCAGGCGGTGTACCTGATGGTT-3′) as described previously (24, 25). After amplification by PCR, the *porA* genes of meningococcal strains were digested with restriction enzyme *Ecl*XI-*Kpn*I and used for replacement of the corresponding part of plasmid pCO14K (17). The cloned PCR products were verified by DNA sequencing on an Applied Biosystems automatic sequencer with double-stranded plasmid DNA templates and a cycle sequencing protocol.

Construction of isogenic strains. The construction of the six isogenic H44/76 PorA strains was described previously (12). These strains carry the individual PorAs as present in the vaccine. The amino acid sequences of the P1.5,10 and P1.7,16 wild-type strains are listed in Table 1 (17, 20). Reference strain 870227, subtype P1.5^c,10, is from our own collection and was used as a gene donor strain in the production of the hexavalent vaccine and for the construction of isogenic strain H44/76 P1.5^c,10 (2, 12). Wild-type meningococcal strains carrying the *porA* genes encoding subtypes P1.5^c,− (strain 2208, spontaneous loop 4-deficient isolate [17]), P1.5^a,10^a (strain 2400), and P1.5^a,10^f (strain 2403) were kindly provided by M. Maiden (National Institute of Biological Standards and Control, Hertfordshire, England). Previously, it was demonstrated that P1.5- and P1.7-related epitopes were associated with loop 1 and that P1.10- and P1.16-related epitopes were associated with loop 4 of the proposed eight-loop topology model of PorA (8, 20, 22). Transformation of meningococci was carried out as described elsewhere (24) with the plasmid constructs as mentioned above. The development of the isogenic P1.7,16 target strains derived from reference strain H44/76 (B:15:P1.7,16) was described previously (17). In a similar way, the *porA* genes from two P1.5,10 variants, showing heterogeneity in the variable regions VR1 (apex loop 1) and/or VR2 (apex loop 4) of PorA, were transformed into an isogenic H44/76 background through homologous recombination of gene constructs made in *E. coli* (references 2, 17, and 25 and this study). Allelic exchange of the appropriate part of *porA* was ensured by checking the presence or absence of the VR1 and VR2 epitopes with MAbs, as well as by selecting for the Kan^r marker located downstream of *porA* on plasmid pCO14K.

The loop 1-deficient, P1.−,10 strain was obtained through transformation of H44/76 P1.−,16 (17) with a plasmid carrying the complete PorA P1.5^c,10 of strain 870227 (12). Before transformation, the plasmid was linearized with *Eco*RI with a restriction site in between VR1 and VR2 in order to ensure exchange of only the loop 4 region.

Immunological methods. After transformation, screening for colonies with the appropriate phenotype was performed by colony blotting (23). The presence or absence of the desired PorA epitopes on whole cells of the constructed strains was verified by ELISA with MAbs for meningococcal typing as described previously (reference 17 and data not shown). Further characterization of the constructed strains was carried out with specific MAbs for LPS and Opc to screen for L3-positive and Opc-negative meningococcal phenotypes. Bactericidal sensitivity or resistance of the constructed strains was verified in the bactericidal assay with an LPS-specific MAb. Equal bactericidal sensitivity was found with the use of the anti-LPS MAb MN15A17F12 (data not shown), which is directed against the L3 immunotype expressed by all constructed strains. The PorA-specific MAbs used were MN22A9.19 (α-P1.5) and MN20.4F17 (α-P1.10). Other MAbs used were MN15A17F12 [α-L3,(,7,9) immunotype], MN4A8B2 (α-L3), MN43F8.10 (α-L8), and B306 (α-Opc). MAb B306 was kindly provided by M. Achtman (Berlin, Germany).

Vaccine preparation. The construction of the trivalent PorA vaccine strains is described by van der Ley et al. (25). Production, characterization, and control of the hexavalent PorA OMV vaccine are described by Claassen et al. (2). The safety and immunogenicity of the hexavalent PorA OMV vaccine are described by Peeters et al. (12). Briefly, this vaccine contains OMV preparations of two *N. meningitidis* H44/76-derived vaccine strains, PL16215 (PorA P1.7,16,5,2,19,15; CPS and PorB deficient) and PL10124 (PorA P1.5^c,10,12,13,7^h,4; CPS and PorB deficient).

Immunization of volunteers. Three adult volunteers were recruited and inducted into the study by regular informed consent. The volunteers were immunized three times, at 0, 2, and 11 months, with 100 μg of the PorA OMV vaccine. Blood samples and PBLs were collected at time points indicated in Fig. 1 and 2, to study both B- and T-cell responses in ELISA, bactericidal assay, and lymphocyte stimulation assay. Serum was collected and stored at −20°C. Each time, PBLs were collected freshly as described below under "Lymphocyte stimulation assay."

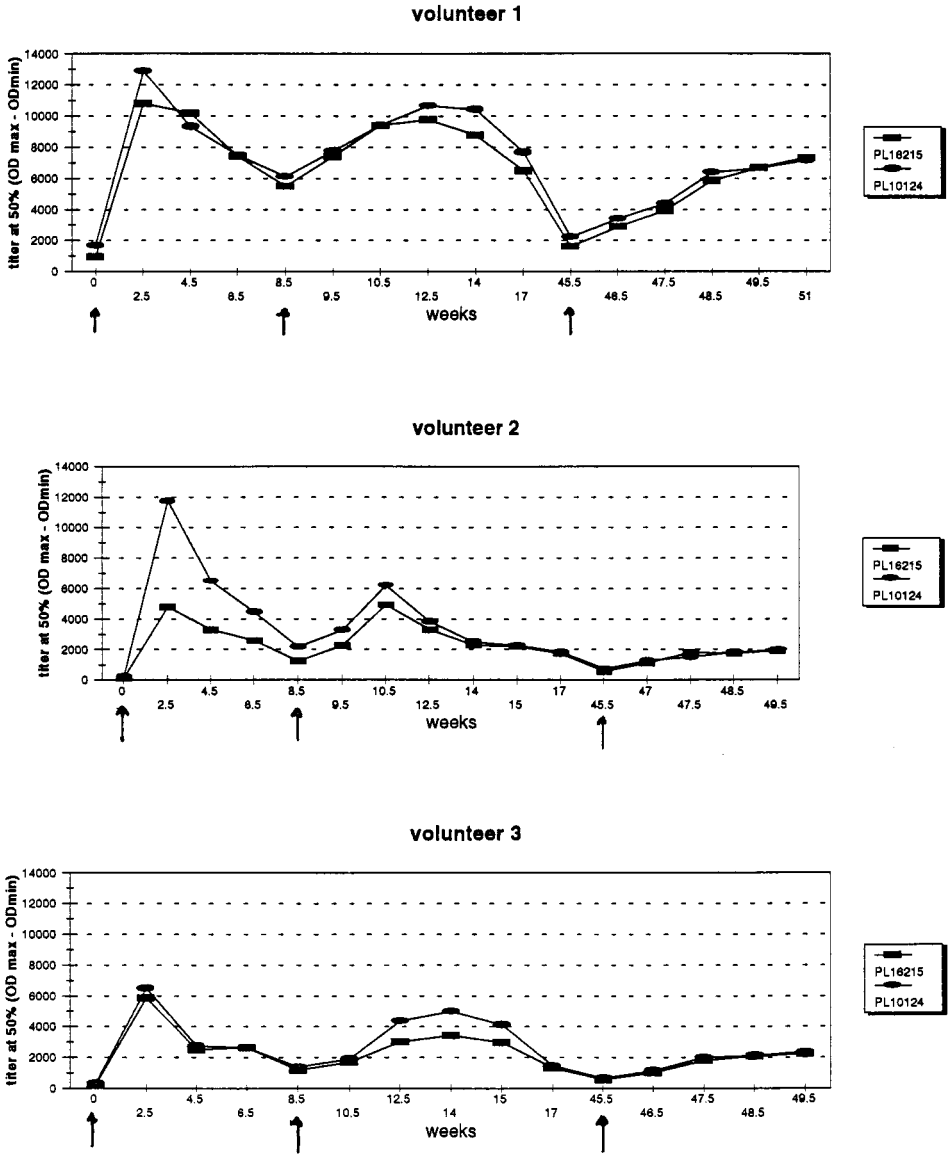

FIG. 1. Immunoglobulin G antibody responses of three immunized adult volunteers as detected in ELISA with the trivalent vaccine OMVs PL16215 (P1.7,16; P1.5,2; and P1.19,15) and PL10124 (P1.5^c,10; P1.12,13; and P1.7^h,4). Arrows indicate the time of immunization. Titers are shown at 50% of maximum optical density − minimum optical density (OD max − OD min) at optical density at 450 nm.

OMV ELISA. The ELISA was performed with the two trivalent vaccine vesicles, PL16215 and PL10124, as coat antigen (5 μg of OMV per ml of phosphate-buffered saline) as described previously (2, 12).

Bactericidal assay. Bactericidal activity of samples was determined against the isogenic target strains as described previously (17). To check for any day-to-day variation, the isogenic H44/76 strains carrying either the original PorA P1.7,16 or P1.5^c,10, as present in the hexavalent PorA OMV vaccine, were included in each day's experiment when the H44/76 mutant strains having loop deletion or point mutations in their PorA were used. Selection for target strains with the appropriate LPS and Opc phenotype was performed with colony blotting and whole-cell ELISA with a panel of specific typing MAbs as described in "Immunological methods" (data not shown).

Lymphocyte stimulation assay. Prior to immunization and at time points indicated in Fig. 2. PBLs were freshly obtained from healthy immunized and nonimmunized donors by sedimentation of heparinized blood on Lymphoprep (Nycomed Pharma AS, Oslo, Norway). PBLs were cultured in 96-well round-bottom microtiter plates (Greiner) at a density of 1.5×10^4 per well in 150 μl of RPMI 1640 (Gibco, Paisley, United Kingdom) supplemented with 5% heat-inactivated human serum, 100 U of penicillin per ml, and 100 mg of streptomycin per liter. OMV preparations from strains H44/76 and HI5 (PorA-deficient H44/76 isogenic variant) were added in the doses 0.01, 0.03, and 0.1 μg per well. The PBLs were incubated for 4 days at 37°C and 5% CO_2 and pulsed with 0.5 μCi of [^{3}H]thymidine over the last 18 h of culture. Cells were harvested, and the incorporated radioactivity was counted in a liquid scintillation counter (Pharmacia LKB). Results are expressed as the mean counts per minute of triplicate cultures.

RESULTS

OMV ELISA. The longitudinal antibody responses of the three adult volunteers against the six PorAs as present in the vaccine were analyzed in ELISA with the trivalent vaccine OMVs as the antigen (12). Each immunization (weeks 0, 8.5, and 45.5) induced a B-cell response resulting in high immunoglobulin G levels in OMV ELISA, although no booster responses were observed (Fig. 1). On the contrary, the immune responses tended to wane after each consecutive immunization.

TABLE 2. Bactericidal responses in three adult volunteers after three immunizations with 100 μg of a hexavalent PorA OMV vaccine

Volunteer and immunization	Wk	Titer for H44/76 PorA isogenic target strain[a]:						
		7,16	5,2	19,15	5[c],10	12,13	7[h],4	HI5
1								
First	0	2	16	4	2	8	2	<2
	2	64	256	256	64	128	128	<2
	4	32	128	128	32	64	64	<2
Boost	8	16	64	64	32	32	32	<2
	10	32	128	128	32	64	64	<2
	12	32	128	128	32	64	64	<2
	17	16	64	128	16	32	32	<2
Boost	46	8	32	64	8	16	16	<2
	47	8	64	64	16	32	32	<2
	48	16	128	64	16	32	32	<2
2								
First	0	<2	<2	<2	<2	<2	<2	<2
	2	<2	8	128	512	8	<2	<2
	4	<2	4	64	256	4	<2	<2
Boost	8	<2	2	8	128	2	<2	<2
	10	<2	2	64	128	2	<2	<2
	12	<2	<2	64	128	2	<2	<2
	17	<2	<2	16	64	<2	<2	<2
Boost	46	<2	<2	4	16	<2	<2	<2
	47	<2	<2	16	64	<2	<2	<2
	48	<2	<2	16	64	<2	<2	<2
3								
First	0	<2	<2	<2	<2	<2	<2	<2
	2	16	64	8	64	8	8	<2
	4	8	32	8	16	4	4	<2
Boost	8	2	16	2	8	2	<2	<2
	10	2	16	2	8	2	<2	<2
	12	8	32	4	32	2	2	<2
	17	4	16	4	32	2	<2	<2
Boost	46	2	8	2	8	<2	<2	<2
	47	4	16	4	16	2	<2	<2
	48	4	16	8	16	2	<2	<2

[a] Bactericidal titers are reported as reciprocal serum dilutions showing >90% killing. 7,16, 5,2, and 19,15 are the PorAs in trivalent OMV PL16215; 5[c],10, 12,13, and 7[h],4 are the PorAs in trivalent OMV PL10124.

Bactericidal assay with six isogenic PorA target strains. Six previously constructed isogenic PorA target strains derived from H44/76, each carrying a different PorA in the same meningococcal background, were used to investigate the induced bactericidal immune responses against individual PorAs (12). The bactericidal activities of the sera from the three volunteers receiving multiple immunizations are shown in Table 2. The induced bactericidal activity was completely PorA dependent, since there was no bactericidal activity when a PorA-deficient H44/76 variant, HI5, was used. The first immunization did induce at least a fourfold increase in bactericidal activity against the majority (four to six) of the PorA vaccine antigens compared to prevaccination titers (Table 2, weeks 0 and 2). Immunodominance of one or two of the three PorAs expressed by the trivalent vaccine strains was observed in the bactericidal assay with respect to volunteers 2 and 3 (i.e., higher titers were observed in the bactericidal assay against one or two isogenic strains carrying individual PorAs). After each consecutive immunization, a recurrent immunodominance for the same PorAs was observed, and in time, the bactericidal titers declined.

Epitope specificity and cross-reactivity of induced bactericidal antibodies. Previously, the bactericidal activities of sera from the three adults were determined against the isogenic H44/76 P1.7,16 and P1.5[c],10 strains with a first batch of human complement (Table 2). To study the specificities and/or cross-reactivities of the induced antibodies, a second batch of the same human complement was used. The human complement source was the same individual but at a different time point. The bactericidal activities of the same sera were determined again against H44/76 P1.7,16 and P1.5[c],10 to obtain a fair comparison with the isogenic H44/76 PorA P1.7,16 and P1.5,10 epitope deletion and point mutation variants. The previously determined titers were somewhat lower, but the tendency of the bactericidal activities of the samples remained the same (Table 2, columns 1 and 4; Table 3, column 1; Table 4, column 1).

PorA P1.7,16. The epitope specificities of the induced antibodies (of the three volunteers) were studied in the bactericidal assay with the two sets (P1.7,16 and P1.5,10) of isogenic H44/76-derived PorA mutant strains as targets. The PorA P1.7,16 target strains are homologous with respect to the P1.7 epitope (loop 1); the heterogeneity is present in loop 4, P1.16 (Table 1). With these variants, the induced bactericidal antibodies were found to be directed mostly towards loop 1, P1.7 (Table 3), as was previously found (17). Therefore, the heterogeneity in loop 4, P1.16, did not have a great impact on the bactericidal activity. Only the data prior to immunization (week 0) and 2 weeks after the first immunization are shown, since the specificity did not alter after additional immunizations. Deletion of the P1.16 epitope (loop 4) sometimes increased the bactericidal sensitivity of the strain. Deletion of both P1.7 and P1.16 epitopes (loops 1 and 4) did not eliminate all bactericidal activity (Table 3).

PorA P1.5,10. The antibody specificity was also studied in the bactericidal assay with the P1.5,10 variants. The bactericidal activities of the samples from the three volunteers were directed mainly against loop 4, but loop 1 is also involved, as determined with the isogenic H44/76 strains carrying PorA P1.5[c],−, P1.−,10, or P1.−,− (Table 4, columns 2, 3, and 4). Deletion of loop 4 (P1.5[c],−) strongly diminished the bactericidal activities of all samples, especially from volunteers 2 and 3 (column 2). Deletion of loop 1 (P1.−,10) influenced the bactericidal activity only partly (column 3), particularly for volunteer 1. Deletion of both loops 1 and 4 (P1.−,−) led to a further decrease of bactericidal activity for some samples, although some remaining activity was observed (column 4).

The cross-reactivities of the bactericidal antibodies were studied with the P1.5[a],10[a] and the P1.5[a],10[f] variants (Table 1).

TABLE 3. Bactericidal titers for three adult volunteers as characterized by using target strains with P1.7,16 loop 1 and 4 mutations

Volunteer	Wk	Titer for H44/76 P1.7,16 isogenic PorA loop mutation strain[a]:					
		7,16	7,−	−,16	−,−	7,16[b]	7,16[c]
1	0	4	16–32	<2	<2	<4	8
	2	>128	>128	8	8	32	64
2	0	<2	<2	<2	<2	ND[b]	ND
	2	2	32–64	<2	<2	<4	<4
3	0	<2	<2	<2	<2	<4	<4
	3	64	>128	16	32	16	32

[a] Bactericidal titers are reported as reciprocal serum dilutions showing >90% killing.

[b] ND, not determined.

TABLE 4. Bactericidal titers for three adult volunteers as characterized by using target strains with P1.5,10 loop 1 and 4 mutations

Volunteer and immunization	Wk	Titer for H44/76 P1.5,10 isogenic PorA loop-mutation strains[a]:					
		5[c],10	5[c],–	–,10	–,–	5[a],10[a]	5[a],10[f]
1							
First	0	32	4	<2	<2	<2	16
	2	>128	16	16	8	4	64
Boost	8	32	16–32	16	4	2	16–32
	10	32–64	16–32	8	4	2	32
	17	32	16	8	4	2	32
Boost	46	16	8	8	4	2	8
	48	64	16	16	2–4	4	32
	51	64	32	32	8	2	64
2							
First	0	<2	<2	<2	<2	<2	<2
	2	>128	2	>128	<2	<2	2
Boost	8	>128	<2	32	<2	<2	<2
	10	64	<2	64	<2	<2	<2
	17	64	2	ND[b]	ND	<2	<2
Boost	46	16	2	8	<2	<2	<2
	48	64	<2	16	<2	<2	<2
	51	ND	ND	ND	ND	ND	ND
3							
First	0	<2	2	<2	<2	<2	<2
	2	>128	16–32	128	32	4	64
Boost	8	64	8	8	2	<2	16
	10	32–64	8	32	16	<2	16
	17	64	4	ND	ND	<2	32
Boost	46	16	4	8	2	<2	8
	48	64	4	16	<2	<2	16–32
	51	ND	ND	ND	ND	ND	ND

[a] Bactericidal titers are reported as reciprocal serum dilutions showing >90% killing.
[b] ND, not determined.

With strain H44/76 P1.5[a],10[a], the bactericidal activities were strongly diminished for all samples from the three volunteers (Table 4, columns 5 and 6). The samples of volunteers 1 and 3 (column 6) kept bactericidal activity to the isogenic variant carrying the P1.5[a],10[f] epitopes. When the sera of our previous study (17) (one immunization only) were analyzed, the predominance of P1.10 over P1.5 was confirmed (data not shown).

T-cell immunity. Lymphocyte proliferation assays were carried out to study the T-cell responses in both vaccinated (three volunteers) and nonvaccinated (one volunteer) donors. OMV preparations from strain H44/76 (PorA proficient) and from strain HI5 (H44/76 PorA deficient) were used to study the antigen-specific stimulation of human PBLs. The results of the proliferation assays are shown in Fig. 2. The nonvaccinee did not respond in this assay. T-cell proliferation was observed after the first and/or second immunization for all three vaccinated volunteers with OMVs from H44/76 as antigen. The third immunization did not induce a detectable T-cell response in the three vaccinees. The OMVs derived from strain HI5 showed diminished responses compared to OMVs from strain H44/76. This suggests PorA to be responsible for part of the detected T-cell responses.

DISCUSSION

This study with a meningococcal hexavalent PorA OMV vaccine in adult volunteers confirmed and extended our previous findings. Bactericidal antibodies induced against PorA P1.7,16 were found to be directed predominantly against loop 1 (P1.7) of the proposed PorA topology model, although loop 4 (P1.16) is involved as well (17, 22). Results as found with the P1.5,10 isogenic H44/76 target strains showed that both loops 1 (P1.5) and 4 (P1.10) were involved in the development of bactericidal antibodies, but in this case, most of the antibodies were directed against the P1.10 epitope within loop 4 (Table 4) (data after one immunization not shown). Therefore, the heterogeneity in loop 4 of PorA P1.5,10 had a much greater impact on the induced bactericidal activity compared to the findings with the P1.7,16 target strains. The relative importance of loop 1 in the case of P1.7,16 and of loop 4 in the case of P1.5,10 probably relates to the differences in lengths of loop 1 containing either epitope P1.7 or P1.5. The lengths of loop 4 containing epitopes P1.16 or P1.10 are similar (17, 20, 22). According to their amino acid sequences and the proposed

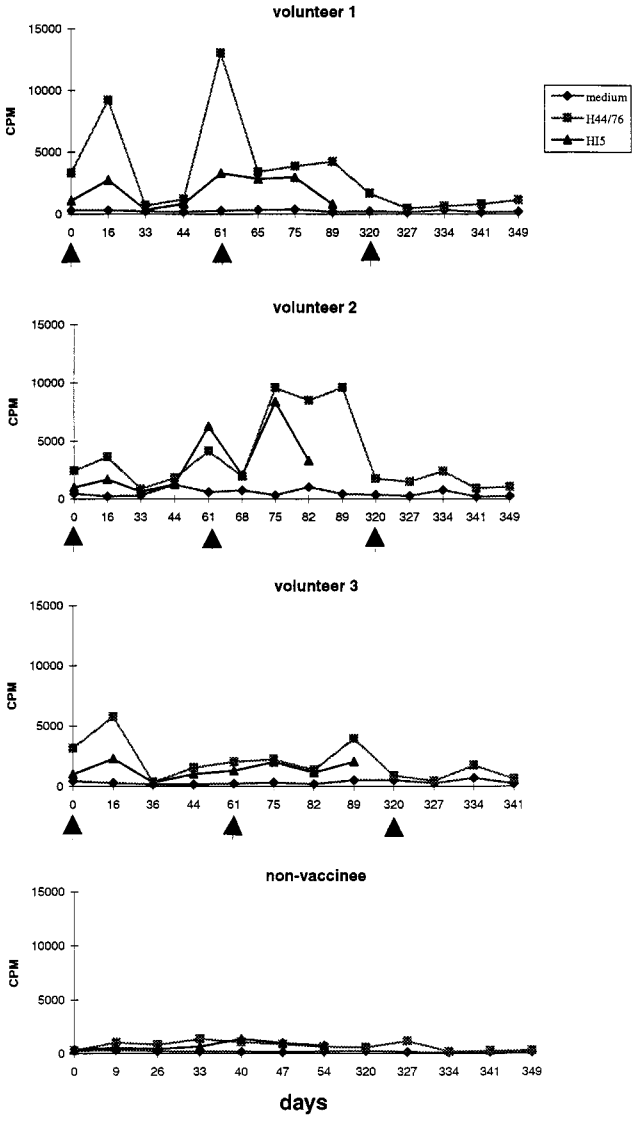

FIG. 2. T-cell responses of three immunized adult volunteers and a nonimmunized volunteer as detected in a lymphocyte proliferation assay. The antigens used for stimulation are OMVs from strains H44/76 and HI5 at a concentration of 0.03 μg per well. Arrows indicate the times of immunization. Results are expressed as the mean counts per minute of triplicate cultures.

topology model of PorA, the entire loop 1 P1.7 is 7 amino acids (aa) longer than the sequence of loop 1, P1.5 (22). This elongation is located in the apex of loop 1 (22). It should be noted that the P1.5^{a},10^{a} and P1.5^{a},10^{f} variants differ in both loops 1 and 4 from the sequence of the PorA P1.5^{c},10 and P1.5,2 (20) included in the hexavalent vaccine (Table 1). The P1.5^{a} epitope, located in the apex of loop 1, differs by 2 and 1 aa with respect to epitope P1.5^{c} (Table 1) and P1.5 (reference 20 and this study), respectively. Both epitopes are present within the vaccine, i.e., in PorA P1.5^{c},10 and in PorA P1.5,2 (Table 2) (2, 25). The P1.10^{a} epitope as expressed by strain H44/76 P1.5^{a},10^{a} showed diminished susceptibility to bactericidal anti-loop 4 antibodies, whereas the P1.10^{f} variant remained sensitive to anti-loop 4 P1.10 antibodies (Table 4, columns 5 and 6) compared to titers obtained with the isogenic H44/76 strain expressing the vaccine antigen PorA P1.5^{c},10 (column 1). Markedly, compared to the sequence of the original P1.10 epitope, the greatest sequence variation is found within the P1.10^{f} epitope (Table 1). P1.10^{a} differs only by one amino acid substitution from the original vaccine P1.10 epitope. The P1.10^{f} epitope contains a 6-aa-long insertion before the putative epitope and, in addition to this, the same amino acid substitution located within the epitope as that found in P1.10^{a}. Conceivably, elongation of the P1.10^{f} epitope by six additional amino acid residues might result in an altered loop structure, which could be more surface exposed than the original P1.10 and variant P1.10^{a} epitopes. The induced anti-P1.10 antibodies which could previously not recognize the altered P1.10^{a} epitope apparently can recognize the P1.10^{f} epitope containing the same sequence as P1.10^{a} plus an additional set of 6 aa.

In some cases, PorA loop deletions resulted in a higher sensitivity of the strain to bactericidal active antibodies compared to strains expressing intact PorA (Table 3, columns 1 and 2) (17). When both loops 1 and 4 containing the variable regions of PorA were deleted, remaining bactericidal activity was observed (Tables 3 and 4, column 4) (17). With the knowledge that the PorA-deficient H44/76-derived isogenic strain HI5 (Table 2) was resistant to killing, such findings indicate the existence of antibodies against conserved PorA regions, which can become active in case loop 1 and/or 4 is deleted.

Another finding from this study relates to the observed immune responses after multiple immunizations. The first immunization results in a marked rise in antibody levels, probably in relation to priming due to nasopharyngeal carriage with meningococci. A second and third immunization did not result in further rise of the antibody responses. In fact, diminished antibody and T-cell responses were observed (Fig. 1 and 2). Such diminished B- and T-cell responses suggest tolerance-generating mechanisms, which may relate to the high dose used. This is in contrast to published results from Norwegian vaccine trials (16). In the Norwegian studies, volunteers were immunized two times (6-week interval) with a monovalent vesicle vaccine (25 μg/dose) derived from wild-type strain H44/76. A third dose administered after 4 to 5 years induced booster responses (16). This wild-type case isolate is CPS proficient, expressing PorB and wild-type L3 LPS. Our vaccine strains are based on a CPS- and PorB-deficient variant of H44/76, expressing truncated GalE LPS (21, 25).

The observation of an immunodominance skewing the immune response towards certain PorAs present in the vaccine, the influence of the heterogeneity of functional epitopes on the bactericidal activity, and the observation of the absence of booster responses in adults indicate the need for further investigations with this vaccine.

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REFERENCES

1. **Bjune, G., E. A. Høiby, J. K. Grønnesby, O. Arnesen, J. H. Frederiksen, A. Halstensen, et al.** 1991. Effect of an outer membrane vesicle vaccine against group B meningococcal disease in Norway. Lancet **338:**1093–1096.
2. **Claassen, I., J. Meylis, P. van der Ley, C. Peeters, H. Brons, J. Robert, D. Borsboom, A. van der Ark, I. van Straaten, P. Roholl, B. Kuipers, and J. Poolman.** 1996. Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine. Vaccine **14:**1001–1008.
3. **Finne, J., D. Bitter-Suerman, C. Goridis, and U. Finne.** 1987. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J. Immunol. **138:**4402–4407.
4. **Frasch, C. E.** 1985. Status of a group B *Neisseria meningitidis* vaccine. Eur. J. Clin. Microbiol. **4:**533–536.
5. **Frasch, C. E.** 1989. Vaccines for prevention of meningococcal disease. Clin. Microbiol. Rev. **2:**S134–S138.
6. **Holten, E.** 1979. Serotypes of *Neisseria meningitidis* isolated from patients in Norway during the first six months of 1978. J. Clin. Microbiol. **9:**186–188.
7. **Maiden, M. C. J., J. A. Byrgraves, J. McCarvill, and I. M. Feavers.** 1992. Identification of meningococcal serosubtypes by polymerase chain reaction. J. Clin. Microbiol. **30:**2835–2841.
8. **McGuinness, B., A. K. Barlow, I. N. Clarke, J. E. Farley, A. Anilionis, J. T. Poolman, and J. E. Heckels.** 1990. Deduced amino acid analysis of class 1 protein (PorA) from three strains of *Neisseria meningitidis*: synthetic peptides define the epitopes responsible for serosubtype specificity. J. Exp. Med. **171:**1871–1882.
9. **McGuinness, B. T., I. N. Clarke, P. R. Lambden, A. K. Barlow, J. T. Poolman, D. M. Jones, and J. Heckels.** 1991. Point mutation in meningococcal *porA* gene associated with increased endemic disease. Lancet **337:**514–517.
10. **Mead, D. A., and B. Kemper.** 1988. Chimeric single-stranded DNA phage-plasmid cloning vectors, p. 85–102. *In* R. L. Rodriguez and D. T. Denhardt (ed.), Vectors: a survey of molecular cloning vectors and their uses. Butterworth, Boston, Mass.
11. **Milagres, L. G., S. R. Ramos, C. T. Sacchi, C. E. Melles, V. S. Vieira, H. Sato, G. S. Brito, J. C. Moraes, and C. E. Frasch.** 1994. Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect. Immun. **62:**4419–4424.
12. **Peeters, C. C. A. M., H. C. Rümke, L. C. Sundermann, E. M. Rouppe van der Voort, J. Meulenbelt, M. Schuller, A. J. Kuipers, P. van der Ley, and J. T. Poolman.** 1996. Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine **14:**1009–1015.
13. **Peltola, H.** 1984. Meningococcal disease: still with us. Rev. Infect. Dis. **5:**71–91.
14. **Poolman, J. T.** 1995. Development of a meningococcal vaccine (review). Infect. Agents Dis. **4:**13–28.
15. **Rosenqvist, E., E. A. Høiby, E. Wedege, D. A. Caugant, L. O. Frohølm, B. T. McGuinness, J. Brooks, P. R. Lambden, and J. E. Heckels.** 1993. A new variant of serosubtype P1.16 in *Neisseria meningitidis* from Norway, associated with increased resistance to bactericidal antibodies induced by a serogroup B outer membrane protein vaccine. Microb. Pathog. **15:**197–205.
16. **Rosenqvist, E., E. A. Høiby, E. Wedege, K. Bryn, J. Kolberg, A. Klem, E. Rønnild, G. Bjune, and H. Nøkleby.** 1995. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect. Immun. **63:**4642–4652.
17. **Rouppe van der Voort, E., P. van der Ley, J. van der Biezen, S. George, O. Tunnela, H. van Dijken, B. Kuipers, and J. Poolman.** 1996. Specificity of human bactericidal antibodies induced with a hexavalent meningococcal outer membrane vesicle vaccine. Infect. Immun. **64:**2745–2751.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Scholten, R. J. P. M., H. A. Bijlmer, J. T. Poolman, A. J. Kuipers, D. A. Caugant, L. van Alphen, J. Dankert, and H. A. Valkenburg.** 1993. Meningococcal disease in The Netherlands, 1958–1990. Steady increase of the incidence rate since 1982 partially caused by new serotypes and subtypes of *Neisseria meningitidis*. Clin. Infect. Dis. **16:**237–246.
20. **Suker, J., I. M. Feavers, and M. C. J. Maiden.** 1996. Monoclonal antibody recognition of members of the meningococcal P1.10 variable region family: implications for serological typing and vaccine design. Microbiology **142:**63–69.

21. **Tommassen, J., P. Vermeij, M. Struyvé, R. Benz, and J. T. Poolman.** 1990. Isolation of *Neisseria meningitidis* mutants deficient in class 1 (PorA) and class 3 (PorB) outer membrane proteins. Infect. Immun. **58:**1355–1359.
22. **van der Ley, P. A., J. E. Heckels, M. Virji, P. Hoogerhout, and J. T. Poolman.** 1991. Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect. Immun. **59:**2963–2971.
23. **van der Ley, P. A., and J. T. Poolman.** 1992. Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. Infect. Immun. **60:**3156–3161.
24. **van der Ley, P. A., J. van der Biezen, P. Hohenstein, C. Peeters, and J. T. Poolman.** 1993. Use of transformation to construct antigenic hybrids of the class 1 outer membrane protein in *Neisseria meningitidis*. Infect. Immun. **61:**4217–4224.
25. **van der Ley, P. A., J. van der Biezen, and J. T. Poolman.** 1995. Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *porA* gene for use in the production of a multivalent outer membrane vesicle vaccine. Vaccine **13:**401–407.
26. **Wedege, E., R. Dalseg, D. A. Caugant, J. T. Poolman, and L. O. Frøholm.** 1993. Expression of an inaccessible P1.7 subtype epitope on meningococcal class 1 proteins. J. Med. Microbiol. **38:**23–28.
27. **Wyle, F. A., M. S. Artenstein, B. L. Brandt, E. C. Tramont, D. L. Kasper, P. L. Altieri, S. L. Berman, and J. P. Lowenthal.** 1972. Immunological response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. **126:**514–522.

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