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Specificity of Human Bactericidal Antibodies against PorA P1.7,16 Induced with a Hexavalent Meningococcal Outer Membrane Vesicle Vaccine

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A set of isogenic strains was constructed from the meningococcal reference strain H44/76 (B:15:P1.7,16) which differed only in their outer membrane protein (OMP) compositions. First, three isogenic strains lacking the expression of either class 3 (PorB) or class 4 (RmpM) OMP or both were obtained. Second, three isogenic class 1 OMP loop-deficient strains of H44/76 lacking the predicted loop 1 or 4 or both of class 1 OMP (PorA) were obtained. Third, three isogenic class 1 OMP strains which differed by point mutations in the predicted loop 4 of subtype P1.16 were constructed. Strains were constructed through transformation with gene constructs made in *Escherichia coli* and their homologous recombination into the meningococcal chromosome. This study describes the contribution of one of the six class 1 OMPs, PorA P1.7,16, in the development of bactericidal antibodies after a single immunization of adult volunteers with 50 or 100 μg of protein within a hexavalent PorA outer membrane vesicle vaccine. PorA-, PorB-, and RpmM-deficient isogenic strains were used to define the human immune response against PorA. The loop-deficient isogenic strains were used to define the contribution of loops 1 and 4 of PorA in the development of bactericidal anti-PorA antibodies. The isogenic strains carrying a point mutation in loop 4 were used to study the cross-reactivity of the induced bactericidal antibodies against target strains showing microheterogeneity. The results indicate that a single immunization with the hexavalent PorA vaccine induced a dose-dependent bactericidal immune response, which is directed mainly against PorA. The epitope specificity of antibodies is directed mostly against loop 1, although loop 4 and as-yet-unidentified epitopes of PorA P1.7,16 are also involved.

Neisseria meningitidis is a human pathogen and one of the major causes of bacterial meningitis (23). Serogroups A, B, and C, and to a lesser extent W-135 and Y, are responsible for most cases of meningococcal disease. Current meningococcal vaccines are based on the capsular polysaccharides of serogroup A, C, W-135, and Y meningococci. These vaccines provide protection of short duration against meningococcal disease. However, no protection can be induced in infants, the age group at highest risk (11, 12). Above all, the available polysaccharide vaccines give no protection against serogroup B meningococcal disease, the most prevalent serogroup in many countries. A vaccine based on group B polysaccharide appears hard to realize because of its poor immunogenicity, probably due to structural similarities with human tissues (10). In addition to the potency issue, safety aspects of group B polysaccharide-based vaccines need to be investigated, especially the possibility of inducing an autoimmune response (10). At this moment, vaccines based on the outer membrane proteins (OMPs) of group B meningococci are under investigation. The subtyping of meningococci is based on differences between the PorAs (1, 2). Although this protein displays antigenic heterogeneity, it is expressed by almost all meningococcal isolates. Together with PorB, they form the basis of the sero-subtyping system of meningococci and function as porin proteins in the outer membrane (1, 2). Murine monoclonal antibodies (MAbs) have been developed for epidemiological purposes to type meningococcal strains (1, 2). Previous research showed that PorA is a vaccine candidate because MAbs are highly bactericidal and give protection against infection in an animal model (29, 30). Immunization of mice with outer membrane vesicles (OMV) induced bactericidal activity which was directed mainly against PorA (36). In field trials, experimental vaccines based on meningococcal OMVs showed partial protection against group B meningococcal disease (4, 7). The in vitro bactericidal activity of the induced antibodies appeared to be partly strain specific and directed against PorA of the homologous strain (15, 26). The presence of bactericidal antibodies is generally accepted as a marker for protection against infection (11, 13). The prediction of the structure of PorA by a two-dimensional topology model showed that PorA is a membrane-spanning protein with eight surface-exposed loops (6, 35). Comparison of amino acid sequences of several PorAs showed that antigenic variation occurred in two discrete regions, variable regions 1 and 2 (VR1 and VR2, respectively), corresponding to loops 1 and 4 in the model (35). Pepscan studies showed that VR1 and VR2 were recognized by bactericidal subtyping PorA MAbs (17). A hexavalent PorA group B OMV vaccine covering more than 80% of the meningococcal subtypes isolated in many countries, for example, The Netherlands, was developed (8, 31, 38). A phase I safety study was carried out in adult volunteers (22). This report describes the construction of several isogenic meningococcal strains derived

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TABLE 1. Meningococcal isogenic target strains

Strain[a]	Relevant characteristic(s)	Reference
H44/76	B:15:P1.7,16	16
H44/76-Δ7	26-residue deletion in loop 1	This study
H44/76-Δ16	25-residue deletion in loop 4	This study
H44/76-Δ7,16	Deletions in both loops 1 and 4	This study
H44/76-Δcl3	Class 3 OMP-deficient mutant, *porB*::*ermC*	This study
H44/76-Δcl4	Class 4 OMP-deficient mutant, *rpmM*::*kanR*	This study
H44/76-Δcl3,4	Class 3 and 4 OMP-deficient mutant, *porB*::*ermC rpmM*::*kanR*	This study
HI5	Spontaneous PorA-deficient mutant	34
TR52-7,16[b]	Exchange with P1.7,16[b] allele	This study
TR52-7,16[c]	Exchange with P1.7,16[c] allele	This study
TR52-7,16[d]	Exchange with P1.7,16[d] allele	This study
TR52	Exchange with P1.5,2 allele	36, 37

[a] All strains are derivatives of H44/76.

from reference strain H44/76 (B:15:P1.7,16) to investigate the contribution of PorA P1.7,16 in the development of bactericidal antibodies. Sero-subtype P1.7,16 is one of the six PorAs present in the hexavalent OMV vaccine. The epitope specificity of the induced bactericidal PorA P1.7,16 antibodies was investigated through construction of deletions or point mutations within PorA P1.7,16 focusing on loops 1 and 4 out of the predicted eight surface-exposed loops (35).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *N. meningitidis* strains used in this study are listed in Table 1. Bacteria were grown overnight at 37°C on gonococcal (GC) medium base (Difco) supplemented with IsoVitaleX in a humid atmosphere containing 5% CO_2. The antibiotics used for selection of meningococcal transformants were erythromycin (5 μg/ml) and kanamycin (100 μg/ml). Transformation of meningococci was carried out as described previously (36). *Escherichia coli* NM522, used for the propagation of plasmids, was grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml). Wild-type meningococcal strains carrying the *porA* genes encoding for P1.16[b] (strain MC58) and P1.16[c] (strain 41/90) were kindly provided by J. Heckels (Southampton, England) and E. Rosenqvist (Oslo, Norway). The meningococcal strain IHN76342, carrying the P1.16[d] epitope, was isolated in Finland during this study. The wild-type strain carrying the *porA* gene with a spontaneous loop 4 deletion, P1.5[c] (strain 2208), was kindly provided by J. Suker and M. Maiden (Hertfordshire, England).

Recombinant DNA techniques. Plasmids were constructed by standard recombinant DNA techniques (28). 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). The 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.

Complete *porA* genes were obtained by PCR with primers 8 (5′-GGAAGCT TGGTTGCGGCATTTATCAGATATTTGTTCTG-3′) and 7 (5′-CGTCAGGC GGTGTACCTGATGGTT-3′) as described previously (37, 38). Sequence analysis was performed with an Applied Biosystems automatic sequencer on double-stranded plasmid DNA templates and with a cycle sequencing protocol.

Construction of plasmids. The plasmid vector used for all constructs was pTZ19R (19). Plasmid pKTH3144, carrying the *porB* gene from strain H44/76, was kindly provided by S. Butcher (Helsinki, Finland) and has been described previously (5).

The erythromycin resistance marker, *ermC*, was derived from pIM13 (20) and cloned in pBluescript between the *Hin*dIII and *Cla*I sites, resulting in plasmid pER2.

(i) PorB (class 3 OMP) deficiency. To obtain PorB-deficient strains, plasmids pRV1 and pRV2 were constructed. For the construction of plasmid pRV1, a 1.6-kb *Pvu*I-*Pvu*I fragment carrying the *ermC* gene was isolated from pER2. Plasmid pKTH3144 was digested once with *Bsi*WI, a restriction site located within the *porB* gene, and sticky ends were flushed with T4 DNA polymerase. Both fragments were ligated to interrupt *porB* with the *ermC* gene. Transformation experiments with the resulting plasmid pRV1 to meningococci failed, presumably because the DNA uptake sequence (DNA-us) was not present on this construct. Therefore, a 1.1-kb *Xho*I restriction fragment was isolated from pCO14 (37); this fragment constituted the downstream part of the *porA* gene of strain 2996, which contained the DNA uptake sequence for *porA* (14). This fragment was ligated behind the *ermC* gene into the *Xho*I site of pRV1, resulting in pRV2 (Fig. 1a). Linearized plasmid was used to transform strain H44/76 to erythromycin resistance (36).

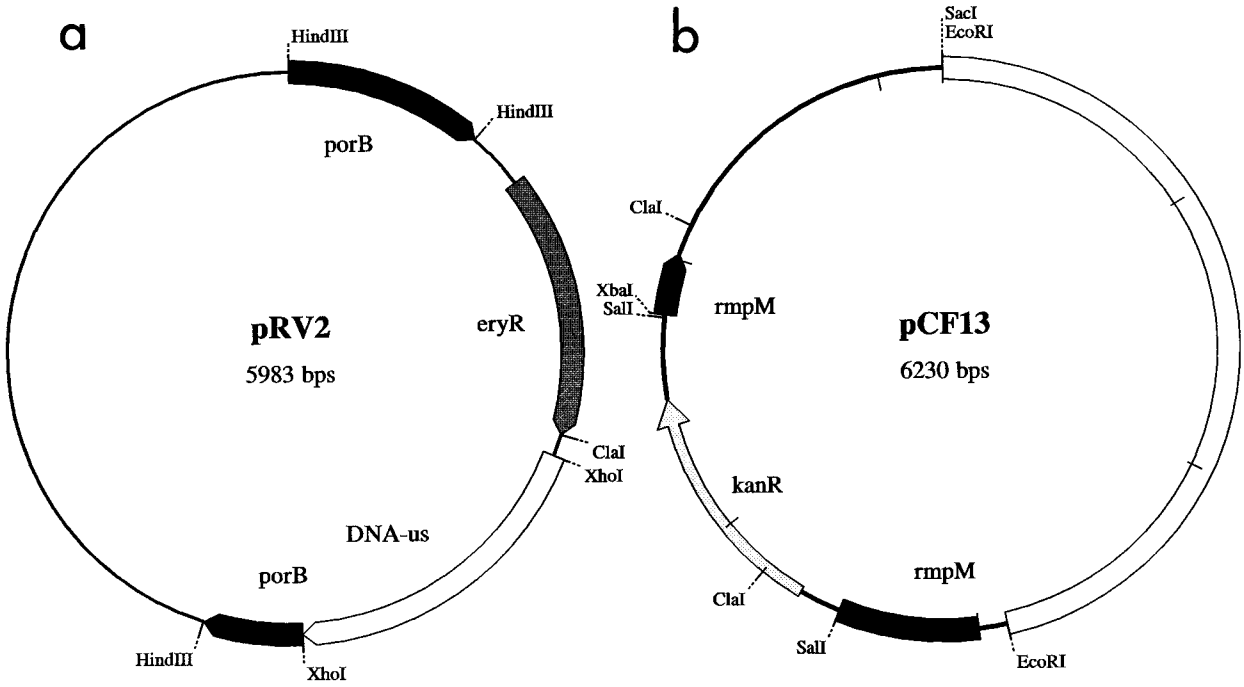

FIG. 1. Restriction maps of plasmids used for making *porB* (a) and *rmpM* (b) mutants. Arrowheads indicate the direction of transcription. DNA-us, DNA uptake sequence derived from pCO14 (38).

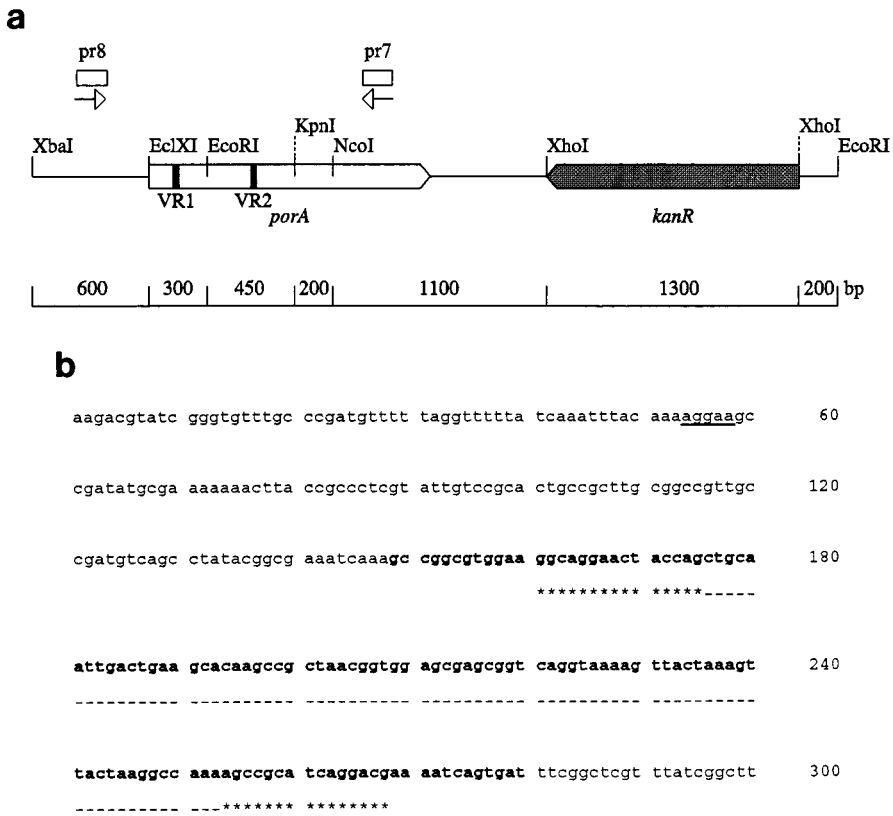

FIG. 2. (a) Restriction map of the *porA* insert in plasmid pCO14K. *Ecl*XI-*Kpn*I-digested PCR products of different *porA* genes were ligated in *Ecl*XI-*Kpn*I-digested pCO14K. (b) The first 300 DNA bases of the *porA* gene sequence of strain H44/76, with the sequence of the mutagenic oligonucleotide primer DL1 indicated (*). DL2 is the complementary strand of DL1. The sequence encoding loop 1 is shown in bold, while missing bases are indicated by a dash.

(ii) RmpM (class 4 OMP) deficiency. Plasmid pCF13 was derived from pCF10 (38) by deleting a 2.2-kb *Xba*I fragment carrying *porA*, which interrupted the *rmpM* gene. Ligation of the digested plasmid DNA resulted in pCF13 carrying the complete *rmpM* gene with an inserted kanamycin resistance gene in the same orientation (Fig. 1b). Linearized plasmid was used to transform strain H44/76 as described previously (36) to kanamycin resistance.

(iii) Construction of loop deletions in PorA (class 1 OMP). Plasmid pCO14 (37) carries the complete *porA* gene from strain 2996. A *kanR* cassette was inserted into the *Xho*I site located downstream of *porA*, resulting in plasmid pCO14K (Fig. 2a). The *Ecl*XI-*Kpn*I fragment encoding both VR1 and VR2 was replaced with a similarly digested PCR product of strain 2208, a group A isolate carrying a spontaneous 26-residue deletion in loop 4 (33). The resulting plasmid was termed pCO14KΔ4. For making a deletion in loop 1, a mutagenic PCR procedure was used (28). Primer DL1 and its complement DL2, spanning the sequence to be deleted, were designed (Fig. 2b). First, PCR products were generated with primers DL1 and -7 and DL2 and -8. The products were gel purified, mixed, and allowed to anneal, after which PCR was performed with primers 8 and 7. The resulting product was digested with *Ecl*XI-*Kpn*I and used to replace the corresponding part of pCO14K, resulting in pCO14KΔ1. Sequence analysis of the VR1 and VR2 regions of plasmids pCO14KΔ1 and pCO14KΔ4 confirmed that the deletions had been constructed correctly (Fig. 3). Exchange of *Eco*RI fragments was used to construct plasmid pCO14KΔ1,4 with deletions in both loops 1 and 4. To transfer these alleles into an isogenic meningococcal background, transformation of H44/76 to kanamycin resistance and screening for colonies with the appropriate P1.7,16 phenotype was performed (7^+16^- for Δloop 4, 7^-16^+ for Δloop 1, and 7^-16^- for Δloops 1,4).

(iv) Subtype P1.16 point mutations. Naturally occurring P1.16 point mutation variants have been isolated in England (18), Norway (27), and Finland (this study) and designated P1.16[b] (YYTKNTNNNLTL), P1.16[c] (Y-TKDTNNN LTL), and P1.16[d] (YYTKHTNNNLTL), respectively (Fig. 3) (mutations denoted with underlining). The wild-type strains carrying these point mutations showed diminished or absent reactivity with P1.16-specific MAbs compared with strains carrying the original P1.16 epitope (data not shown) (references 18 and 27 and present study). The P1.16[b] and P1.16[c] genes of strains MC58 (18) and 41/90 (27) were transferred into H44/76 as follows. *Ecl*XI-*Kpn*I-digested PCR products were used to replace the corresponding part of plasmid pCO14K (Fig. 2a). The resulting plasmids, termed pMC58K and p41/90K, were used to transform strain TR52 (36, 37), an isogenic strain derived from H44/76 with a P1.5,2 phenotype, to kanamycin resistance and a P1.7-positive and P1.5,2-negative phenotype. For transfer of the P1.16[d] allele of strain IHN76342, a slightly different approach was used. First, this strain was transformed to kanamycin resistance with plasmid pCO14K linearized with *Nco*I (Fig. 2a). Second, chromosomal DNA of the resulting strain was used to transform TR52 (36, 37) to kanamycin resistance and a P1.7-positive and P1.5,2-negative phenotype. A comparison of the amino acid sequences of the produced loops 1 and 4 is shown in Fig. 3.

Immunological methods. Expression of class 1, class 3, or class 4 OMP epitopes on whole cells of the constructed strains was verified by colony blotting (36) or enzyme-linked immunosorbent assay (ELISA) (1, 2, 31, 32) and Western blotting (immunoblotting) (35, 37) with MAbs for meningococcal typing (31, 32). Bactericidal sensitivity or resistance of the constructed strains was verified by a bactericidal assay with a panel of epitope-specific meningococcal typing MAbs. The class 1-specific MAbs used were MN14C11.6 (α-P1.7), MN5C11G (α-P1.16), MN22A9.19 (α-P1.5), and MN16C13F4 (α-P1.2). Other MAbs used were MN15A17F12 (α-L3,7,9 immunotype), MN15A14H6 (α-class 3 OMP, serotype 15), MN2D6D (α-class 4 OMP [25]), and MN33H7.6 (α-loop 5 of PorA P1.7,16). The specific PorA, α-loop 5 MAb was prepared during this study. BALB/c mice were immunized with a conjugate of a cyclic peptide derived from loop 5 of PorA P1.7,16 from strain H44/76. Cyclo-[LFLIGSGSDQACPA] was conjugated to (bromoacetylated) tetanus toxoid through its side chain of the cysteine residue. Fusion experiments resulted in the production of a nonbactericidal MAb of the immunoglobulin G2b isotype, specifically recognizing loop 5 of native PorA P1.7,16 in a whole-cell ELISA and colony blot (16b).

Phase I study. A group of 30 healthy adults with negative anamnesis for meningococcal disease participated in a phase I study (22). Volunteers were immunized once, with either a placebo (aluminum phosphate, $n = 10$) or two doses (total protein, 50 μg [$n = 10$] or 100 μg [$n = 10$]) of the hexavalent PorA OMV vaccine (8, 22). The contribution of each class 1 OMP present in the vaccine was estimated to be 15% (8). Thus, the amount of class 1 OMP P1.7,16 used for immunization of volunteers was approximately 7.5 or 15 μg (8). Blood samples were taken on day 0 prior to immunization and 14 days after immunization. Sera were collected and stored at −20°C.

Bactericidal assay. The bactericidal activity of antibodies against isogenic variants of strain H44/76 was determined as described by Bartoloni et al. (3) and Peeters et al. (22), with some adaptations to increase the sensitivity. Serum samples and bacteria were allowed to incubate for 10 to 15 min at room temperature (20 to 22°C) prior to the addition of complement. A final concentration

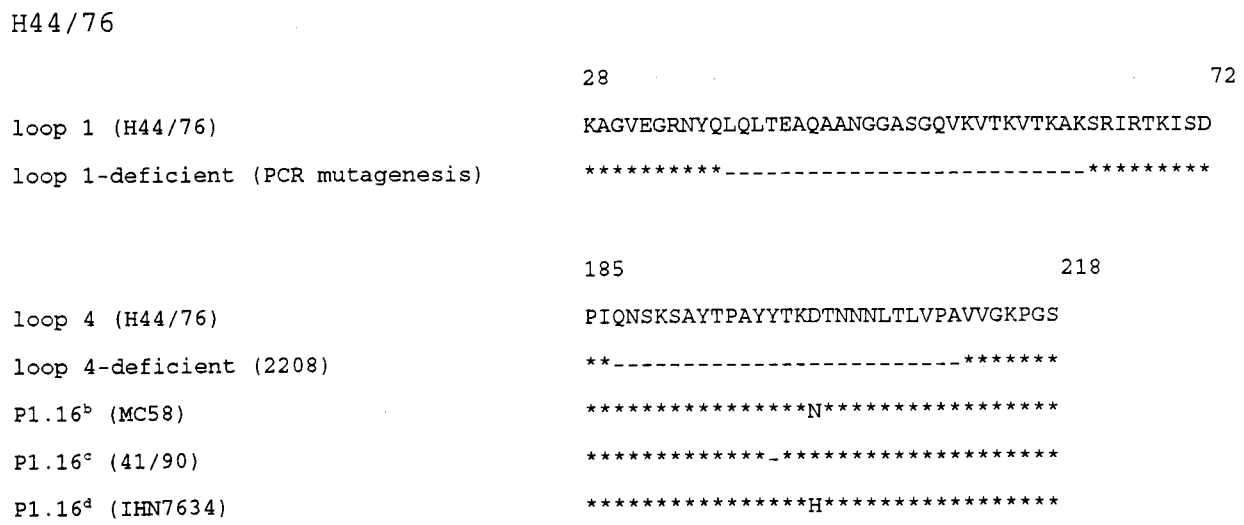

FIG. 3. Amino acid sequences of loops 1 and 4 of PorA variants used in this study as present in an identical H44/76 meningococcal background. The origins of the different PorAs are given in parentheses (references 18, 27, and 33, and present study).

of 10% human complement was used as an exogenous complement source. The bactericidal assay was carried out in an end volume of 24 μl per well. The bactericidal titer is reported as the highest reciprocal serum dilution showing killing of the bacteria (by visual inspection).

RESULTS

Verification of constructed strains. First, Western blotting with PorA (class 1 OMP)-specific murine MAbs was used to check the presence or absence of desired epitopes as well as the level of PorA expression in the constructed strains (Fig. 4). The lane numbers of Fig. 4 correspond with the strain numbers as mentioned in Table 2. Demonstration of the presence or absence of PorB (class 3 OMP) was not possible by Western blotting (9) and was therefore verified by a whole-cell ELISA (data not shown). The presence or absence of RmpM (class 4 OMP) was also verified by a whole-cell ELISA (data not shown).

Deletion of loop 1 (P1.7) or 4 (P1.16) or both resulted in a PorA with a lower molecular weight (Fig. 4c, lanes 2 to 4) than that of the complete PorA, as indicated by an arrow (Fig. 4, lane 1). The simultaneous loss of loops 1 and 4 resulted in a PorA with a molecular weight almost identical to that of PorB. The presence or absence of the P1.7 or P1.16 epitope was verified with MAbs MN14C11.6 (α-P1.7) and MN5C11G (α-P1.16) as shown in Fig. 4a and b. PorAΔ7,16, lacking loops 1 and 4, showed no reaction with these MAbs, as expected (Fig. 4a and b, lane 4). Identification of PorAΔ7,16 was possible by Western blotting with a recently developed MAb, MN33H7.6 (see Immunological methods above), recognizing loop 5 of PorA P1.7,16 (Fig. 4c, lane 4).

The isogenic strains, as listed in Table 2, carrying a point mutation in loop 4 within the predicted P1.16 epitope ($P1.16^b$ and $P1.16^d$) as determined by McGuinness et al. (17, 18) could not be detected with MAb MN5C11G (Fig. 4b, lanes 9 and 11). $P1.16^c$, carrying a point mutation outside the predicted epitope, was detectable as was the original P1.16 epitope of H44/76 (Fig. 4b, lanes 1 and 10). This is in accordance with previous findings with the wild-type strains from which the *porA* genes were derived (see Subtype P1.16 point mutations above). The P1.7 epitope, identical in the latter four strains, could be demonstrated with MAb MN14C11.6 (Fig. 4a, lanes 1 and 9 to 11). HI5, the PorA-deficient strain, did not react with the PorA-specific MAbs (Fig. 4a to c, lanes 8). Second, the isogenic strains were tested in a bactericidal assay for their sensitivity for bactericidal MAbs. The data, as listed in Table 2, show that the removed epitopes resulted in bactericidal resistance for the particular epitope-specific MAb, while sensitivity remains while the epitope is present. Like the wild-type strains, the isogenic strain carrying the $P1.16^b$ epitope shows a low MN5C11G susceptibility and the strain with the $P1.16^c$ epitope shows an intermediate MN5C11G susceptibility in comparison with that of the original P1.16 strain (27). The isogenic strain carrying the $P1.16^d$ epitope was found to be resistant to the bactericidal activity of MAb MN5C11G, as was found with the wild-type strain IHN76342 (16a).

Specificity of bactericidal activity of sera from immunized volunteers. Sera from the volunteers were tested for bactericidal activity against the isogenic H44/76 strains. Previously, an increase in bactericidal activity against the parental strain H44/76 was investigated (22). The volunteers of the control group (0 μg of total protein, $n = 10$), who received aluminum phosphate only, showed no increase in bactericidal activity against H44/76 (0 of 10 volunteers). Of the volunteers immunized with a single dose of the hexavalent PorA OMV vaccine (50 μg of total protein, $n = 10$), four vaccinees (40%) showed at least a fourfold increase in bactericidal activity (Table 3). Of the volunteers immunized with a double dose of the hexavalent PorA OMV vaccine (100 μg, $n = 10$), seven vaccinees (70%) showed at least a fourfold increase (Table 3).

The sera of the volunteers showing increased bactericidal activity against the original strain H44/76 (22) were tested in the bactericidal assay against the H44/76 isogenic strains (Table 3). To control day-to-day variation, the original strain H44/76 was included in each day's experiment, when sera of the phase I study were tested against the isogenic strains. The bactericidal assay was reproducible and showed a minimal day-to-day variation (less than one dilution step; all samples were tested at least five times against the parental strain H44/76). It has been suggested that RmpM (class 4 OMP), as present in the OMV preparations, is capable of inducing blocking antibodies, which decrease the bactericidal activity of other antibodies (21). Therefore, RmpM was removed from two target strains (H44/76-Δcl4 and H44/76-Δcl3/4). Strains HI5 (PorA deficient), H44/76Δcl3 (PorB deficient) and H44/76-Δcl3/4

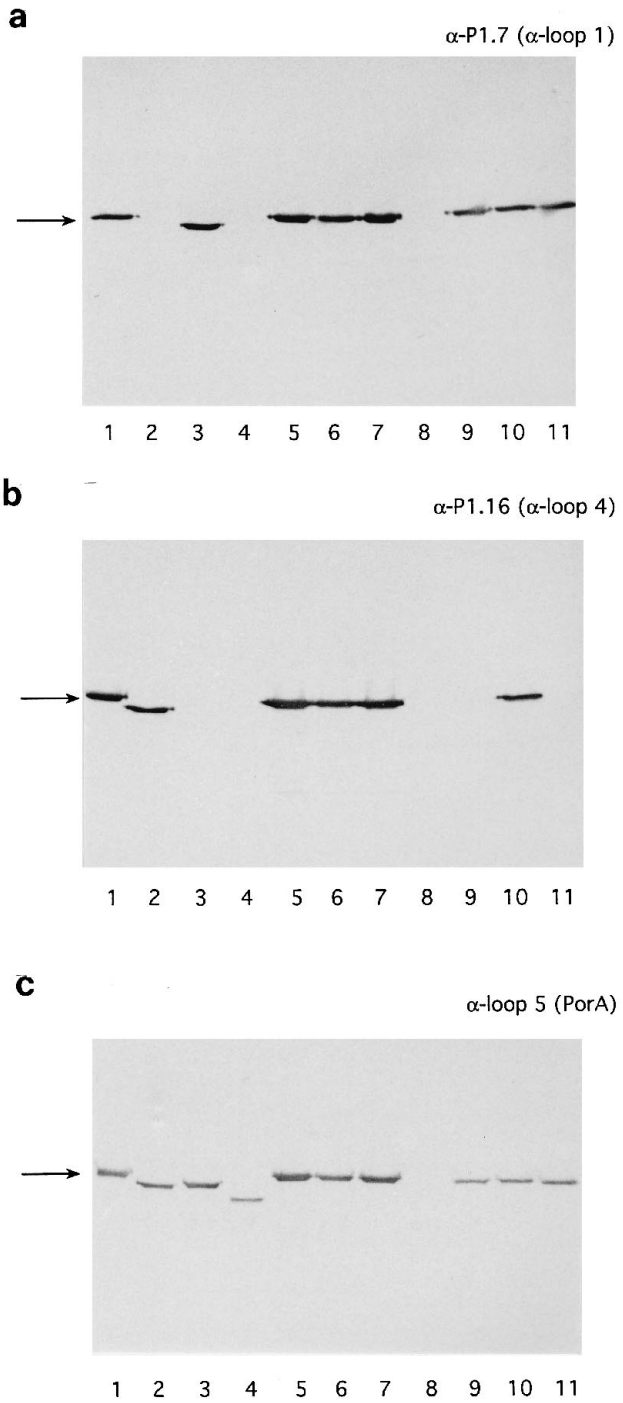

FIG. 4. Western blots with isogenic target strains derived from H44/76 (B:15:P1.7,16). Immunoblots were incubated with specific PorA MAbs, i.e., MN14C11.6, α-P1.7 (a), MN5C11G, α-P1.16 (b), and MN33H7.6, α-loop 5 (c). The numbers of the lanes correspond to the numbers of the strains as listed in Table 2. The arrow in each panel indicates the position of the original PorA P1.7,16.

(PorB and RmpM deficient) were used to determine the bactericidal activity against PorA. Elimination of RmpM within the target strains did not increase the bactericidal activity of sera in comparison with titers found with the parental strain H44/76 (RmpM proficient). Some postimmunization sera revealed an increase in bactericidal activity when PorB was eliminated from the target strains. The increase in bactericidal activity after one immunization of all tested sera vanished when HI5, the PorA-deficient strain, was used. The epitope specificity and cross-reactivity of the induced PorA-dependent bactericidal antibodies was further investigated by use of isogenic strains carrying loop deletions or point mutations in their PorA (Table 3). A number of patterns can be recognized, as follows. In pattern I, bactericidal activity depends on PorA but not on loops 1 and 4 (samples 6 and 21). Neither deletion of loop 1 or 4 or both abolished the bactericidal activity which depends upon PorA. In pattern II, bactericidal activity depends on loops 1 and 4 (samples 7, 16, 20, and 34). Deletion of both loops was necessary to eliminate the increase in bactericidal activity. In pattern III, bactericidal activity depends particularly on loop 1 (samples 8, 15, 30, 31, and 35). Deletion of loop 1 appears to result in complete loss of the increase in bactericidal activity, whereas deletion of loop 4 actually results in even higher titers. In pattern IV, bactericidal activity depends particularly on loop 4 (sample 11). Deletion of loop 4 was found to have an impact on bactericidal activity of sample 11. The putative specificity of the induced antibodies is given in Table 4. When the P1.16 point mutation variants were used, in general, the bactericidal activities of serum samples remained equal compared with titers against parental strain H44/76, except for sample 11 activity, which was reduced. This differentiation must be considered to indicate major types of responders.

DISCUSSION

Previous research indicated that PorA, one of the major OMPs of meningococci, is a potential vaccine component (15, 26, 29, 30). Recently, a phase I study was conducted by us with adult volunteers by use of a hexavalent PorA meningococcal OMV vaccine, of which the protein content was 90% PorA (8, 22, 38). Previous studies have shown that induction of bactericidal antibodies was optimal when PorA was in its natural conformation as present on living bacteria or OMVs (24). This hexavalent OMV vaccine contains class 1 OMP P1.7,16 as one of the six PorAs. Isogenic strains derived from meningococcal strain H44/76, which differed in their OMP compositions, have been constructed to create an identical meningococcal background. The collection of isogenic strains was used to investi-

TABLE 2. Bactericidal sensitivities of isogenic H44/76 strains to a panel of bactericidal murine MAbs

Strain[a]	Sensitivity[b] to:			
	MN14C11.6, α-PorA (P1.7)	MN5C11G, α-PorA (P1.16)	MN15A14H6, α-PorB serotype 15	MN15A17F12, α-LOS 3, 7, 9 immunotype
1. H44/76	+++	+++	+	+++
2. H44/76-Δ7	−	+++	ND[c]	+++
3. H44/76-Δ16	+++	−	ND	+++
4. H44/76-Δ7,16	−	−	ND	+++
5. H44/76-Δcl3	+++	+++	−	+++
6. H44/76-Δcl4	+++	+++	+	+++
7. H44/76-Δcl3,4	+++	+++	−	+++
8. HI5	−	−	ND	+++
9. TR52-7,16[b]	+++	±	ND	+++
10. TR52-7,16[c]	+++	++	ND	+++
11. TR52-7,16[d]	+++	−	ND	+++

[a] All strains are derivatives of H44/76.
[b] Symbols: −, not sensitive; ±, low sensitive, titer of 1/150; +, sensitive, titer of 1/500; ++, sensitive, titer of 1/5,000; +++, sensitive, titer of >1/10,000.
[c] ND, not determined.

TABLE 3. Bactericidal activity of serum samples from immunized volunteers against the isogenic target strains

Dose (μg)	Sample no.[a]	Titer against strain[b]: H44/76		HI5		H44/76-Δ7		H44/76-Δ16		H44/76-Δ7,16		TR52-P1.7,16[b]		TR52-P1.7,16[c]		TR52-P1.7,16[d]		H44/76-Δcl3		H44/76-Δcl4		H44/76-Δcl3,4	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
50	6	2	8	2	2	2	16	<2	16	2	8	4	32	2	16	2	16	2	16	2	8	2	16
	8	2	16	<2	2	2	2	4	64	<2	2	2	16	2	16	2	32	2	32	2	16	2	16
	16	4	32	2	2	8	64	16	32	4	4	16	32	8	64	16	64	8	16	4	16	8	32
	20	8	64	2	2	8	16	8	32	2	2	4	32	4	32	4	32	8	>128	4	32	32	>128
100	7	<2	4	2	2	<2	2	2	2	2	2	2	2	<2	<2	<2	2	<2	4	<2	2	<2	4
	11	2	32	2	2	2	16	4	8	2	2	2	8	2	8	2	8	2	32	2	32	2	16
	15	2	64	2	2	8	8	16	128	4	4	8	64	8	>128	8	128	4	>128	8	64	16	>128
	21	2	32	2	2	2	16	4	>128	2	32	2	16	4	64	8	32	2	32	2	32	2	64
	30	16	32	8	8	2	2	32	>128	16	32	16	64	32	64	16	64	32	128	8	64	32	64
	31	2	16	2	2	2	2	8	128	4	16	4	16	2	32	8	32	2	16	2	16	4	32
	34	2	64	<2	<2	2	16	<2	32	2	2	2	>128	<2	>128	2	>128	2	>128	2	64	2	64
	35	<2	16	2	2	8	8	4	64	2	2	8	32	4	16	8	32	2	32	4	16	2	32

[a] Samples from all volunteers but no. 30 showed at least a fourfold PorA-dependent increase in bactericidal activity; the sample from volunteer 30 showed a twofold PorA-dependent increase in bactericidal activity.

[b] Titers are shown as the highest reciprocal serum dilutions showing bactericidal activity upon visual inspection, both before (Pre) and after (Post) vaccination.

gate the contribution of PorA, subtype P1.7,16, in the development of bactericidal antibodies in sera of the vaccinees, with respect to loops 1 and 4.

After a single immunization with an estimated amount of 7.5 or 15 μg of PorA P1.7,16 (8), 40% (4 of 10) and 70% (7 of 10) of the volunteers, respectively, demonstrated at least a fourfold increase in bactericidal antibody titer against strain H44/76 (B:15:P1.7,16:L3,7,9) (Table 3). It has been suggested that RmpM (class 4 OMP), as present in the OMV preparations, is capable of inducing blocking antibodies which decrease bactericidal activity of other antibodies (21). Elimination of RmpM within two strains (H44/76-Δcl4 and H44/76-Δcl3/4) did not increase the bactericidal activity of sera relative to titers found with the parental strain H44/76 (RmpM proficient), which could be expected if blocking antibodies were induced. Abolition of PorB (class 3 OMP) produced meningococcal strains with increased serum sensitivity in the case of four postvaccination serum samples. Previous research showed that elimination of PorB did not increase the expression level of PorA (34), which might have explained the higher sensitivity for PorA-specific antibodies. Another explanation for this increased sensitivity might be a better access of PorA antibodies because of the absence of PorB. The observed increase in bactericidal activity was completely dependent upon PorA, as was found by use of the isogenic PorA-negative variant, HI5, as the target strain (34). Within the PorA-dependent bactericidal activity of sera from the converted vaccinees, four different patterns can be distinguished (Table 4). Both loops 1 and 4 are involved in the induction of bactericidal antibodies, as was found with target strains carrying loop deletions in their PorA, although antibodies directed against loop 1 were encountered more frequently. This is in contrast with previous findings in polyclonal mouse sera, in which bactericidal activity seemed mainly directed against loop 4 (37). In some cases, higher bactericidal titers were found when loop 4 was deleted from PorA, as compared to the strains carrying the complete PorA (Table 4, pattern 3). This might be due to shielding of loop 1 epitopes by loop 4. Point mutations in loop 4 were not shown to have a great impact on the bactericidal activity of antibodies. However, one volunteer seemed to have developed bactericidal antibodies directed exclusively against loop 4, with a reduced bactericidal activity when P1.16 point mutation variants were used. Noticeably, mutations in loop 1 do not occur as often as they do in loop 4. Until now, three variants of loop 4, P1.16 epitope, have been encountered in the field (18, 27, this study), whereas only one variant carrying a mutation (deletion of three amino acids outside epitope P1.7) in loop 1 is described (39). A number of volunteers developed bactericidal antibodies which depended on loops 1 and/or 4, as expected. Surprisingly, a few volunteers developed PorA-specific bactericidal antibodies which did not depend on either loops 1 and/or 4. This requires further examination, particularly since this may point to common epitopes.

TABLE 4. Specificity patterns of induced bactericidal activity

Dose (μg)	Sample no.[a]	Putative specificity	Pattern
50	6	PorA, unidentified	I
	8	PorA, loop 1	III
	16	PorA, loops 1 + 4	II
	20	PorA, loops 1 + 4	II
100	7	PorA, loops 1 + 4	II
	11	PorA, loop 4	IV
	15	PorA, loop 1	III
	21	PorA, unidentified	I
	30	PorA, loop 1	III
	31	PorA, loop 1	III
	34	PorA, loops 1 + 4	II
	35	PorA, loop 1	III

[a] Samples from all volunteers showed at least a fourfold increase in bactericidal activity, except for volunteer 30 (twofold increase), after one immunization.

In conclusion, one immunization with the hexavalent PorA OMV vaccine induced at least a fourfold increase in bactericidal PorA P1.7,16-specific antibodies which showed a tendency to be dose dependent (4 of 10 volunteers receiving a single dose, 7 of 10 volunteers receiving a double dose), as was also found for the other sero-subtypes present in the hexavalent PorA OMV vaccine (22). Both loops 1 and 4 of PorA P1.7,16 were involved in eliciting bactericidal antibodies, although no uniform pattern could be distinguished in the epitope specificity among the volunteers. This paper demonstrates the importance of the construction of mutant meningococci for the evaluation of the specificity of a bactericidal

immune response after immunization with OMP-based meningococcal vaccines.

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