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Emergence of a New Virulent Clone within the Electrophoretic Type 5 Complex of Serogroup B Meningococci in Norway

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An increase in B:15:P1.12 meningococci among isolates from patients with *Neisseria meningitidis* infection in Norway in recent years led to further characterization of such strains. Between 1987 and 1992, B:15:P1.12 strains constituted 9.8% (24 strains) of B:15 isolates. The B:15:P1.12 strains belonged to the electrophoretic type 5 (ET-5) complex, but 17 (71%) strains were a new clone (ET-5c) not found elsewhere in the world. All but one strain of ET-5c were responsible for a localized outbreak of systemic meningococcal disease in western Norway. A novel monoclonal antibody (202,G-12), developed against the unknown variable region 2 on the class 1 protein of one of these strains, bound to 19 of the 15:P1.12 strains, 4 strains bound the subtype P1.13 reference monoclonal antibody MN24H10.75, and the remaining strain showed no reaction. Sequencing of *porA* genes demonstrated a series of nine threonine residues in the deduced variable region 2 of the latter strain, while four and five threonine residues were found in the corresponding regions of strains reacting with the monoclonal antibodies 202,G-12 and MN24H10.75, respectively. Epitope mapping with synthetic peptides showed that 202,G-12 bound to a sequence of 11 amino acids which included the four threonine residues specific for subtype P1.13a. Immunoglobulin G antibodies against the P1.7,16 subtype protein, induced in volunteers after vaccination with the Norwegian meningococcal vaccine, did not cross-react on immunoblots with the subtype protein of clone ET-5c. Thus, postvaccination class 1 protein antibodies, assumed to be protective, may not be effective against infection with the new clone.

Neisseria meningitidis isolates are characterized serologically by the different porins expressed in the outer membrane. The serotype is defined by the class 2 or class 3 protein, and the subtype is defined by the class 1 protein (10). The class 1 proteins form cation-selective pores and the class 2 and class 3 proteins form anion-selective pores (35), and all are multispanning molecules in the outer membrane with eight surface-exposed loops (21, 36). Several *porA* genes encoding the class 1 proteins, or PorA proteins (15), have been sequenced. Nearly all antigenic variation in these proteins is confined to two variable regions (VRs), called VR1 and VR2, located on loops 1 and 4, respectively, generating two independent subtypes of the class 1 porins (3, 20, 21, 26, 28, 36).

While recombinational genetic exchange of all or parts of the *porA* gene has been proposed as an important mechanism for antigenic variation (9, 19), minor variations within VR1 and VR2 have also been described. Two different point mutations in the P1.16 subtype epitope, located in VR2, have been found among isolates from patients with *N. meningitidis* infections in England and Norway, respectively (27, 30). A deletion in VR1 of three amino acids outside the epitope for the P1.7 subtype reference monoclonal antibody (MAb), which leads to a masking of this epitope, has also been reported in strains from various countries (20, 28, 42, 44).

Several studies have demonstrated that class 1 proteins are important vaccine antigens. After vaccination of human volunteers with a meningococcal vaccine, a positive correlation between the bactericidal activity of sera from the volunteers and antibody activity against the class 1 protein was found (45). Class 1 protein antibodies were also demonstrated in sera from both patients and vaccinated volunteers (14, 23, 40).

The dominant serogroup B meningococcal clone causing systemic disease in Norway has been designated electrophoretic type (ET) 5 (ET-5) (7). This clone was mainly associated with strains expressing serotype 15 and subtype P1.7,16 proteins. An outer membrane vesicle preparation from a strain with these characteristics (strain 44/76) was used to vaccinate 172,000 teenagers in a placebo-controlled protection trial in Norway from 1988 to 1991 (5). This study was followed by vaccination of about 49,000 of the previous placebo controls in an open trial in 1991 and 1992 (4).

Since the late 1980s, an increase in B:15 case isolates that expressed a new class 1 protein with subtype P1.12 was observed (43). Here, we report that the majority of such B:15:P1.12 strains stemmed from a new clone of the ET-5 complex that caused a localized outbreak of systemic meningococcal disease in western Norway. Subtype P1.12 is located in VR1 (21), and the unknown VR2 of the P1.12 proteins was characterized by nucleotide sequencing of *porA* genes and reaction with a newly developed VR2-specific MAb. Evidence is presented that human immunoglobulin G (IgG) antibodies against the P1.7,16 subtype protein, induced after vaccination with the Norwegian serogroup B vaccine, did not cross-react with the class 1 protein of the new clone. Given the important role of the class 1 protein antibodies, these findings suggest that the Norwegian meningococcal vaccine may offer reduced protection against infection with the new clone.

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MATERIALS AND METHODS

Meningococcal strains. The strains studied consisted of 432 isolates collected from patients in Norway between 1987 and 1992. This collection included all

TABLE 1. Distribution of subtypes among B:15 meningococcal case strains isolated in Norway between 1987 and 1992

Subtype	No. of strains in year:							Percent
	1987	1988	1989	1990	1991	1992	Total	
P1.1,15	1	1					2	0.9
P1.5		1					1	0.4
P1.5,2	1	1		2	3	4	11	4.5
P1.7	1		3	1		1	6	2.5
P1.(7)[a]			3		1		4	1.6
P1.7,2		1		2	1		4	1.6
P1.(7),3		1					1	0.4
P1.7,16	29	29	26	28	22	21	155	63.5
P1.(7),16	5	4	9	4	5	2	29	11.9
P1.12	1	2	3	6	4	8	24	9.8
P1.15	1						1	0.4
P1.−[b]			1	1	3	1	6	2.5
Total of B:15 strains	39	40	45	44	39	37	244	100

[a] Designates the masked P1.7 epitope (44).
[b] Nonsubtypeable with the MAbs used.

isolates from teenage patients in the vaccination trial, supplemented with about 50 consecutive patient isolates received annually at the National Reference Laboratory for Meningococci, Akershus Central Hospital. The serological characteristics of most of these strains have been reported previously (43). In addition, 149 strains isolated from patients between 1976 and 1987 were included in our study. The strains in both collections were isolated from patients throughout Norway. A total of 338 strains from *N. meningitidis* carriers were also analyzed. One collection (133 isolates) was obtained in 1987 from military recruits from throughout Norway enrolled in a camp near the city of Stavanger in western Norway (46). The second group of 114 carrier isolates was collected in 1992 from college students in connection with an outbreak of meningococcal disease near the city of Bergen in western Norway (13), and the third group was from randomly selected individuals living near Oslo in 1991 (8). All strains were typed by dot blotting (46) with the specific MAbs listed below.

Human sera. Acute- and convalescent-phase serum samples were obtained from participants in the Norwegian group B vaccination trial who fell ill with meningococcal disease (16). Late-convalescent-phase serum samples were also obtained between 6 months and 2 years after the occurrence of the disease (11).

MAbs. (i) Production of 202,G-12. Strain 51/90, isolated from a placebo control subject in Bergen who fell ill with systemic meningococcal disease, was chosen as a representative B:15:P1.12 isolate. BALB/c mice were immunized subcutaneously with 20 μg of Li-extracted outer membrane vesicles (29) from this strain in Freund's complete adjuvant and were then given a booster dose 2 weeks later with the same amount of antigen in phosphate-buffered saline. A similar dose was given 14 weeks later. Fusion between spleen cells and NSO myeloma cells was performed 4 days later by standard techniques. Hybridoma culture supernatants were screened in an enzyme-linked immunosorbent assay (ELISA) (46) against whole-cell suspensions from strain 51/90 and reference strain S3032 (NT:P1.12,16). Those testing positive with 51/90 but negative with S3032, thus omitting antibodies against subtype P1.12 expressed by both strains, were selected and analyzed further by immunoblotting and dot blotting. Hybridoma cells were cloned by limiting dilution and injected into Pristane-primed mice to obtain ascites. MAb 202,G-12 was purified on protein A-Sepharose (Pharmacia, Uppsala, Sweden). Cell culture medium was isotyped with a kit from Zymed Laboratories Inc., San Francisco, Calif.

(ii) Specificities and sources of reference MAbs. MAbs against serogroup B (2-1-B), serotype 15 (3-1-P15), and subtypes P1.1 (9-1-P1.1), P1.2 (3-1-P1.2), P1.3 (12-1-P1.3), P1.15 (2-1-P1.15), and P1.16 (3-1-P1.16) were supplied by W. D. Zollinger, Walter Reed Army Institute of Research, Washington, D.C. MAbs against serotypes 14 (MN5C8C) and 16 (93E9.1) and subtypes P1.4 (MN20B9.34), P1.5 (MN22A9.19), P1.6 (MN19D6.13), P1.7 (MN14C11.6), P1.9 (MN5A10F), P1.10 (MN20F4.17), P1.12 (MN20A7.10), P1.13 (MN24H10.75), and P1.14 (MN21G3.17) were supplied by J. T. Poolman, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Gel electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 12% acrylamide gels with whole-cell suspensions of heat-inactivated meningococci (46) or outer membrane vesicle preparations (29), which were boiled in sample buffer, as antigens (46). Nitrocellulose filters for immunoblotting were incubated overnight with human sera diluted 1:200 and were developed for 2 h with a 1:500 dilution of rabbit anti-human IgG conjugated to horseradish peroxidase (Dakopatts a/s, Glostrup, Denmark) (46). MAbs were developed with a 1:1,000 dilution of rabbit anti-mouse Ig conjugated to horseradish peroxidase (Dakopatts a/s). Incubation of primary antibodies was always performed either without or with the detergent Empigen BB (Albright & Wilson, Whitehaven, United Kingdom) to increase the renaturation of the boiled antigens (41). Human sera and MAbs were incubated with 0.15 and 0.25% Empigen BB, respectively.

Multilocus enzyme electrophoresis. The isolates were characterized by their combination of alleles at 14 enzyme loci as described previously (6). Distinctive multilocus genotypes were designated ETs. Isolates were assigned to the ET-5 complex if they differed from the ET-5 reference strain 44/76 (B:15:P1.7,16) at 2 or fewer of the 14 enzyme loci.

Amplification and sequencing of variable regions of *porA* genes. Isolation of chromosomal DNA and amplification of *porA* genes from 11 isolates were performed as described previously (44) with primers P-10/27s and P-1182/1159as. PCR products were purified by centrifugation through Chroma Spin + TE 100 columns (Clontech Labs, Palo Alto, Calif.). Sequencing reactions were carried out with primers E and H described by McGuinness et al. (28). Sequencing primers were labelled with [γ-^{33}P]ATP (Amersham International, Buckinghamshire, United Kingdom). Sequencing reactions were performed with an AmpliTaq cycle sequencing kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's recommendations, except that the annealing temperature was set at 42°C. Sequencing gels were run and radioautographed as reported previously (44).

Peptide synthesis and epitope mapping. For mapping of the epitope reacting with MAb 202,G-12, two sets of peptides were synthesized in duplicate on solid polyethylene pins with an Epitope Scanning Kit from Cambridge Research Biochemicals, Cambridge, United Kingdom. The first set consisted of 17 peptides, each of 10 amino acids which overlapped by 9 amino acids, and the second set consisted of 19 peptides, each of 15 amino acids which overlapped by 14 amino acids. Both covered the deduced VR2 amino acid sequence of strains reacting with MAb 202,G-12. Synthesis was carried out as described previously (12, 26). After synthesis, the N terminus of each peptide was acetylated for 90 min at 30°C with *N*,*N*-dimethylformamide–acetic anhydride–triethylamine (5:2:1; vol/vol/vol) as described in the manufacturer's manual. Side chain deprotection and neutralization were performed for 4 h at room temperature in trifluoroacetic acid-anisole-ethanedithiol (95:2.5:2.5; vol/vol/vol); this was followed by sonication in 0.1% HCl in methanol-water (1:1; vol/vol) for 15 min at room temperature. For the ELISA, pins were blocked for 90 min in physiological saline containing 2% bovine serum albumin and 0.1% Tween 20 before incubation overnight with MAb 202,G-12 or MN24H10.75. Antibody binding was detected as described previously (39) with a 1:1,000 dilution of rabbit anti-mouse Ig conjugated to horseradish peroxidase (Dakopatts a/s).

Bactericidal assays. Bactericidal assays were performed in wells of microtiter plates as described previously in detail (17). Each well contained 25 μl of antibody dilution and 12.5 μl of inoculum (corresponding to 80 to 100 CFU) and was incubated with 12.5 μl of human complement for 30 min at 37°C. The complement source itself gave no reduction in the number of CFU of the inoculum after 60 min of incubation. A twofold dilution series of each MAb was used starting with a final dilution of 1:8. The titers in Table 3 are given as reciprocals of final dilutions of the MAbs resulting in >50% killing of the meningococci counted as the number of CFU.

Nucleotide sequence accession numbers. The sequences encoding the 22 VR1 and VR2 in Table 2 have EMBL accession numbers Z48012 to Z48033.

RESULTS

Subtype distributions of meningococcal isolates. The subtypes of the B:15 strains isolated from patients between 1987

TABLE 2. Deduced amino acid sequences of the meningococcal class 1 protein variable regions VR1 and VR2 from case isolates

Strain	Serological classification	VR1	VR2
98/90	B:15:P1.12,–[a]	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTTTTTTFV
24/90	B:15:P1.12,13	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTTT----FV
131/92	B:15:P1.12,13	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTTT----FV
139/92	B:15:P1.12,13	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTTT----FV
44/92	B:NT:P1.(7),13[b]	NYQLQLTEAQAANGGASGQVKVTK---AK	YWTTVNTGSATTTTT----FV
51/90	B:15:P1.12,13a[c]	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTT-----FV
28/90	B:NT:P1.12,13a	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTT-----FV
49/92	B:16:P1.12,13a	NYQLQLTEKPSSTNAKTGNKVEV--TKAK	YWTTVNTGSATTTT-----FV
3/89	B:14:P1.7,13a	NYQLQLTEAQAANGGASGQVKVTKVTKAK	YWTTVNTGSATTTT-----FV
23/89	B:15:P1.(7),13a	NYQLQLTEAQAANGGASGQVKVTK---AK	YWTTVNTGSATTTT-----FV
138/91	B:15:P1.13a	NIQLQLTEPPSKGQT--GN-KVTK---GK	YWITVNTGSATTTT-----FV
S3032[d]	B:NT:P1.12,16	NIQLQLTEKLSSTNAKTGNKVEV--TKAK	YTKDTNNNLT---------LV
M990[d]	B:6:P1.18,25	NIQLQLTEPPSKGQT--GN-KVTK---GK	YTVDSSGVVT---------PV

[a] Isolate 98/90 did not react with the P1.13 reference MAb MN24H10.75 or MAb 202,G-12.
[b] P1.(7) designates the masked P1.7 epitope with a three-amino-acid deletion (44).
[c] P1.13a subtype strains react with MAb 202,G-12.
[d] Sequences and subtype designations of reference strains published by Maiden et al. (21) and McCarvil et al. (25), respectively.

and 1992 are given in Table 1. During that period, 244 (56%) of the 432 strains studied were B:15. Together, the dominant subtype, subtype P1.7,16, and the variant with the three-amino-acid deletion outside the P1.7 epitope, designated P1.(7),16 (44), constituted 75% of the B:15 strains. They were followed by 24 (9.8%) strains of subtype P1.12. The nine remaining subtypes and the nonsubtypeable strains each constituted between 0.4 and 4.5% of the strains. The proportion of subtype P1.12 among the B:15 strains increased from 2.6 to 22% between 1987 and 1992, while that of subtypes P1.7,16 and P1.(7),16 strains decreased from 87 to 62%. B:15:P.12 strains were not detected among patient isolates recovered before 1987.

Production of the VR2-specific MAb 202,G-12. Subtype P1.12 is located in VR1 on the class 1 protein (21). To characterize the unknown VR2 of the 15:P1.12 strains, a MAb against the VR2 epitope was produced by using strain 51/90 (B:15:P1.12) as an immunogen. On immunoblots, this MAb (202,G-12, isotype IgG2b) reacted only with the class 1 protein of strain 51/90 and not with that of any of the reference strains described by Frasch et al. (10). Therefore, this MAb most probably bound to the unknown subtype-specific VR2 region of strain 51/90 and not to a common determinant on the class 1 proteins.

Characterization of B:15:P1.12 strains with VR2-specific MAbs. MAb 202,G-12 and a new reference MAb against subtype P1.13 (MN24H10.75) further differentiated among the B:15:P1.12 strains. On blots, 19 of the 24 strains reacted with 202,G-12, while 4 strains reacted with the P1.13 reference MAb and so expressed a P1.12,13 subtype protein. MAb 202,G-12 cross-reacted weakly with the latter strains on dot blots, but not on immunoblots. The remaining strain (strain 98/90) did not bind any of the VR2-specific MAbs and had a class 1 protein of slightly higher molecular mass in SDS-polyacrylamide gels than those of the other strains.

The two VR2-specific MAbs displayed different binding patterns on immunoblots. The P1.13 reference MAb bound only to the class 1 proteins when it was incubated together with the detergent Empigen BB, whereas the binding of 202,G-12 was detergent independent. These results imply that the P1.13 reference MAb probably bound to a conformational epitope and 202,G-12 bound to a linear one.

Deduced amino acid sequences of VRs. The divergent binding of these two subtype MAbs indicated different amino acid sequences in the VR2s of the class 1 proteins. *porA* genes from 15:P1.12 strains that reacted with either the P1.13 reference MAb or 202,G-12 and from the single strain that did not react with the MAbs (strain 98/90) were amplified by the PCR technique and sequenced. In addition, *porA* genes from case strains with other subtypes in VR1 were also analyzed. The deduced amino acid sequences of VR1 and VR2 are given in Table 2 together with published sequences of the reference strains S3032 and M990 (21, 25).

Strains reacting with the P1.13 MAb had a sequence of five threonine residues at the end of VR2, whereas those reacting with MAb 202,G-12 had four threonine residues (Table 2). Thus, except for one threonine residue, the latter sequence was similar to that of strains that bound the P1.13 reference MAb. It was found to be identical to one recently designated P1.13a (34), and henceforth, MAb 202,G-12 will also be named a P1.13a subtype-specific MAb.

Strain 98/90, which did not react with the P1.13 or P1.13a MAb, had a succession of nine threonine residues in VR2. This explained the higher molecular mass of its class 1 protein in SDS-polyacrylamide gels, estimated to a difference of about 500 Da, compared with those of the other P1.12 subtype proteins. The threonine sequence in this and the other strains was coded by repeating ACT triplets.

The results in Table 2 show that isolates with other typing characteristics, which reacted with the P1.13a and P1.13 MAbs, also had identical VR2 sequences with four and five threonine residues, respectively. Strain 138/91 (B:15:P1.13a) was the only one with a substitution of threonine with isoleucine in the N-terminal part of VR2. The VR1s of the P1.12 strains were similar to that of P1.12 reference strain S3032 except for two amino acids. P1.12 strains isolated in Norway had a tyrosine and a proline residue in the first part of VR1 (amino acids 2 and 10 in Table 2), whereas the reference strain expressed an isoleucine and a leucine in the corresponding positions. These substitutions did not affect binding of the P1.12 reference MAb, implying that these amino acids were not part of that epitope. VR1 of strain 138/91 was similar to one designated subtype P1.18 present in reference strain M990 (21, 25).

Epitope mapping. Synthetic peptides that covered VR2 of the P1.13a protein were used to map the epitope for the P1.13a MAb 202,G-12. This MAb did not bind to the set of peptides

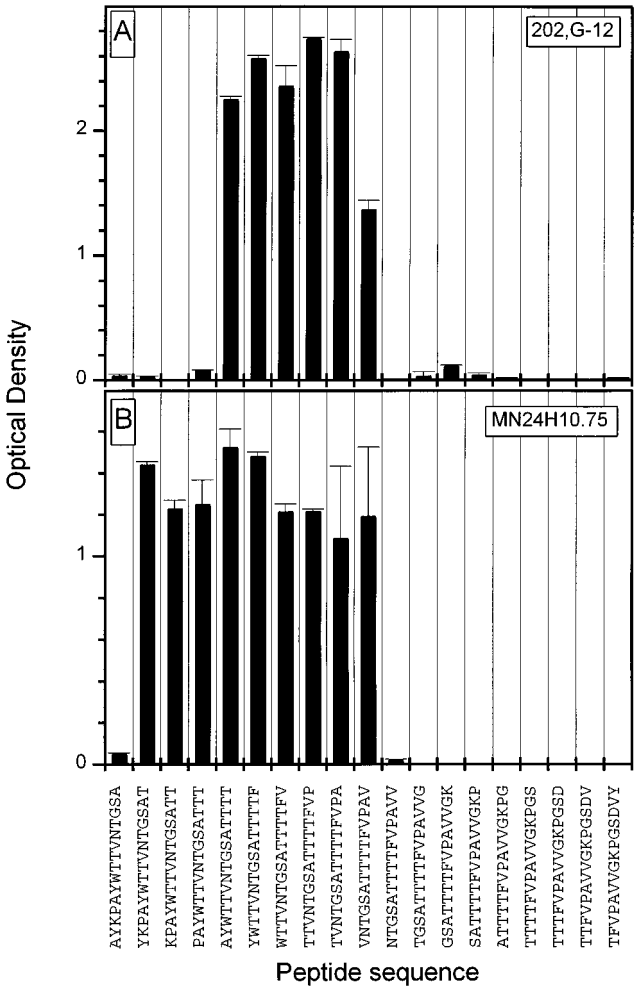

FIG. 1. Epitope mapping of the subtype-specific MAbs P1.13a (202,G-12) (A) and P1.13 (MN24H10.75) (B) with the same set of synthetic peptides (15-mers overlapping by 14 amino acids). MAb P1.13a was used as culture supernatant and was diluted 1:5; MAb P1.13 was diluted 1:1,000. Antibody binding was detected in ELISA with peroxidase-conjugated antibodies. Bars represent the mean A_{405} from two separate experiments with two sets of pins; T-shaped symbols indicate the standard deviations of the mean.

with 10 amino acids, in which the adjacent peptides differed in 1 amino acid, spanning a sequence of 26 amino acids in VR2 (data not shown). In contrast, the set of peptides with 15 amino acids revealed an antibody-binding sequence of 11 amino acids (TVNTGSATTTT) (Fig. 1A). When the N-terminal threonine of this sequence was absent, the results showed a reduced level of binding of MAb P1.13a. Because it did not react with the corresponding decapeptide (VNTGSATTTT), this threonine must contribute to the epitope. With the same two sets of peptides, the P1.13 reference MAb MN24H10.75 recognized the sequence VNTGSAT (Fig. 1B).

Bactericidal activity of the P1.13a and P1.13 subtype-specific MAbs. Previous studies have shown that subtype-specific MAbs are bactericidal and protective in animal models (32). The bactericidal activity of the P1.13a and P1.13 MAbs was therefore tested with meningococci expressing different class 1 proteins (Table 3). None of the MAbs killed the Norwegian vaccine strain 44/76 with the P1.7,16 subtype protein or strain 98/90 with nine threonine residues in VR2. The P1.13a MAb 202,G-12 demonstrated the highest titers against the two

TABLE 3. Bactericidal activity of the P1.13a and P1.13 subtype-specific MAbs

Strain	Serological characterization	Bactericidal titer of MAbs[a]	
		P1.13a (202,G-12)	P1.13 (MN24H10.75)
51/90	B:15:P1.12,13a	1,024	<8
148/91	B:15:P1.12,13	64	256
98/90	B:15:P1.12,−[b]	<8	<8
23/89	B:15:P1.(7),13a	512	<8
44/76	B:15:P1.7,16	<8	<8

[a] The titer is given as the reciprocal of the final MAb dilution showing >50% killing of the inoculum. The lowest final dilution tested was 1:8.
[b] VR2 contained nine threonine residues (strain 98/90).

P1.13a subtype strains. Some bactericidal activity was seen with strain 148/91 (15:P1.12,13), which also reacted weakly with this antibody on dot blots. This strain was the only one killed by the P1.13 reference MAb.

Multilocus enzyme electrophoresis. All 24 B:15:P1.12 strains belonged to the ET-5 complex, but they represented four different ETs within that complex, as shown in Table 4. Seventeen (71%) of the strains were a new clone of the ET-5 complex, designated ET-5c. None of the strains isolated before 1989 were ET-5c. Two of the isolates of this clone expressed subtype P1.12,13, and the remaining 15 strains expressed subtype P1.12,13a. Two strains with subtype P1.12,13a belonged to ET-5 itself. Both subtypes were found among strains of clone ET-5a, whereas the single strain with nine threonine residues in VR2 (98/90) and one P1.12,13 strain were ET-5b. Clone ET-5c differed from ET-5 by its allele at the gene coding for malic enzyme, whereas clone ET-5a was different at the isocitrate dehydrogenase locus and clone ET-5b was different at both this and the peptidase loci.

Geographical localization of B:15:P1.12 strains of ET-5c. B:15:P1.12 strains of ET-5c showed a strict localization in that 16 of the 17 ET-5c strains in Table 4 were from patients admitted to hospitals in Bergen (12 patients) and Stavanger (4 patients), two larger cities 160 km apart in two neighboring counties on the western coast of Norway. Two of the patients in the Bergen region were students at the same school who had been vaccinated in the Norwegian protection trial. Both 15:P1.12,13 isolates of ET-5c, recovered in 1991 and 1992, were also from patients in this area. The four patients in the Stavanger region were infected in 1992; three of these were a grandmother and her two grandchildren who fell ill within about a week. The remaining strain of ET-5c, without this geographical restriction, was from a patient in Kirkenes, one of

TABLE 4. Distribution of B:15:P1.12 meningococci within the ET-5 complex

ET	No. of strains				Allele at enzyme locus for[b]:		
	Total	15:P1.12,13	15:P1.12,13a	15:P1.12,−[a]	ME	PEP	IDH
ET-5	2	0	2	0	1	7	8
ET-5a	3	1	2	0	1	7	5
ET-5b	2	1	0	1	1	8	5
ET-5c	17	2	15	0	4.5	7	8
Total	24	4	19	1			

[a] VR2 contained nine threonine residues (strain 98/90).
[b] Enzyme abbreviations: ME, malic enzyme; PEP, phenylalanyl-leucine peptidase; IDH, isocitrate dehydrogenase. Alleles at the other 11 enzyme loci were identical for all 24 strains.

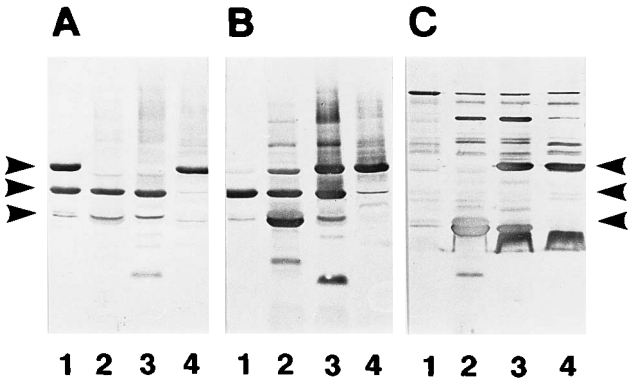

FIG. 2. Immunoblot analyses of IgG antibody responses against class 1 proteins and their VR1s and VR2s in sera from patients infected with B:15:P1.12,13a strains of clone ET-5c. Antigens were boiled outer membrane vesicles from meningococci with different subtype combinations in their class 1 proteins: lane 1, B:15:P1.7,16 (strain 44/76); lane 2, B:15:P1.(7),13a; lane 3, B:15:P1.12,13a; and lane 4, B:NT:P1.12,16 (strain S3032). Convalescent-phase sera (1:200) were taken 2 years after the occurrence of disease from a vaccinee (A) and a placebo control (B) and 18 days after the occurrence of disease from a nonparticipant (C). IgG binding was detected with peroxidase-conjugated antibodies. The arrowheads point to the positions of the class 1 (43,000-Da), 3 (37,000-Da), and 5 (30,000-Da) proteins, from top to bottom, respectively.

the most northern cities in Norway. We were unable to determine if this patient had connections to western Norway. None of the remaining seven strains of the other ET-5 clones were from western or northern Norway, but were mainly recovered from patients living in the region around the capital of Norway, Oslo.

Antibody responses of patients infected with B:15:P1.12 strains. In the blinded and open part of the Norwegian protection trials, five vaccinees and four placebo controls were infected with B:15:P1.12 strains. All vaccinees and two controls fell ill with infections caused by ET-5c strains. From seven of these patients acute- and convalescent-phase sera, taken up to 45 days after the onset of disease, were obtained. None of the sera showed distinct IgG antibody binding on immunoblots to the P1.7,16 subtype protein in the vaccine or to the P1.12,13 or P1.12,13a proteins of their own infecting strains, although antibody activity against other outer membrane proteins was seen (data not shown).

However, with sera taken 2 years after the occurrence of disease, one vaccinee and one control demonstrated IgG antibodies against the subtype proteins. Serum from the vaccinee bound distinctly to the P1.7,16 protein (Fig. 2A). When this serum sample was probed against B:15 strains with other subtype combinations in VR1 and VR2, the antibodies also reacted with the P1.12,16 subtype protein but not with the P1.7,13a and P1.12,13a proteins (Fig. 2A), implying that the IgG response was most probably mounted against the P1.16 epitope in VR2. The late-convalescent-phase sample from the placebo control reacted with protein P1.12,13a of the infecting strain. In a similar cross-blot experiment (Fig. 2B), IgG antibodies bound strongly to the P1.12,13a and P1.12,16 proteins, weakly to the P1.7,13a protein, and not at all to the P1.7,16 protein. Therefore, the majority of subtype antibodies seemed to be directed against the P1.12 epitope in VR1. These antibodies showed no binding to an immunoblot with strains representing all other known subtypes (data not shown), further demonstrating the subtype specificity of the serum. When this serum sample was incubated with the synthetic 15-mer peptide set, its weaker P1.13a activity recognized the same epitope of 11 amino acids as that recognized by the P1.13a MAb 202,G-12.

Sera were also obtained from four of the six nonparticipants in the trials infected with B:15:P1.12 strains. Only one convalescent-phase serum sample, taken 18 days after the onset of disease, bound to the P1.12,13a protein. As found for the placebo control, these antibodies were directed against the P1.12 epitope (Fig. 2C). Both P1.12-responding serum samples reacted equally well with the P1.12 protein of strain 98/90 with nine threonine residues in VR2, indicating that the longer VR2 did not affect binding to VR1 (data not shown). Figure 2 shows that the convalescent-phase sera reacted with several other antigens, among them the serotype 15 protein and class 5 proteins, in addition to other unknown antigens, mainly with greater molecular masses.

Another experiment also supported the subtype specificity of the class 1 protein antibodies following disease and vaccination. When nine serum samples from other vaccinees, infected with B:15:P1.7,16 strains and showing a good IgG response against the P1.7,16 protein on immunoblots, were probed against outer membrane vesicles from strain 51/90 (15:P1.12,13a), none bound to the P1.12,13a protein. Only one of the serum samples showed weak binding to protein P1.12,13a when it was incubated with detergent to enhance antigen renaturation (data not shown).

Occurrence of subtypes P1.13a and P1.13 among other isolates from patients and carriers. We also studied if the subtype epitopes characteristic of clone ET-5c were expressed by case strains recovered before 1987 and by carrier strains. Subtype P1.13a was first demonstrated on a B:15:P1.(7),13a strain isolated from a patient in 1980. The combination with subtype P1.12 was seen in 1987, the first year that this subtype was observed among isolates from patients in Norway. Subtype P1.13 was first detected in 1990 on a B:15:P1.12,13 isolate. In all, subtype P1.13a was expressed by 30 (5.2%) strains isolated from patients between 1976 and 1992, and subtype P1.13 was expressed by 5 (0.9%) strains.

With regard to the carrier strains, three P1.13a strains, but no P1.13 strains, were found among 133 isolates from the throats of military recruits near Stavanger. The corresponding numbers among samples collected later from 91 volunteers living near Oslo were seven and three strains, respectively; one of these (NG:15:P1.13a) was the only ET-5 isolate in that collection. Among 114 throat samples from college students living near Bergen, where most of the cases of infection with ET-5c strains occurred, 6 P1.13a strains and 2 P1.13 strains were found. This collection contained the only ET-5c carrier isolate (B:15:P1.12,13) observed. The frequencies of subtypes P1.13a and P1.13 among the carrier isolates were thus 4.7 and 1.5%, respectively.

DISCUSSION

In the present study we characterized a new clone of B:15:P1.12 meningococci within the ET-5 complex which was responsible for a localized outbreak of systemic disease in Norway. The clone expressed a subtype protein different from that of the prevalent strain B:15:P1.7,16 being used as vaccine antigen in the Norwegian protection trials (4, 5).

Between 1987 and 1992, about 10% (24 strains) of the B:15 case isolates expressed a class 1 protein with subtype P1.12, and this was the third most frequent subtype in Norway. The B:15:P1.12 isolates all belonged to the ET-5 complex (7), but 17 of the strains (71%) showed a new genotype, named ET-5c (Table 4). This genotype first emerged in Norway in 1989 and has never been seen among about 4,500 meningococcal strains

from other countries analyzed by multilocus enzyme electrophoresis. Strains of ET-5c revealed a striking geographical localization in that all but one isolate were from patients living in two neighboring counties in western Norway. The new clone, which was made up of only B:15:P1.12 strains and no strains with other serotypes and subtypes, differed from ET-5 in its allele for malic enzyme. The remaining seven B:15:P1.12 isolates, distributed among three other clones of the ET-5 complex, were from patients in other parts of the country. Interestingly, the single ET-5c strain detected among the carrier isolates was from an individual living in the country where most of the cases of infection with ET-5c strains occurred.

Demonstration of the new virulent clone of B:15:P1.12 meningococci led to characterization of the undefined VR2 of the class 1 proteins in which subtype P1.12 is located in VR1 (21). For this purpose, a new MAb (MAb 202,G-12), specific for VR2 was developed, and *porA* genes of representative isolates were sequenced. Nineteen of the 24 B:15:P1.12 strains expressed a class 1 porin with a sequence of four threonine residues in VR2 which reacted with MAb 202,G-12. Four isolates bound the reference MAb for subtype P1.13 (MN24H10.75 from J. T. Poolman) and showed one more threonine residue in VR2. The epitope with four threonine residues was thus rather similar to that reacting with the P1.13 reference MAb and has recently been designated P1.13a by Suker et al. (34). So, MAb 202,G-12 is a P1.13a subtype-specific MAb. Its specificity was also supported by the reaction with African serogroup A strains of subgroup IV-1 (unpublished data) whose VR2s contained the P1.13a sequence (34). No VR2-specific MAb has been available until now to characterize this subgroup, and attempts to raise such a MAb by using a synthetic P1.13a peptide sequence as antigen were unsuccessful (34).

Although the VR2s of the P1.13 and P1.13a proteins deviated by only one threonine residue, the two subtyping MAbs seemed to be directed against different epitopes. Immunoblot analyses indicated that P1.13 MAb bound to a conformational epitope since it only reacted with P1.12,13 proteins when detergent was present to enhance antigen refolding (22, 41). The P1.13a MAb (MAb 202,G-12) bound to denatured P1.12,13a proteins, implying a reaction with a linear epitope or one that was easily renatured during electrotransfer or subsequent incubations. Epitope mapping showed that the P1.13a MAb recognized a linear sequence of 11 amino acids (TVNTGSA TTTT) that included the four threonine residues specific for P1.13a subtype strains (Fig. 1A and Table 2). The same sequence was also recognized by IgG antibodies in one serum sample from a patient (Fig. 2B). This epitope is longer than those described for other VR1- and VR2-specific MAbs (26, 28). Identical sequences were also present in P1.13 subtype strains and in the single strain (strain 98/90) with nine threonine residues in VR2, which did not bind MAb P1.13a in the blotting assays, demonstrating that the additional threonine residues abolished antibody binding to the epitope. We have also previously reported that one amino acid deletion outside an epitope, defined by peptide mapping, affected antibody binding (30).

Surprisingly, epitope mapping demonstrated that the P1.13 reference MAb reacted with the sequence VNTGSAT, a smaller part of that binding MAb P1.13a (Fig. 1) and present in the three different P1.12 proteins (Table 2). Because the blotting methods showed that the P1.13 MAb only bound to P1.13 strains with five threonine residues in VR2, but not when four or nine threonine residues were present, the five amino acids must in some way contribute to the epitope, demonstrated by immunoblotting to be conformationally dependent. Identification of a linear epitope for the P1.13 reference MAb, which did not include the five threonine residues specific for P1.13 subtype strains, shows that epitope characterization with synthetic peptides may give misleading results as discussed by Laver et al. (18). One possible explanation for the puzzling results is that high peptide concentration on pins favors cross-reactivity (38).

Demonstration of three different P1.12 subtype proteins with four, five, and nine threonine residues in the C-terminal flank of loop 4 (Table 2) implies duplication of a different number of ACT triplets at a specific site in the *porA* genes. The VR2 sequences of subtypes P1.13a and P1.13 (Table 2) were longer than other known VR2 sequences (20, 21, 26, 36). This was also observed by McGuinness et al. (28) for a nonsubtypeable strain (strain MC71) seen to express a VR2 identical to that of P1.13a. To our knowledge, the single P1.12 strain that we found with nine threonine residues in VR2 (strain 98/90 in Table 2) seemed to have the longest VR2 hitherto reported. This strain was resistant in bactericidal assays with the P1.13a and P1.13 MAbs (Table 3).

The *porA* genes, which encode the class 1 proteins, possess a mosaic structure that is thought to have arisen from the horizontal exchange of gene fragments between strains (9, 24). Parts of *porA* genes, including those encoding the VR1 and VR2 regions, may be reassorted independently, and antigenic variation, particularly for the serogroup B strains, is further increased by substitutions, deletions, and insertions possibly driven by the selection pressure from the immune system, as reviewed by Maiden (19). Evidence has also been presented that meningococci of different serogroups have a common global *porA* gene pool (34). Subtype P1.13a is found in clone IV-1 of serogroup A strains, which have been endemic in the African meningitis belt since the 1960s (1, 34). Our results indicated that this epitope has been expressed by Norwegian group B isolates since 1980. Although subtype P1.13a was associated with the masked P1.7 epitope in isolates recovered in 1980, it was later most often paired with subtype P1.12 first detected among group B isolates in 1987. In The Netherlands, subtype P1.12 has been found since 1958 on group B meningococci (33), and it was also expressed by group B strains isolated in Canada between 1987 and 1989 (2). Thus, blocks of *porA* genes encoding the P1.12 and P1.13a subtypes seemed to be present in case isolates in other countries long before they were found among case isolates in Norway.

The model of the P1.7,16 subtype protein shows that loop 1 with subtype P1.7 is longer than loop 4 with subtype P1.16 (36). We have previously reported that the dominant class 1 protein antibody activity in volunteers immunized with a 15:P1.7,16 vaccine was directed against the P1.16 subtype loop (30, 40). For nonvaccinated individuals infected with 15:P1.12,13a meningococci, the dominant class 1 protein activity, in those who responded, was directed against subtype P1.12 located on loop 1 (Fig. 2). Thus, loop 4 seemed to be more immunogenic than loop 1 in protein P1.7,16, whereas the response was reversed for the P1.12,13a protein.

Because the class 1 subtype protein is an attractive vaccine candidate, a major question is whether antibodies raised against the P1.7,16 subtype protein in the Norwegian meningococcal vaccine will protect against infection with clone ET-5c expressing a different subtype protein. On immunoblots, we observed no distinct IgG binding to the P1.7,16 protein with acute- and convalescent-phase sera from vaccinees and placebo controls infected with 15:P1.12,13a strains, although strong antibody activity against other vaccine antigens was seen. A few of the serum samples gave weak immunoreactive class 1 protein bands. This is consistent with the low class 1

protein antibody levels in ELISAs of acute- and convalescent-phase sera from participants in the Norwegian vaccine trial reported by Guttormsen et al. (14). However, a serum sample drawn from one of the vaccinees 2 years after the occurrence of disease showed distinct IgG antibody binding to the P1.7,16 protein (Fig. 2A). These antibodies, as well as corresponding IgG antibodies in sera from other vaccinees infected with B:15: P1.7,16 strains, did not bind to the P1.12,13a subtype protein on immunoblots. Nor did antibodies against the P1.12,13a protein, mounted after infection of nonvaccinees with B:15: P1.12,13a strains of ET-5c, cross-react with the P1.7,16 protein (Fig. 2B and C). Immunoblot analyses therefore implied that the distinct class 1 protein antibody activity was subtype specific. However, the immunoblotting method is restricted in that it will probably show antibody binding only to denatured or more linear epitopes and to conformational ones partly renatured during the experimental procedure. With these methodological limitations in mind, our data indicated that vaccine-induced antibodies against the P1.7,16 protein will probably not be effective against strains of clone ET-5c. We have previously found that only a small change in VR2 of the P1.7,16 protein led to increased resistance to vaccine-induced bactericidal activity (30). The lack of cross-reactivity between subtype-specific bactericidal antibodies was also the rationale behind the multivalent class 1 protein vaccine reported by van der Ley and Poolman (37). However, other antigens, e.g., the Opc protein in the Norwegian vaccine, which gives bactericidal antibodies in humans (30, 31), may contribute to protection. The emergence of a new clone of virulent group B meningococci may have consequences for future vaccine development and emphasizes the importance of close epidemiological surveillance of meningococcal strains.

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