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Antigenic Diversity of Meningococcal Outer Membrane Protein PorA Has Implications for Epidemiological Analysis and Vaccine Design

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The currently used serological subtyping scheme for the pathogen *Neisseria meningitidis* is not comprehensive, a proportion of isolates are reported as not subtypeable (NST), and few isolates are fully characterized with two subtypes for each strain. To establish the reasons for this and to assess the effectiveness of DNA-based subtyping schemes, dot blot hybridization and nucleotide sequence analyses were used to characterize the genes encoding antigenic variants of the meningococcal subtyping antigen, the PorA protein. A total of 233 strains, including 174 serologically NST and 59 partially or completely subtyped meningococcal strains, were surveyed. The NST isolates were chosen to be temporally and geographically representative of NST strains, isolated in England and Wales, and submitted to the Meningococcal Reference Unit in the period 1989 to 1991. The DNA-based analyses demonstrated that all of the strains examined possessed a *porA* gene. Some of these strains were serologically NST because of a lack of monoclonal antibodies against certain PorA epitopes; in other cases, strains expressed minor variants of known PorA epitopes that did not react with monoclonal antibodies in serological assays. Lack of expression remained a possible explanation for serological typing failure in some cases. These findings have important implications for epidemiological analysis and vaccine design and demonstrate the need for genetic characterization, rather than phenotypic characterization using monoclonal antibodies, for the identification of meningococcal strains.

Neisseria meningitidis continues to cause frequently fatal childhood and adult infections worldwide (9, 30), and no satisfactory childhood vaccine that protects against all meningococcal strains is available, despite more than 20 years of research (3, 16, 20, 35, 44). The failure to develop effective vaccines can be attributed, at least in part, to the antigenic variability of the meningococcus, which has evolved numerous genetic mechanisms for generating antigenic variation (14, 28, 33). The resultant antigenic diversity has, however, been exploited for the purposes of strain identification, and a strain characterization scheme, based on several antigens, has been developed (15, 17, 34, 41). Meningococcal groups (designated A, B, C, etc.) are defined by capsular polysaccharides (4, 41), and types and subtypes are based on the major outer membrane proteins (OMPs). The class 1 OMP (PorA) defines the subtypes, while the mutually exclusive class 2 or 3 OMPs (PorB) define the types (2, 17, 38). The lipooligosaccharide component of the outer membrane is less frequently used in strain identification to define immunotypes. Various immunological reagents against these components, including polyclonal sera and monoclonal antibodies, are available (1, 15, 34, 41).

The majority of meningococci isolated from cases are group A, B, or C; a small number of cases are caused by group Y and W-135 organisms. Within these groups, however, the type and subtype antigens are diverse, and these characteristics are widely used to provide detailed epidemiological information. The monoclonal antibody-based system currently available for serotype and serosubtype identification is not comprehensive, and many isolates remain not typeable or not subtypeable (NST) or only partially typed and subtyped.

Interest in PorA was stimulated by the development of vaccines that included this OMP, and the molecular basis of its antigenic variation has been established by comparative sequence (7, 24, 26, 36, 39) and immunological analyses (26, 27). PorA has three variable regions (VRs) (VR1 to VR3), with the most variation in the first two regions, VR1 and VR2, which form surface loops I and IV of the proposed structure of this porin protein (24, 39), respectively. Many of the subtype-specific monoclonal antibodies react with linear peptide epitopes located in these putative loops (26, 27).

Subtypes were originally defined by reaction of particular monoclonal antibodies and indicated by a number with the prefix P1., e.g., P1.16. This system was introduced without the benefit of detailed knowledge of the antigenic structure of the PorA protein, and three refinements to the scheme have recently been proposed (36). First, as the peptide sequences of the PorA VRs are now known, the numbers should refer to peptide sequences, not antibody reactivities. This has a number of advantages, including the ability to incorporate new variants systematically. Second, each VR is a member of a family of related variants: these minor variants are indicated by the addition of a lowercase letter as a suffix to the number, e.g., P1.7, P1.7a, and P1.7b. Third, the location of the antigen in VR1 or VR2 should be indicated by the order of the subtype number after the P1 prefix. For example, a strain designation of P1.7,16 indicates the PorA protein with a sequence belonging to family 7 in VR1 and family 16 in VR2. Two subtypes identified by monoclonal antibodies, 6 and 14, are probably determined by sequences in VR3 and should be given last, if

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quoted (24, 37). Characterization of a meningococcal strain for subtype should include two numbers representing VR1 and VR2; the VR3 location can be excluded for most strains. As subtype information can be obtained by genetic means, the present work will use the term serosubtype to indicate characterization serologically and the term DNA subtype to indicate genetic characterization.

A DNA dot blot subtyping system, which utilized DNA-DNA hybridization to identify the VR-encoding regions of the *porA* gene of a given isolate, was developed to overcome the problem of serologically NST meningococci (23). In the present work, a total of 233 meningococcal isolates were examined by using this system and nucleotide sequence analyses to establish: (i) the effectiveness of DNA-based characterization; (ii) the reasons for the failures of the antibody-based subtyping; and (iii) the antigenic variability of NST strains. Of the 233 strains examined, 174 were serologically NST, chosen to be geographically representative of the 1,669 such strains, isolated in England and Wales in the period 1989 to 1991. The remaining 59 strains were subtyped immunologically for at least one VR. The resultant data established the effectiveness of DNA-based typing and identified all the PorA antigenic variants present in the meningococcal strains examined.

MATERIALS AND METHODS

Bacterial strains. A total of 233 strains were supplied to National Institute for Biological Standards and Control by the Meningococcal Reference Unit (MRU) on Dorset egg agar slopes (Technical Service Consultants, Lancaster, United Kingdom) (22). These strains were derived from a collection of meningococcal cultures referred from England and Wales to the MRU for epidemiological characterization. The 174 NST strains included were chosen to be representative of the 1,669 NST meningococcal strains submitted to the MRU in the period 1989 to 1991 (18, 19).

Subtyping with monoclonal antibodies. All of the strains were characterized at the MRU by an immunological dot blot method with ascitic fluid containing the following subtype-specific antibodies, provided by J. T. Poolman of the RIVM, Bilthoven, The Netherlands, and National Institute for Biological Standards and Control: MN14C2.3 (P1.1), MN16C13F4 (P1.2), MN20B9.34 (P1.4), MN19D6.13 (P1.6), MN14C11.6 (P1.7), MN5A10F (P1.9), MN20F4.17 (P1.10), MN20A7.10 (P1.12), MN21G3.17 (P1.14), MN3C5C (P1.15), and MN5C11G (P1.16) (31). A swab of the meningococcal strain to be tested was taken from a culture grown overnight on Kellog's agar (19a) plates, emulsified in sterile saline, and pasteurized by heating to 56°C for 30 min. Samples (2 μl) of these suspensions were applied to nitrocellulose membrane strips and air dried. The strips were then blocked with blocking buffer (skim milk [4%, wt/vol] in phosphate-buffered saline) and then incubated with the appropriate ascitic fluid diluted 1:200 to 1:1,500 in blocking buffer with 0.5% (vol/vol) Tween 20. Membranes were washed with saline containing 0.05% (vol/vol) Tween 20, incubated with protein A-peroxidase conjugate (Sigma), washed again, and developed with 4-chloro-1-napthol substrate (0.05 mg/ml in 17% ethanol [vol/vol]–0.05% H_2O_2 [vol/vol]).

DNA extraction and PCR amplification. Chromosomal DNA was extracted directly from meningococcal cells on Dorset agar slopes with an Isoquick DNA preparative kit (Microprobe Corp.). A loopful of cells was scraped from the agar slope and distributed evenly into 0.5 ml of TE (Tris-EDTA) buffer (25), and the resultant cell suspension was centrifuged at 12,000 × *g* in a microcentrifuge for 2 min to sediment the cells. The cell pellet was extracted with the kit following the manufacturer's instructions. The DNA was finally resuspended in deionized water (50 μl).

For PCR amplification, a 5-μl sample of meningococcal chromosomal DNA was added to the following reaction mixture to a total volume of 100 μl: reaction buffer (10 mM Tris-Cl [pH 8], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin); dATP, dCTP, dGTP, and dTTP (200 μM each); the required primers (2 μM each); and 0.5 U of *Taq* polymerase (Cetus Corp.) per 20-μl reaction mixture. The components were incubated for 30 cycles in a programmable heat block (PHC-2; Techne Instruments Ltd.), with 1 cycle consisting of 2 min at 94°C, 2 min at 70 or 75°C, and 3 min at 72°C. After 30 cycles, the reaction mixtures were incubated for 3 min at 72°C. Primers 21 and 22 were used for amplifying the complete class 1 OMP genes (24).

DNA subtyping. The DNA subtyping of isolates was done as described previously (23). Briefly, a reference collection of VR-encoding DNA samples cloned into bacteriophage M13mp19 were bound onto nylon membranes. This collection contained 16 unique VR sequences from nine reference strains and represented most of the known epitopes, including some VRs for which no serological reagent had been produced. PCR-amplified *porA* genes from the meningococcal strains to be typed were radioactively labelled with ^{32}P and individually hybridized to the reference membranes at 50°C in 4× SET buffer (25) plus 0.1% sodium pyrophosphate, 50% formamide, 0.2 mg of heparin per ml, and 2% sodium dodecyl sulfate (SDS). After hybridization, the reference membranes were washed at 65°C in 0.1× SET buffer (25) with 0.1% SDS. The reactivity of a given *porA* gene with the reference collection of VR-encoding M13 DNA samples was established by autoradiography of the filter. In the majority of cases, the labelled *porA* gene hybridized to only one VR1 and one VR2 M13 reference DNA. Ambiguous and negative results were investigated by nucleotide sequence analysis of the relevant *porA* gene.

Nucleotide sequence analysis. Direct nucleotide sequence analysis of PCR products was done by a modified version of the method of Embley (13). The PCR products were purified from reaction components by precipitation at 37°C with 20% (wt/vol) polyethylene glycol 8000 in 2.5 M NaCl and washing in 80% (vol/vol) ethanol. The pellets were dried and resuspended in water (25 μl for a 100-μl amplification). Cycle sequencing was done with an appropriate primer and radiolabelled by T4 polynucleotide kinase with [γ-^{32}P]ATP and *Taq* polymerase and termination mixes from the *Taq*uence sequencing kit (United States Biochemicals Corp.). Each sequence was determined at least once on each strand. The additional primers used for nucleotide sequencing were primers 8U (24), 103L, and 122L (36).

Assignment of VR sequences to families and definition of subvariants. The scheme of Suker et al. (36) was used to define epitope families and to assign names to novel VR1 and VR2 peptide sequences, with reference to all known *porA* gene nucleotide sequences present in GenBank. The DNA sequences encoding the VRs were translated, and the peptide sequences were aligned. These sequences were used to generate a distance matrix as previously described (36). Sequences that were 80% identical were considered members of the same VR family and assigned a VR subvariant name.

RESULTS

Characterization of isolates by DNA-based typing. A PCR-amplified *porA* gene was obtained for all strains included in the study, indicating the presence of a *porA* gene in each of these isolates. The amplified products were initially characterized by DNA dot blot analysis, and if this technique resulted in incomplete or ambiguous characterization for either VR, the nucleotide sequences of the appropriate parts of the *porA* gene (VR1, VR2, or both) were determined on both DNA strands. The success rates for determining the subtypes encoded by VR1 and VR2 over all 233 strains examined were 83.8% for VR1, 69.1% for VR2, and 56.7% for VR1 and VR2 when the DNA dot blot analysis alone was used. When dot blot and nucleotide sequence analyses were combined, the success rates were 97% for VR1, 97.9% for VR2, and 95.3% for VR1 and VR2. For the set of 174 NST isolates, 166 (95.4%) were characterized in either VR1 or VR2 by DNA dot blot analysis, with 149 (85.6%) characterized in VR1 and 173 (64.94%) in VR2. A total of 96 (55.2%) isolates were characterized at both locations. The inclusion of nucleotide sequence data resulted in either VR1 or VR2 being determined for all 174 NST isolates, with 173 (99.4%) strains characterized for VR1 and 171 (98.3%) for VR2. Characterization of both VRs was achieved for 170 (97.7%) of the NST strains.

Identification and definition of epitope families. The definition of VR epitope families used was that of Suker et al. (36). This scheme, which is based on amino acid sequence similarity and defines minor variants on the basis of amino acid identity, allowed the assignment of all possible VR sequences to families and was independent of protein reactivity with monoclonal antibodies. The VR1 and VR2 designation of each strain were assigned to a particular VR family on the basis either of an unambiguous result with the dot blot or of the translated nucleotide sequence of the VR. The peptide sequences of the VR families and variants found in all the 233 strains examined are listed in Table 1.

Two novel VR2 families were present in this data set and assigned the numbers P1.26 and P1.28 by using the criteria of Suker et al. (36). Table 1 also includes a proposed comprehensive nomenclature which is consistent with the previous scheme and with that of Suker et al. In addition, a number of novel

TABLE 1. VRs identified in the present study with published and proposed nomenclature as appropriate

Location	VR no.	Peptide sequence	Reference(s)
VR1	5	PLQNIQPQVTKR	24
	5c	PLPNIQPQVTKR	36
	7	AQAANGGASGQVKVTKVTKA	26
	7b	AQAANGGASGQVKVTKA	36
	12	KLSSTNAKTGNKVEVTKA	24
	12a	KPSSTNAKTGNKVEVTKA	23
	17	PPQKNQSQPVVTKA	24
	18	PPSKGQTGNKVTKG	24
	18a	PPSQGQTGNKVTKG	36
	19	PPSKSQPQVKVTKA	24
	19a	PPSKSQSQVKVTKA	This work
	21	QPQVTNGVQGNQVKVTKA	36
	22	QPSKAQGQTNNQVKVTKA	24
	22a	QPSRTQGQTSNQVKVTKA	36
VR2	1	YVAVENGVAKKVA	24
	2	HFVQQTPKSQPTLVP	24
	2b	HFVQQPPKSQPTLVP	This work
	2c	HFVQQTPQSQPTLVP	36
	4	HVVVNNKVATHVP	23
	9	YVDEQSKYHAA	24
	10	HFVQNKQNQRPTLVP	23
	10a	HFVQNKQNQPPTLVP	This work; 37
	10b	HFVQDKKGQPPTLVP	This work; 37
	10c	HFVQNKQNQQPTLVP	This work; 37
	10d	HFVQNKQNKQNQPPTLVP	This work; 37
	10f	HFVQNKQNQQNQQNQPPTLVP	This work; 37
	10g	HFVQNKQNKPPTLVP	This work; 37
	13	YWTTVNTGSATTTTTFVP	This work
	13a	YWTTVNTGSATTTTFVP	37
	13b	YWTTVNTGSATTTFVP	This work
	13c	YWTTVNTGSATITTFVP	This work
	13d	YYTTVTQGSATTTTFVP	This work
	15	HYTRQNNADVFVP	24
	15b	HYTRQNNIDVFVP	This work
	16	YYTKDTNNNLTLVP	24
	16b	YYTKNTNNNLTLVP	8
	24	TLANVANTNIGVP	23
	25	TYTVDSSGVVTPVP	24
	26	HFVADSQGKITRVP	This work
	27	YVDEKKMVHA	23
	27a	YVDEKQVSHA	This work
	28	YYYTTATNSSTSTTFVP	This work

variants of existing VR families were observed and assigned new numbers, including six in the P1.10 family (P1.10a, P1.10b, P1.10c, P1.10d, P1.10f, and P1.10g), two in the P1.13 family (P1.13b and P1.13c) and a P1.27 variant, P1.27a.

Strains not characterized by the DNA dot blot method. Of the 25 VR1s not identified by the dot blot method, 9 failed to give a result and 16 gave ambiguous results. One strain could not be identified by sequencing and of the remaining 24, 19 were variants of VRs that differed from those in the reference DNA set, including 1 P1.5c, 9 P1.7b, 1 P1.12a, 1 P1.18, 2 P1.19, and 5 P1.22a VRs. Two were P1.21, for which there was no reference in the set of cloned VR-encoding DNA samples used (23). There were three examples from 233 tests where the technique had failed to identify epitopes that were present in the reference set, one P1.19, one P1.22, and one P1.5.

Of the 63 VR2s not identified by the DNA dot blot method, 41 gave negative results and 22 gave ambiguous results. Nucleotide sequence analysis showed that the negative results were from three strains that could not be characterized, one strain with a deletion of 45 bp including VR2 and the following VR families that were not represented in the set of reference DNA samples: 24 P1.13, 1 P1.26, 10 P1.27, and 1 P1.28. The final result was a P1.4 epitope that should have been identified. The latter result represented the only failure of the DNA dot blot typing procedure to identify a VR2 variant that was included in the reference set from the 233 strains. All of the 22 ambiguous results were associated with the related P1.2 and P1.10 epitopes. One P1.2c-containing strain cross-reacted with the P1.10 reference DNA, and 21 P1.10 variants cross-reacted with the P1.2 reference DNA. In the strain with a deletion of VR2, the deletion extended from base position 506 to 550 (numbering after the aligned nucleotide sequences in reference 24), which was distinct from the previously reported deletion (36), showing that the loss of VR2 has occurred and persisted in the meningococcal population sufficiently long to be identified on at least two separate occasions.

The 11 isolates not fully characterized by dot blot or nucleotide sequence analysis gave a result indicating two sequences for one or both VRs. There are two possible explanations for these results: (i) the samples contained two discrete meningococcal strains, and (ii) each of the isolates contained two *porA* genes. There have been no studies designed to investigate colonization or infection of individuals with multiple meningococcal strains, but the frequency of carriage in the United Kingdom (10) suggests that such colonizations may occur. To date, no single strain containing two *porA* genes has been described, but this remains a possible explanation.

Antigenic characteristics of NST strains. The dot blot and sequencing results from the NST isolates permitted calculation of the frequencies of VR families present in the NST strains from England and Wales in 1990 and 1991. These data, summarized in Table 2, permit the completion of the epidemiological picture for this period. There were differences in the PorA epitopes present in serogroup C and serogroup B isolates (Table 3). The *porA* genes of the serogroup C isolates were comparatively uniform, 26 of 41 (63.4%) of these isolates had the P1.5,10 VR family combination, and a further 9 (22%) had the related P1.5,2 VR family combination. All of the serogroup C isolates with the P1.5,10 VR family combination were sulfonamide sensitive, and those with the P1.5,2 combination were sulfonamide resistant. The remaining six isolates had *porA* genes encoding the following VR family combinations: P1.7,9, P1.7,13, P1.18,1, and P1.19,15; all of these strains were sulfonamide sensitive. By contrast, the *porA* genes of serogroup B isolates were more diverse than the serogroup C meningococci. The most common VR family combination among serogroup B isolates was P1.7,4 (26.2% of strains), followed by P1.19,15 (18.6% of strains) and P1.5,10 (14% of strains). There were 25 other VR family combinations among the serogroup B isolates, with frequencies ranging from 0.6 to 5.2%. There was no association of sulfonamide resistance with particular VR family combinations among the serogroup B isolates.

Ambiguities between DNA and serological subtyping. A total of 59 serosubtyped strains were included in this study, 19 isolates that were characterized at VR1 and 56 that were characterized for VR2. There was an ambiguity with one VR1 result, which was serologically typed as P1.12 but which gave an ambiguous result with DNA dot blot subtyping. Nucleotide sequence analysis showed that this strain was a minor variant, P1.12a, of this epitope. There were six conflicts between DNA dot blot and serological subtyping. In these cases, the nucleotide sequence analysis result was consistent with the dot blot result.

Nucleotide sequences encoding epitope variants. Nucleotide sequence analyses revealed minor variants of a number of VR families, particularly the P1.10 and P1.13 families. These se-

TABLE 2. Frequencies of epitope families in 174 serologically NST strains and the effectiveness of DNA dot blot subtyping in the characterization of these strains

Epitope family[a]	No. of strains identified by DNA dot blot subtyping	No. of strains resolved by sequence analysis	Effectiveness of DNA dot blot subtyping (%)	Total no. of strains	Frequency of epitope family (%)
VR1					
P1.5	54	2	96.43	56	32.37
P1.7	48	9	84.21	57	32.95
P1.12	7	1	87.50	8	4.62
P1.17	1	0	100.00	1	0.58
P1.18	9	1	90.00	10	5.78
P1.19	24	3	88.89	27	15.61
P1.21*	0	2	0.00	2	1.16
P1.22	6	6	50.00	12	6.94
VR2					
P1.1	1	0	100.00	1	0.58
P1.2	11	1	91.67	12	7.02
P1.4	39	1	97.50	40	23.39
P1.9	1	0	100.00	1	0.58
P1.10	24	21	53.33	45	26.32
P1.13*	0	24	0.00	24	14.04
P1.15	16	0	100.00	16	9.36
P1.16	3	0	100.00	3	1.75
P1.24	11	0	100.00	11	6.43
P1.25	5	0	100.00	5	2.92
P1.26*	0	1	0.00	1	0.58
P1.27*	0	10	0.00	10	5.85
P1.28*	0	1	0.00	1	0.58
Del	0	1	0.00	1	0.41

[a] The VR families not included in the reference set of DNA samples used for DNA subtyping are indicated with an asterisk.

quences are presented in Table 4. Although the sequences of these VR2 families were diverse, there were several similarities in the mechanisms of antigenic change in these families. Variation had occurred by both base substitution and duplication of single codons (in the P1.13 family) and runs of codons (in the P1.10 family). All of the base changes observed in these VRs gave rise to changes in amino acid sequence, and a relatively high proportion of the sequenced examples belonged to variants other than the prototype in both of these VR2 families. For the P1.10 family, the 29 sequenced isolates contained variants at the following frequencies: 8 prototype P1.10 (27.5%), 13 P1.10a (44.5%), 2 P1.10b (7%), 1 P1.10c (3.5%), 3 P1.10d (10%), 1 P1.10f (3.5%), and 1 P1.10g (3.5%). For the 27 members of the P1.13 epitope family sequenced, the following frequencies of epitope variants were observed: 4 prototype P1.13 (15%), 16 P1.13a (59%), 5 P1.13b (19%), 1 P1.13c (3.5%), and 1 P1.13d (3.5%).

DISCUSSION

These data establish the value of genetic procedures in the determination of antigenic variability of the class 1 OMP of the meningococcus and illustrate the great range of PorA antigenic variants present in the United Kingdom population of meningococci. These findings are of interest both in the study of meningococcal antigens and as a model for other antigenically variable bacteria.

Antigenic diversity is a feature of many microorganisms that live in close association with human tissues. Presumably this diversity is the result of coevolution of the host immune system with bacterial strategies for evading immune attack (6), although structural diversity of meningococcal surface antigens has also been implicated in other pathogenic processes (42). From a medical perspective, antigenic variability requires the development of complex typing schemes and nomenclature and also presents problems for vaccine design. To overcome this variability and develop vaccines that will protect against most or all meningococcal strains, the following approaches have been adopted: (i) the use of cocktails of several antigenic variants and (ii) the identification of conserved antigens. The design and assessment of vaccines based on these approaches require precise identification of the antigenic variants expressed by the prevalent disease-causing meningococci and a knowledge of their mechanisms and relative rates of change of the putative vaccine components.

PorA protein vaccines provide an instructive example of the principles and problems associated with the design of protein component vaccines directed against a variable antigen and illustrate the importance of accurate and comprehensive strain characterization in vaccine design. PorA proteins or sequences derived from them were proposed as components of novel antimeningococcal vaccines for two reasons (32): (i) their ability to elicit a T-cell-dependent, anamnestic immune response and (ii) their relative antigenic stability, as revealed by monoclonal antibody subtyping. To date, two vaccines containing PorA proteins as major components have been used in field trials with variable success (3, 35), a new multivalent PorA vaccine has been developed (40), and various approaches for the delivery of PorA proteins or epitopes in vaccines have been reported elsewhere (11, 21, 29). The choice of PorA epitopes to be included in multivalent PorA vaccines has depended largely on serosubtyping data obtained with monoclonal antibodies.

While serosubtyping has been used extensively for routine epidemiological studies and has played an important part in the development of meningococcal vaccines, the present work demonstrates that monoclonal serosubtyping antibodies are not completely reliable for the identification of antigenic variants of PorA. This is because (i) the current reagent panel is not comprehensive, with no reagents for several major VR

TABLE 3. Frequencies of VR family combinations present in the strains characterized at both VR1 and VR2

Epitope family combination	All isolates		Isolates in serogroup:					
			B		C		Other	
	No.	%	No.	%	No.	%	No.	%
P1.5,2	14	6.31	4	2.33	9	21.95	1	11.11
P1.5,10	56	25.23	24	13.95	26	63.41	6	66.67
P1.5,13	1	0.45	1	0.58				
P1.7,1	3	1.35	3	1.74				
P1.7,2	1	0.45	1	0.58				
P1.7,4	45	20.27	45	26.16				
P1.7,9	1	0.45			1	2.44		
P1.7,10	1	0.45	1	0.58				
P1.7,13	11	4.95	9	5.23	2	4.88		
P1.7,16	2	0.90	2	1.16				
P1.7,27	1	0.45	1	0.58				
P1.7,28	1	0.45	1	0.58				
P1.7,Del	1	0.45	1	0.58				
P1.12,1	1	0.45	1	0.58				
P1.12,4	2	0.90	2	1.16				
P1.12,13	8	3.60	8	4.65				
P1.17,9	1	0.45	1	0.58				
P1.18,1	1	0.45			1	2.44		
P1.18,13	1	0.45	1	0.58				
P1.18,24	4	1.80	3	1.74			1	11.11
P1.18,25	6	2.70	5	2.91			1	11.11
P1.19,9	1	0.45	1	0.58				
P1.19,13	4	1.80	4	2.33				
P1.19,15	34	15.32	32	18.60	2	4.88		
P1.19,24	7	3.15	7	4.07				
P1.21,16	3	1.35	3	1.74				
P1.22,4	1	0.45	1	0.58				
P1.22,9	1	0.45	1	0.58				
P1.22,26	1	0.45	1	0.58				
P1.22,27	8	3.60	8	4.65				
Total	222	100	172	100	41	100	9	100

families, and (ii) there are numerous minor variants of some VR families and we have recently shown that the specificity of the monoclonal antibodies for such variants is not predictable (37). Lack of expression and masking (43) can also result in loss of data, and there can be difficulties in the interpretation of serological subtyping experiments. These difficulties are illustrated by the differences between some of the serosubtyping results and the DNA subtyping and sequencing results. Typing failures result in inaccurate strain identification and incomplete epidemiological data. Few strains are immunologically subtyped for both VR1 and VR2, and as the loops encoded by these regions may fold to form conformational epitopes recognized by the human immune system, these associations may be important to human immunity. Further, it should be noted that the serosubtyping monoclonal antibodies are not necessarily a good model for human immune reactions against PorA (12).

A comprehensive analysis of the antigenic diversity of PorA in the serogroup A meningococci has been published previously (36), but the present work represents the most complete data set from serogroup B and C meningococci produced to date, with both VR1 and VR2 determined for 222 isolates. The isolates included in the present study were derived from cases of endemic disease in England and Wales and comprised both serogroup B and serogroup C strains. The uniformity of the *porA* genes of the serogroup C strains isolated at diverse geographical locations, together with the association of sulfonamide resistance with the isolates with the P1.2,5 VR family combination, suggested that the serogroup C strains circulating in the United Kingdom were of limited genetic diversity and recombined with other meningococci relatively infrequently. By contrast, the serogroup B isolates were diverse with a greater range of VR families and combinations of VR families, with no association of particular *porA* genes with sulfonamide resistance in this sample of strains. Some VR families were particularly promiscuous, for example, the P1.7 epitope family in VR1, which occurred with VR2 epitopes 1, 2, 4, 9, 10, 13, 16, 27, and 28 and the P1.13 family in VR2, which was found with diverse VR1 families, namely, 5, 7, 12, 18, and 19.

The development of effective antimeningococcal vaccines has proved to be difficult, and the failure of traditional approaches has led to the design of increasingly sophisticated vaccines, none of which has resulted in a satisfactory product to date. Molecular techniques for the analysis of variable antigen genes provide precise data that enable an accurate appreciation of the biological reasons for these difficulties, which is essential for rational vaccine design and evaluation. The data presented here indicate the high degree of PorA antigenic

TABLE 4. Nucleotide sequences encoding members of the P1.10 and P1.13 VR families

Epitope variant	DNA sequence[a]
P1.10 family	
P1.10	CAT TTT GTT CAG AAT AAG CAA AAT ... ... CAG CGG CCT ACT CTC GTT CCG
P1.10a	CAT TTT GTT CAG AAT AAG CAA AAT ... ... CAG **C**C**G** CCT ACT CTC GTT CCG
P1.10b	CAT TTT GTT CAG **G**AT AAG **A**AA **GG**T ... ... CAG **C**C**G** CCT ACT CTC GTT CCG
P1.10c	CAT TTT GTT CAG AAT AAG CAA AAT ... ... CAG C**A**G CCT ACT CTC GTT CCG
P1.10d	CAT TTT GTT CAG AAT AAG CAA AAT ... ... CAA AAT CAG C**C**G CCT ACT CTC GTT CCG
P1.10e	CAT TTT GTT CAG AAT AAG CAA A**G**T ... ... CAG CGG CCT ACT CTC GTT CCG
P1.10f	CAT TTT GTT CAG AAT AAG CAA AAT CAG CAA AAT CAG CAA AAT CAG C**C**G CCT ACT CTC GTT CCG
P1.10g	CAT TTT GTT CAG AAT AAG CAA AAT ... ... AAG C**C**G CCT ACT CTC GTT CCG
P1.13 family	
P1.13	TAT TGG ACT ACT GTG AAT ACC GGT AGT GCT ACT ACT ACT ACT ACT TTC GTT CCG
P1.13a	TAT TGG ACT ACT GTG AAT ACC GGT AGT GCT ACT ACT ACT ... TTC GTT CCG
P1.13b	TAT TGG ACT ACT GTG AAT ACC GGT AGT GCT ACT ACT ACT ... ... TTC GTT CCG
P1.13c	TAT TGG ACT ACT GTG AAT ACC GGT AGT GCT ACT A**T**T ACT ACT ... TTC GTT CCG
P1.13d	TAT TAT ACT ACT GTG A**C**T **CA**G GGT AGT GCT ACT ACC ACT ACT ... TTC GTT CCG

[a] A period indicates a gap inserted for purposes of alignment. Nucleotides that are different from those of the prototype sequence are shown in bold type and underlined.

variability, generated by several genetic processes, present in endemic meningococci and demonstrate that monoclonal antibody-based subtyping underestimates this variability. The nature of this diversity and the mechanisms of antigenic variation of PorA suggest that a dynamic situation exists within the endemic meningococcal population, with continual mutation and reassortment of epitopes. If this is in fact the case, the effectiveness of subtype-specific vaccines in prevention of disease in countries, such as England and Wales, that suffer from endemic meningococcal disease, is questionable. This hypothesis can be tested in appropriately designed clinical studies of such vaccines.

Before any multivalent PorA vaccines are introduced or included in large-scale trials, it is important to establish the natural rate of antigenic variation of PorA and to determine whether carried or commensal meningococci can act as a source for additional *porA* diversity for disease-causing meningococci by horizontal genetical exchange. This requires further studies using genetically based approaches that would have to include more isolates, a representative proportion of asymptomatic carrier isolates, and strains isolated over a period of at least several decades. There is some evidence that *porA* may vary at a rapid rate during carriage (5), and more studies that address this problem are also required. These data would enable the effect of vaccine introduction on meningococcal antigen diversity and therefore the likely long-term effectiveness of the vaccine to be assessed.

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