Expression of an inaccessible P1.7 subtype epitope on meningococcal class 1 proteins

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Summary. Dot-blot analysis of whole-cell suspensions of meningococci showed that 81 % of B:15:P1.16 strains from patients reacted with a monoclonal antibody (MAb) against subtype P1.7. The remaining strains, which did not react on dot-blot or in ELISA, demonstrated the P1.7 subtype epitope on immunoblots after denaturation of the cells with sodium dodecyl sulphate. The monomeric class 1 proteins of the two P1.16 subtype variants had slightly different mol. wts, but bound the P1.7 antibody equally well. These results were explained by a deletion of three codons in the gene encoding the first variable region of the P1.16 class 1 protein. The deletion accounted for the non-exposure of the P1.7 epitope on native cells. Other patient strains, with subtypes P1.3, P1.9 or without any known subtype, also showed a binding site for the P1.7 MAb, which became available only after denaturation. Demonstration of inaccessible epitopes may have consequences for subtype designations and vaccine development.

Introduction

The class 1 protein is one of the major outer-membrane proteins of *Neisseria meningitidis*, and antigenic variations of this protein define the subtype of a meningococcal strain.¹ To date, monoclonal antibodies (MAbs) have been made against c. 15 different subtypes,^{1–8} and the binding of two different subtype MAbs to some class 1 proteins has been demonstrated.^{4, 8–10}

Expression of the class 1 protein is controlled by the *porA* gene.¹¹ The predicted amino-acid sequence of the gene shows homology with the gonococcal PI pore proteins,^{12, 13} and a pore function with a slight cationic selectivity has been demonstrated *in vitro*.¹⁴ Two major variable regions are present in the amino-acid sequences of different class 1 proteins in which the subtype-specific epitopes are localised.^{13, 15} Thus, the subtype P1.7,16 protein of the prototype strain 44/76 has the epitopes for the subtypes P1.7 and P1.16 MAbs in the first (VR1) and the second (VR2) variable region, respectively.¹⁵ Both regions are exposed on the cell surface, since the MAbs bind to whole cells and are bactericidal.¹⁶

A dot-blot study of whole-cell suspensions of meningococci¹⁷ showed that many B:15:P1.16 strains from Norwegian patients also reacted with the P1.7 subtype MAb. This was similar to the prototype strain 44/76 of serotype 15, which was originally isolated in Norway.¹⁸ The remaining B:15:P1.16 strains did not react, but preliminary studies with a few such strains

indicated binding of the P1.7 MAb after denaturation and immunoblotting.¹⁷

In this study, we examined the reactions of more B:15:P1.16 strains and strains of other subtypes with the P1.7 MAb in dot-blotting, ELISA and immunoblotting. The reason for the inaccessibility of the epitope in some strains was examined by DNA sequencing.

Materials and methods

Meningococcal strains

The serotypes and subtypes of 50 patient and 133 carrier strains, collected in Norway during 1987–1988, have been described previously.⁹ One hundred and one strains from Norwegian patients, collected during 1988–1990, 87 strains from various other parts of the world,¹⁹ and three patient strains from Chile were also studied.

Monoclonal antibodies

P1.Ham MAb was supplied by Dr F. E. Ashton. MAbs against serotype 15 (3–1–P15), subtypes P1.3 (12–1–P1.3) and P1.16 (3–1–P1.16) were supplied by Dr W. D. Zollinger. MAbs against subtypes P1.6 (MN19D6.13), P1.7 (MN14C11.6) and P1.9 (MN5-A10F) were supplied by author J.T.P.

Dot-blotting and immunoblotting

These procedures were performed with whole-cell suspensions as described previously.⁹ The cells were

initially heat inactivated at 56°C for 30 min in phosphate-buffered saline.²⁰ Binding of the MABs to the blotted antigens was detected with rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dakopatts a/s, Denmark).⁹ Cells used as antigens in immunoblotting were either fully denatured by boiling with sample buffer²¹ containing 2-mercaptoethanol and SDS 0.7% or denatured mildly by mixing with ice-cold sample buffer just before application on to SDS-PAGE gels containing acrylamide 12%. The dilutions of the P1.7 MAB used in dot-blotting and immunoblotting were 1 in 60000 and 1 in 20000, respectively. Mol. wt determinations were performed with a calibration kit from Pharmacia AB, Sweden.

ELISA

Heat-inactivated cell suspensions served as antigens in ELISA, which was performed as described previously.⁹ The P1.7 MAB was diluted 1 in 10000.

Enzyme electrophoresis

Each of the 374 isolates was characterised by multilocus enzyme analysis with starch gel electrophoresis and enzyme-specific staining.²² The assignment of the isolates to the ET-5 complex was based on the analyses of 14 different enzyme loci.²³

Polymerase chain reactions

Chromosomal DNA was isolated as described previously,²⁴ except for an additional treatment of the DNA solution for 2 h with boiled RNAase A (Sigma) 20 µg/ml before the second phenol-chloroform extraction. DNA concentration and purity were determined spectrophotometrically and electrophoretically. The primers, which were synthesised by the Biotechnology Centre, Oslo, were P-1182/1159 as 5'-TTA GAA TTT GTG GCG CAA ACC GAC-3', which corresponded to the anti-sense strand of the region encoding the seven C-terminal amino acids of the class 1 protein and the TAA stop codon,¹³ and P-10/27s 5'-AAA CTT ACC GCC CTC GTA-3', which coded for amino acids 4-9 of the signal peptide.

The polymerase chain reactions (PCRs) were performed as described by Maiden *et al.*,¹³ except that the concentration of the primers was 0.2 µM to prevent nonspecific binding. The annealing temperature was 60°C.

Sequencing of PCR products

PCR products were separated from the primers by centrifugation through Chroma Spin+ TE 100 columns (Clontech Labs, CA, USA). The sequencing primer was P10/27s, which was labelled with (γ-³²P) ATP, and was used without purification as described by Embley.²⁵ The composition of the sequencing reaction mixtures was as reported by Embley,²⁵ but

4 µl of 10 × concentrated *Taq*-polymerase buffer was used; 4 µl of the reaction mixture was added to each of four tubes with 4 µl of termination mixture. This mixture was as follows: for the A reaction—333 µM ddATP, 15 µM dATP and 50 µM each of dGTP, dCTP and dTTP; for the G reaction—250 µM ddGTP, 15 µM dGTP and 50 µM each of dATP, dCTP and dTTP; for the C reaction—333 µM ddCTP, 15 µM dCTP and 50 µM each of dATP, dGTP and dTTP; and for the T reaction—666 µM ddTTP, 15 µM dTTP and 50 µM each of dATP, dCTP and dGTP. Each tube was overlaid with one drop of light mineral oil. The sequencing reactions were performed by PCR.²⁵ The samples were left at 4°C before the addition of 4 µl of formamide stop buffer (Boehringer Mannheim, Germany). One µl of each reaction mixture was analysed in 40-cm lengths of polyacrylamide 6% sequencing gels. The gels were fixed and dried at 80°C²⁵ and autoradiographed for 14 h with Hyperfilm-MP (Amersham International).

Results

15:P1.16 strains

Dot-blotting with specific MABs showed that 15:P1.16 was the most frequent serotype and subtype combination among 151 strains collected from Norwegian patients during 1987–1990. These strains constituted 46% (69 of 151) of the case strains (table); all were serogroup B. Fifty-six (81%) of the 15:P1.16 strains also reacted with the P1.7 MAB on dot-blot and, therefore, had subtype P1.7,16, whereas 13 (19%) of the strains had no reaction (table). In ELISA, the latter strains showed no binding of the P1.7 MAB. These analyses were performed with bacteria that had been inactivated at 56°C for 30 min and stored as cell suspensions at 4°C.²⁰ The binding patterns on dot-blot of strains representing the two different subtypes P1.16 and P1.7,16 with the P1.16 and P1.7 MABs are shown in fig. 1A. All strains expressed similar levels of class 1 proteins in SDS-PAGE gels.

When cell suspensions of strains with the two kinds of subtypes were boiled with SDS-containing sample buffer before gel electrophoresis, the monomeric class 1 proteins reacted equally well with the P1.7 MAB on immunoblots. However, their mol. wts were slightly different (fig. 1B). The P1.7,16 subtype protein had a mol. wt of 42.3×10^3 , whereas that of the P1.16 subtype protein was 41.6×10^3 . Both subtype epitopes were present on the same antigen. This was shown by splitting the nitrocellulose strip from one blotted lane and incubating each half with the P1.7 and P1.16 MABs, respectively (fig. 2). All 13 patient strains with subtype P1.16 showed a binding site for the P1.7 MAB which became available only after denaturation of the antigens (table).

To examine whether less harsh denaturation of the cells before immunoblotting would give the same response as the more native antigens on dot-blot, cells

Table. Reaction of meningococcal strains with the P1.7 subtype-specific MAb in dot-blotting and immunoblotting

Strains	Number of strains	Number of strains with reactions on		
		dot-blots		immunoblots* P1.7+
		P1.7+	P1.7-	
15:P1.16 from patients	69	56	13	13†
15:P1.16 from carriers	3	1	2	2†
15:- from patients	15	3	12	4†
15:- from carriers	7	0	7‡	0
With subtype P1.3 from patients	8	0	8	5§
With subtype P1.3 from carriers	18	0	18	0
With subtype P1.9 from patients	13	1	12	1
Other strains	241	18	223	ND
Total	374			

The strain collection included 151 patient and 133 carrier strains from Norway and 90 patient strains from other countries.

ND, not determined.

* Immunoblotting was performed with all strains not reacting with P1.7 MAb on dot-blots (P1.7- strains).

† Belonged to the ET-5 complex, reacted with P1.Ham.

‡ All had subtype P1.6.

§ Belonged to the ET-5 complex, two strains reacted with P1.Ham.

|| Reacted with P1.Ham.

Fig. 1. Dot-blot (A) and immunoblots (B and C) of whole-cell suspensions of 15:P1.16 strains with surface-exposed or masked P1.7 subtype epitopes, respectively, applied in every second position. Reference strain 44/76 with subtype P1.7,16 was applied in the first position on all blots. Dot-blot was incubated with the P1.7 and P1.16 MAbs and immunoblots with the P1.7 MAb. In B, the cells were denatured by boiling with sample buffer before SDS-PAGE,²¹ and in C, the same cells were denatured mildly by mixing with cold sample buffer before application on to the gel. B shows the two immunoreactive monomeric class 1 proteins of slightly different mol. wts; C shows the polymeric class 1 proteins with traces of the monomeric proteins. MAb binding was detected with peroxidase-conjugated antibodies.

with the two subtype proteins were denatured mildly by mixing with cold sample buffer containing SDS before electrophoresis. Several high mol. wt bands reacting with the P1.7 MAb were observed with a trace of monomeric class 1 proteins (fig. 1C). Strains with the two kinds of subtype proteins showed a similar pattern and intensity of the polymeric immunoreactive bands. Thus, mild denaturation of the antigens with SDS was sufficient to expose or unmask the epitope for binding with the P1.7 MAb.

The finding of an intact P1.7 epitope in such P1.16 strains and also the lower mol. wt of their class 1

protein suggested the possibility of a deletion in the protein. DNA was extracted from three B:15:P1.16 strains with masked P1.7 epitope. The extracted DNA was subjected to PCR and the products were sequenced as detailed above. The three strains had identical nucleotide sequences in VR1 of the protein in which the P1.7 epitope is located.^{15,16} The nucleotide sequence and the resulting amino-acid sequence of VR1 are shown in fig. 3. When the strains with a masked P1.7 epitope were compared to the corresponding amino-acid sequence of the P1.7,16 protein from the reference strain 44/76,¹⁵ all had a deletion of

Fig. 2. Immunoblots of 15:P1.16 (lanes 1 and 2), 15:P1.3 (3 and 4) and 15:P1.9 (5 and 6) strains with masked P1.7 epitopes. Whole-cell suspensions of the strains were boiled with sample buffer before SDS-PAGE. Nitrocellulose strips, obtained by blotting of each strain, were divided in two and each half was incubated with different subtype MABs as shown: lane 1 with P1.16 MAB; 3 with P1.3 MAB; 5 with P1.9 MAB, 2, 4 and 6 with P1.7 MAB. The subtype P1.3 and P1.9 MABs were incubated in the presence of Empigen BB 0.25% to increase antibody binding.^{26, 27}

three amino acids next to the identified P1.7 epitope.^{15, 16}

When the enzyme genotypes of the strains were determined by multilocus enzyme electrophoresis, all Norwegian 15:P1.16 patient strains with the masked P1.7 epitope were shown to belong to the ET-5 complex.²⁸ They also reacted with the P1.Ham MAB on dot-blot (table). The 15:P1.7.16 strains had similar genotype, but 18% of these did not bind the P1.Ham MAB, which suggested further subtype variations within this group of strains. Two 15:P1.16 carrier strains, which also showed an inaccessible P1.7 epitope, were ET-5 and bound the P1.Ham MAB (table).

Strains with other subtypes

We examined other strains which were ET-5 and bound the P1.Ham MAB for carriage of a masked P1.7 epitope. Fifteen Norwegian patient strains of serotype 15 did not react with the P1.16 MAB on dot-blot (designated as 15:- strains in the table). They all belonged to the ET-5 complex and bound the P1.Ham MAB. Three of the strains had a surface-exposed P1.7 epitope as shown by binding of the P1.7 MAB on dot-blot (table). Immunoblotting of the 12 remaining strains showed that four (33%) strains reacted with the P1.7 MAB after denaturation and thus carried a masked P1.7 epitope. In contrast, none of the 15:- isolates from carriers in our collection reacted with the P1.7 reagent on blot (table). They had a different

subtype, previously defined as subtype P1.6;^{4, 7} they did not belong to the ET-5 complex, and only one strain bound the P1.Ham MAB. The serotype 15 protein of these carrier strains had a lower mol. wt in SDS gels than the corresponding class 3 protein from 15:- patient strains, which indicated differences in the serotype 15 proteins.

Some meningococci with other subtypes also expressed a masked P1.7 epitope. Five patient strains of subtype P1.3 from Chile and one Swiss strain with subtype P1.9, which did not bind the P1.7 MAB on dot-blot or in ELISA, reacted after immunoblotting (table). The mobilities of the class 1 protein bands reacting with each pair of MABs were similar, as shown in fig. 2. The strain from Switzerland and two of the Chilean strains bound the P1.Ham MAB, and all the Chilean strains belonged to the ET-5 complex. The other patient or carrier strains with subtypes P1.3 or P1.9 did not bind the P1.7 MAB on immunoblots (table), nor did they belong to the ET-5 complex or react with the P1.Ham MAB.

None of the prototype strains,¹ including that for subtype P1.9 (M982), revealed a masked P1.7 epitope on immunoblots.

Discussion

This report describes an epitope for the P1.7 subtype-specific MAB on some meningococci which is unavailable for antibody binding with intact cells when they are examined by dot-blotting and ELISA methods. The epitope, which was expressed by meningococci of different subtypes, was exposed only after denaturation of the cells with SDS, but not after heat inactivation at 56°C.²⁰ After denaturation, class 1 proteins with the masked P1.7 epitope reacted as strongly with the P1.7 MAB as strains recognising the MAB in the native state, which indicated that the epitope was intact.

For Norwegian B:15:P1.16 patient strains which carried a masked P1.7 epitope, a deletion of three amino acids in VR1 of the class 1 protein was the most likely explanation for these findings. VR1 is located in loop 1 of the monomeric protein.^{13, 16} This loop is the longest and contains the linear epitope for the P1.7 MAB at its tip.¹⁶ A deletion will shorten the loop and pull the epitope somewhat closer to the outer membrane so that it may be masked by the other loops or by other molecules, e.g., LPS.²⁹⁻³¹ SDS altered this

	GCA	CAA	GCC	GCT	AAC	GGT	GGA	GCG	AGC	GGT	CAG	GTA	AAA	GTT	ACT	AAG	...	...	...	GCC	AAA	AGC
P1.16*	A	Q	A	A	N	G	<u>G</u>	<u>A</u>	<u>S</u>	<u>G</u>	Q	V	K	V	T	K	.	.	.	A	K	S
P1.7.16	A	Q	A	A	N	G	<u>G</u>	<u>A</u>	<u>S</u>	<u>G</u>	Q	V	K	V	T	K	V	T	K	A	K	S

* with a masked P1.7 epitope

Fig. 3. Nucleotide and deduced amino-acid sequences of the VR1 domain of the gene coding for the class 1 subtype P1.16 protein with a masked P1.7 epitope. Compared to the amino-acid sequence of VR1 of the subtype P1.7.16 protein,^{15, 16} a deletion of three amino acids is observed. Deleted residues are indicated by dots, and the defined P1.7 epitope^{15, 16} is underlined. Except for the deleted residues, the deduced amino-acid sequence of the P1.16 protein was identical to that of the P1.7.16 protein¹⁵ up to amino acid no. 109, which represents the limit of sequence information obtained with the primer used.

conformation and, as a result, made the epitope available for antibody binding.

Approximately 20% of the Norwegian B:15:P1.16 strains had the subtype variant with the masked P1.7 epitope. There was some evidence for a restricted geographical localisation as three of five such strains, isolated in 1987 and 1988, were from patients in a restricted coastal region in western Norway. Such strains were also isolated from two brothers, who developed meningococcal disease, which suggested that this subtype variant may be a valuable epidemiological marker.

All 15:P1.16 strains from Norway with a masked P1.7 epitope belonged to the ET-5 complex²⁸ which consists of genetically closely related clones of sulphonamide-resistant meningococci, which have been causing epidemics in various parts of the world.³² These strains also bound the MAb against P1.Ham, a class 1 subtype epitope, which is associated with meningococcal strains from Hamilton, Canada.⁵ Most strains with subtype P1.3, P1.9 or non-subtypable serotype 15 isolates which carried a masked P1.7 epitope had the same multilocus enzyme genotype and reacted with the P1.Ham MAb. A previous study of subtype combinations¹⁷ showed an association between the P1.7 and P1.Ham epitopes. These results suggest that the P1.Ham epitope may serve as a marker for most strains with either an exposed or a masked P1.7 epitope.

Previously, a Chilean strain of subtype P1.3 was found to react in ELISA with the P1.7 MAb,⁴ but the binding was weaker than that of various other strains reacting with this MAb. In the present study, five further Chilean strains of subtype P1.3 showed no binding of the P1.7 MAb in ELISA. If the masked P1.7 epitope is located in VR1 of these P1.3 subtype proteins, as has been demonstrated for the accessible

epitope in the P1.7,16 and P1.1,7 subtype proteins,^{13,15} this indicates that the P1.3 subtype epitope is present in VR2. Recently, two Norwegian 4:P1.4 patient strains have also been found to carry masked P1.7 epitopes (unpublished results). Studies are in progress to determine if the masked P1.7 epitope found in the strains with other subtypes is caused by a deletion in VR1 similar to that of the 15:P1.16 strains.

The demonstration of an inaccessible P1.7 epitope may have consequences for the subtype designation of strains with such epitopes. This implies that strains with masked epitopes will not bind subtype MAbs in routine ELISA and dot-blot typing analyses. We propose to use parentheses to indicate a masked epitope, e.g., P1.(7),16. There are also implications for vaccine development. Antibodies against class 1 proteins seem to be important, as class 1 protein-specific MAbs protected infant rats against bacterial challenge.³³ Furthermore, sera from vaccinees and patients contained class 1 protein antibodies,³⁴ and a correlation was observed between the bactericidal activity of post-vaccination sera and class 1 protein antibody activity.³⁵ Thus, it may be of interest to examine whether the P1.7 MAb protects against challenge with strains having a masked P1.7 epitope and also, to determine whether the level of protection afforded by a vaccine with the P1.7,16 subtype protein differs against P1.16 strains with exposed or masked P1.7 epitopes.

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