DEDUCED AMINO ACID SEQUENCES OF CLASS 1 PROTEIN (PorA) FROM THREE STRAINS OF *NEISSERIA MENINGITIDIS*

Synthetic Peptides Define the Epitopes Responsible for Serosubtype Specificity

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Disease caused by *Neisseria meningitidis* is a severe life-threatening infection, with world-wide incidence of sporadic or epidemic cases (1), and for which no fully effective vaccine is currently available. Vaccines containing the capsular polysaccharide provide limited protection against infection caused by strains of serogroups A and C. However, there is no protection against infection caused by strains of serogroups A and C. However, there is no protection against group B meningococci, the predominant cause of infection in many temperate countries, since the group B polysaccharide is poorly immunogenic in humans (2). For these reasons vaccines derived from outer membranes are currently being evaluated for their efficacy in preventing meningococcal disease (3–6). Such vaccines which are composed of a number of outer membrane proteins are heterogeneous in their composition and may induce antibodies directed against nonprotective and highly variable antigenic determinants (4, 6). An alternative approach is to identify individual proteins that can induce protective antibodies and that do not exhibit excessive antigenic heterogeneity. The proteins that appear most promising in this respect are the class 1 and class 2/3 outer membrane proteins.

All meningococcal isolates contain either a class 2 or class 3 protein: which is quantitatively the major outer membrane protein (7). Expression of either protein, which is stable and mutually exclusive for any individual strain, is controlled by the gene locus that has recently been designated porB (8, 9). The proteins function as outer membrane porins and are therefore analogous to the gonococcal proteins PIA and PIB. Both the gonococcal porin proteins and meningococcal class 2/3 proteins from invasive isolates have been shown to spontaneously insert into artificial lipid membranes, suggesting a possible role in tissue invasion (10).

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The class 1 protein is also expressed by almost all meningococcal isolates. Its expression is controlled by the porA gene locus which, unlike those of the other outer membrane proteins, is not also present in gonococci (8, 9). Although no biological role has been unequivocally assigned to the class 1 protein, the predicted amino acid sequence of the *porA* gene (11) shows considerable homology with the gonococcal PI proteins (12, 13), suggesting that the protein may play a similar and additional role to the class 2/3 proteins in meningococcal infection.

Both proteins exhibit differences that generate antigenic diversity between strains. With the advent of mAbs it has been possible to define a consistent scheme for designation of serotype and subtype specificity (14), and to identify the proteins on which the epitopes reside (15, 16). Thus, antigenic differences in the class 2/3 outer membrane protein are responsible for serotype specificity, while antigenic determinants on the class 1 protein generate subtype specificity.

The class 1 protein is unique to meningococci, it is immunogenic and antibodies directed against it are bactericidal in vitro (3, 5). mAbs to the class 1 protein also protect against infection in an experimental animal model (17). Such antibodies are subtype specific and any vaccine designed to induce a broadly protective immune response would probably need to contain a number of different subtype determinants. The structure of the epitopes recognized by subtype-specific antibodies is therefore of considerable interest and importance.

We have previously described the cloning and sequencing of the gene from strain MC50 which is responsible for expression of the class 1 protein (11, 18). In the present paper we describe the cloning and sequencing of the equivalent gene (*porA*) from two further strains of different subtype specificity, and the use of the predicted amino acid sequences to construct synthetic peptides that have been used to define the structure of the epitopes recognized by a series of subtype-specific mAbs.

Materials and Methods

Meningococcal Strains and Monoclonal Antibodies. Meningococcal strains MC50 (Group C, type NT, subtype 16; C:NT:P1.16) and MC51 (C:NT:P1.15) were from patients with meningococcal disease and were isolated by the Public Health Laboratory Service as described previously (19). Strain H44/76 (B:15:P1.7, 16) was originally obtained from Dr. E. Holten (National Institute for Public Health, Oslo, Norway) and is a subtype P1.7, 16 reference strain (14).

The mAbs directed against class 1 protein have been described previously and are reference antibodies for determination of subtype specificity (15, 16).

Cloning and Sequence Determination of the porA Gene. Previous studies in which the complete nucleotide sequence of the porA gene from strain MC50 was obtained revealed that the entire gene was located on a 2.2-kb Xba I fragment. Chromosomal DNA from strain MC51 was digested with Xba I and fragments of 1.5–2.5 kb were purified using GENECLEAN™ (BIO 101 INC., La Jolla, California) and ligated in both orientations into Xba I cleaved M13mp18&19 RF DNA. Recombinant plaques were selected that hybridized with the 442-bp Eco RI–Kpn I fragment from stain MC50 which encompasses the central portion of the porA gene (11). Sequencing was carried out by the dideoxy chain termination method using SEQUENASE™ (U. S. Biologicals, Cleveland, OH) and a previously described series of synthetic oligonucleotide primers (11).

An Eco RI clone from H44/76 carrying the distal two thirds of the class 1 OMP gene in a λgt11 vector was amplified by polymerase chain reaction (PCR) with λgt11 flanking primers. The amplified fragment of ~2 kb was then cloned into Eco RI cut pUC18, and double-stranded DNA from this clone was used as template in a standard dideoxynucleotide sequencing reaction. Multiple synthetic sequencing primers were used to extend the sequence obtained with

the standard pUC forward primer, and in this way the entire coding strand of the Eco RI fragment was determined. Separately, the entire class 1 OMP gene of H44/76 was amplified from a chromosomal DNA preparation as described. This amplified 1,340-bp fragment carrying the class 1 OMP gene was cloned into M13 vectors and both strands of the DNA sequence upstream of the Eco RI site were determined from these clones. In another experiment, a pool of PCR-amplified genomic DNA was sequenced directly and a TYR (TAT) codon was confirmed at amino acid position 203 in contrast to the ASN (AAT) codon seen in the subclone from λgt11 obtained after PCR amplification. The direct sequencing data are probably more representative of the genome sequence than individual clones selected after PCR amplification, which may carry base changes introduced by polymerase misincorporation. This was the only difference seen in over 500 bp of sequence obtained by direct genomic sequencing.

Structural Analysis of the Predicted Protein Sequences. The hydropathy profiles of the predicted protein sequences were calculated using the algorithm of Kyte and Doolittle (20).

Synthesis of Peptides. Solid-phase peptide synthesis was carried out using a commercially available kit (Cambridge Research Biochemicals, Cambridge, UK). The peptides were synthesized on polyethylene rods via a *Nα*-9-fluorenylmethyloxycarbonyl (FMOC) protected *β*-alanine link coupled via polyacrylic acid and hexamethylene diamine to the rods (21, 22). Pentafluorophenyl (pfp) active esters of FMOC-L-amino acids with t-butyl side chain protecting groups were used for the synthesis, except in the case of arginine which had a methoxytrimethylphenylsulphonyl side chain protecting group and serine and threonine in which the oxobenzatriazine active ester was used (Milligen, Watford, UK). All solvents (Romil, Leicester, UK) were of analytical grade and were used without further purification with the exception of piperidine (BDH, Poole, Dorset, UK), which was redistilled before use and *N-N'*-dimethylformamide (DMF) which was stored over molecular sieve (BDH, grade 4A) to remove amine impurities.

Synthesis was carried out as described previously (23). The *β*-alanine was initially deprotected by mild base cleavage with piperidine, following which the rods were washed and the first amino acid was coupled overnight in the presence of hydroxybenzotriazole (as catalyst). Deprotection and coupling were repeated for addition of each amino acid required. After synthesis, the terminal amino groups were acetylated by reaction with acetic anhydride and amino acid side chain protecting groups were removed by cleavage with trifluoroacetic acid. Each synthesis was performed in duplicate and control peptides reacting with a mAb supplied by the manufacturer were included to verify the coupling during each synthesis.

Detection of Immunological Reactivity. Detection of the immunological reactivities of the synthesized peptides on the rods was performed by ELISA as described previously (23). The rods were blocked in PBS containing 1% BSA; fraction V (Sigma, Poole, Dorset, UK), 1% ovalbumin (Sigma; grade II), and 0.1% Tween-20 for 1 h at room temperature followed by incubation with appropriate mAbs diluted 1:1,000 in the same blocking solution for 18 h at 4°C. After washing and reaction with goat anti-mouse IgG-conjugated horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) color was developed using azino-di-3-ethylbenzthiazodinsulphonate (ABTS; Sigma) in substrate buffer (0.1 M disodium hydrogen orthophosphate, 0.08 M citric acid, pH 4.0 with citric acid) as substrate.

The solid-phase peptides were reused after bound antibody was dissociated by sonication of the rods in 1% SDS, 0.1% 2-ME in 0.1 M phosphate buffer at 60°C for 30 min. Immunological reactivity was always observed in duplicate peptides and in assays repeated on at least two occasions.

Results

Comparison of the Predicted Amino Acid Sequences of Class 1 Protein from Three Meningococcal Strains. The predicted amino acid sequences of the class 1 proteins from three meningococcal strains are shown in Fig. 1. The proteins showed considerable structural homology with over 90% of the sequences conserved between the strains. Most of the structural diversity was confined to two major regions, variable region

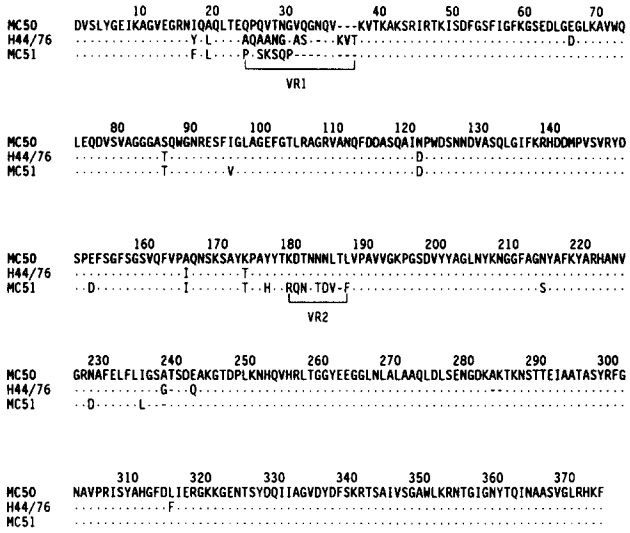

FIGURE 1. Comparative alignment of the predicted amino acid sequences of the class 1 protein from three meningococcal strains of differing serosubtype specificities. Numbers indicate the amino acid position in the mature class 1 protein from strain MC50 (11), dots indicate identities in the class 1 proteins of strains MC51 or H44/76 to the MC50 sequence, dashes indicate padding characters inserted to preserve optimal alignment of the sequences. The two major variable domains (VR1 and VR2) are indicated.

1 (VR1) corresponding to amino acid residues 24–36 of the MC50 sequence and variable region 2 (VR2) corresponding to residues 179–187. Strains MC50 (subtype P1.16) and H44/76 (subtype P1.7,16) showed the greatest similarity with major variations occurring in VR1 but not VR2.

Epitope Localization. Using the predicted amino acid sequence of the class 1 outer membrane proteins a series of solid-phase decapeptides spanning the entire length of each of the three molecules were synthesized, with adjacent peptides overlapping by five residues. Those peptides were reacted in ELISA with pairs of mAbs of three different subtype specificities. In each case the predominant reactivity was seen with peptides corresponding to VR1 or VR2 (Fig. 1).

The P1.16-specific mAbs (MN5C11G and 62-D12) both reacted strongly with the same single decapeptide present in VR2 of both P1.16 strains, corresponding to amino acid residues 176–185 in strain MC50 (P1.16: Fig. 2, *a* and *b*) and residues 178–187 in strain H44/76 (P1.7, 16: Fig. 2, *c* and *d*). In addition, the P1.16-specific mAbs also reacted weakly with peptide 31–40 from VR1 of strain H44/76 but not with any peptides from the quite distinct sequence of VR1 from strain MC50.

Both P1.7-specific mAbs (MN14C11.6 and ADAM 1) reacted with two overlapping decapeptides (26–35 and 31–40) from VR1 of H44/76 (Fig. 2, *e* and *f*), the only P1.7 strain, and not with any other sequences.

Unlike the other pairs of mAbs, which showed very similar reactivities, the two P1.15-specific mAbs (MN3C5C and 93H5) had quite distinct properties. Antibody 93H5 failed to react with any of the linear peptides tested (Fig. 2 *h*). In contrast antibody MN3C5C reacted strongly not only with two overlapping peptides in VR2 of the MC51 sequence (172–181 and 177–186), but also with a number of other peptides towards the COOH terminus in the more conserved regions of the molecule (Fig. 2 *g*).

Epitope Definition. In an attempt to precisely define the epitopes recognized by each of the subtype-specific mAbs a series of solid-phase decapeptides were synthe-

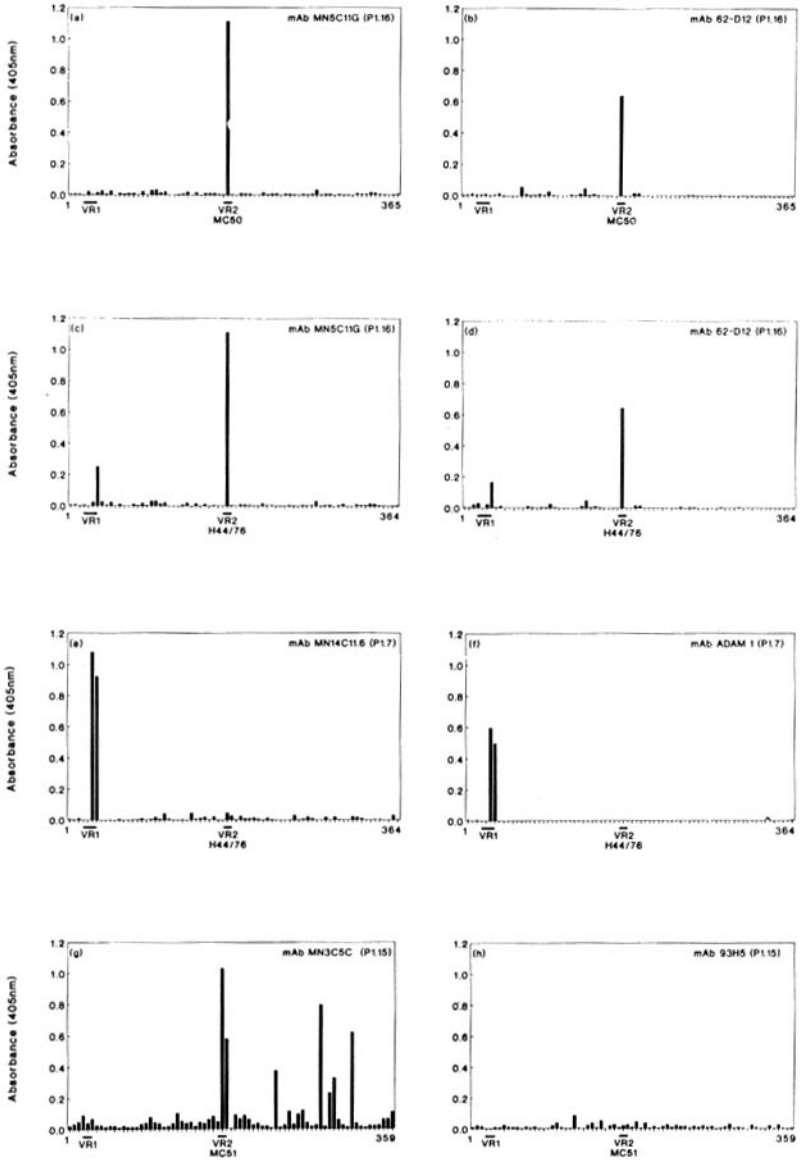

FIGURE 2. Epitope scanning by reaction of mAbs with solid-phase decapeptides spanning the predicted amino acid sequences of class 1 proteins from strains MC50, MC51, and H44/76. Adjacent decapeptides differ by five amino acid residues. Annotations show the strain from which the sequence was derived, the mAb used, and its subtype specificity.

sized spanning each of the VR1 and VR2 sequences, and also the other regions of reactivity in the MC51 sequence, in which adjacent peptides differed by a single amino acid. Subsequently, a further series of smaller peptides covering the regions of interest were synthesized to establish the minimum reactive epitope for each mAb.

(a) Subtype P1.16. The P1.16-specific mAbs reacted with the same five adjacent overlapping decapeptides (Fig. 3, *a–d*) from VR2 of both P1.16 strains. In either case, optimal reactivity was seen with the peptide TKDTNNNLTL and each of the reacting peptides had in common the sequence KDTNNN. A slight difference was seen in the reactivity of the two antibodies with small peptides, while MN5C11G

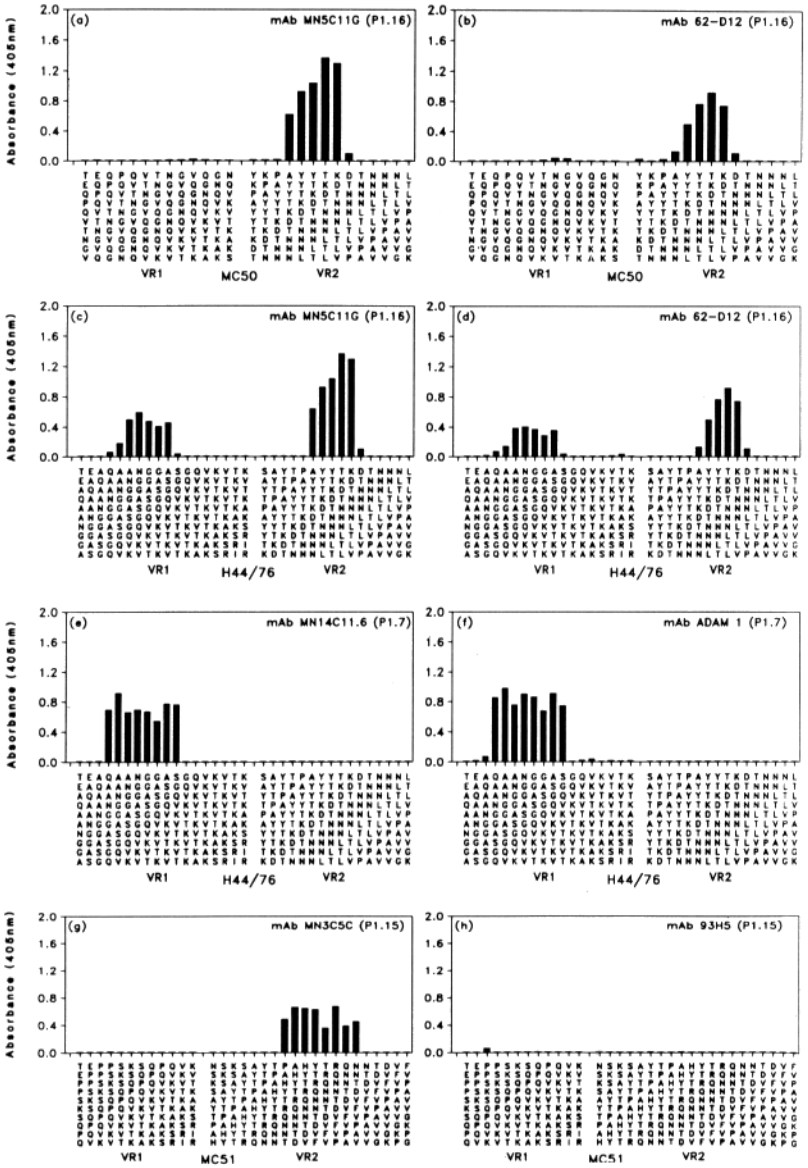

FIGURE 3. Reaction of the mAbs with series of overlapping decapeptides corresponding to variable regions VR1 and VR2, with adjacent peptides differing by a single amino acid residue. Annotations show the strain from which the sequence was derived, the mAb used, and its subtype specificity.

showed significant reactivity with the hexapeptide KDTNNN this was considerably less with 62-D12 (Fig. 4, *a* and *b*). Thus, at the level of resolution possible, it appears that the two P1.16-specific mAbs recognize almost but not quite identical epitopes in VR2 of the P1.16 strains.

The additional weak reactivity of both P1.16-specific mAbs in VR1 of strain H44/76

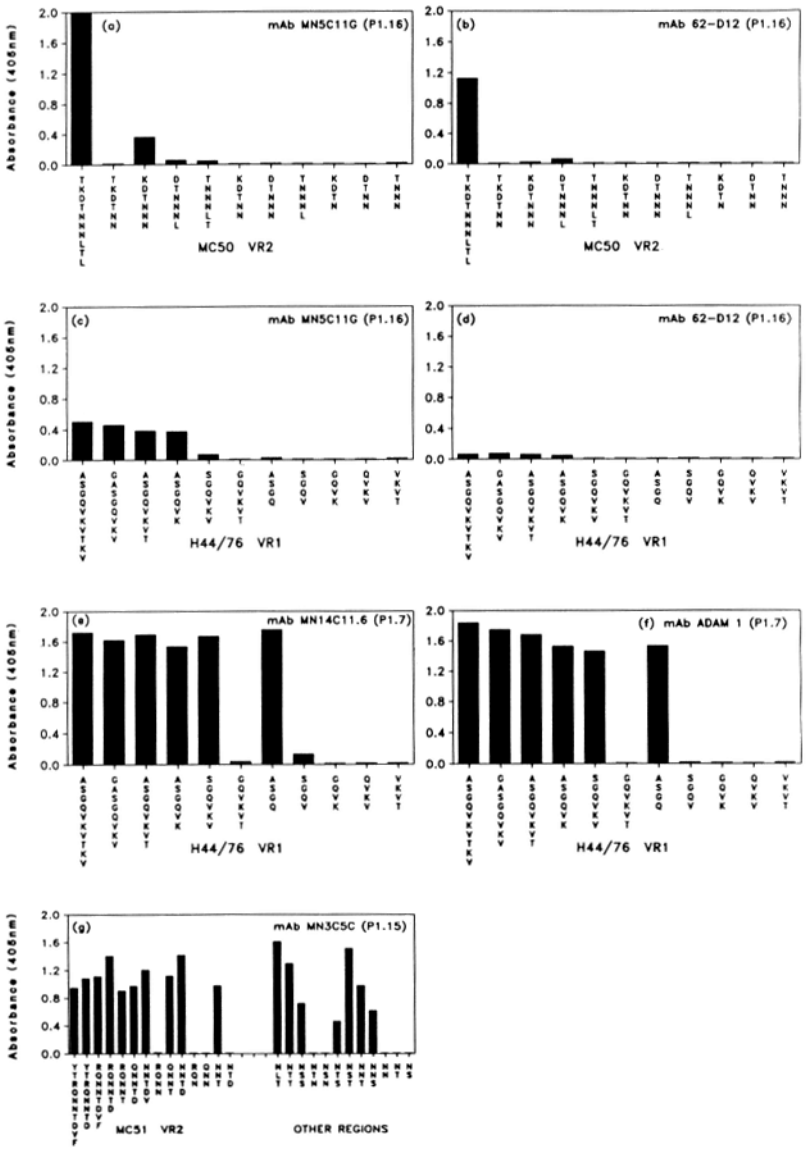

FIGURE 4. Determination of the minimum linear epitopes recognized by mAbs. Each antibody was reacted with a series of oligopeptides contained within the decapeptides found to be recognized in figure 3. Annotations show the relevant variable region, the strain from which the sequence was derived, the mAb used, and its subtype specificity.

was localized to five adjacent overlapping decapeptides containing the common sequence ASGQVK (Fig. 3, *c* and *d*). Although in each case MN5C11G reacted more strongly than 62-D12, the pattern of reactivity with small peptides was identical. Both reacted with the two octapeptides, they reacted strongly with only one of the hexapeptides (ASGQVK) and neither recognized any of the tetrapeptides made of this VR1 (Fig. 4, *c* and *d*).

(b) Subtype P1.7. The two P1.7-specific mAbs both reacted with eight adjacent overlapping decapeptides in VR1 of strain H44/76, containing the common sequence SGQ (Fig. 3, *e* and *f*). Both also reacted strongly with the two octapeptides synthesized and with two of the hexapeptides (ASGQVK and SGQVKV), but not with the third (GQVKVT), and with only one of the tetrapeptides (ASGQ) (Fig. 4, *e* and *f*). Thus, MN14C11.6 and ADAM 1 have very similar specificities for the linear peptides from VR1 of the P1.7 strain, with the sequence SGQ, corresponding to residues 32–34, being necessary although not entirely sufficient for reactivity.

(c) Subtype P1.15. As with the preliminary scanning experiments (Fig. 2 *h*) no reactivity of mAb 93H5 could be seen with any of the overlapping decapeptides corresponding to VR1 or VR2 of strain MC51, suggesting that the epitope recognized is not a simple linear peptide but rather involves a particular conformation of the protein over a more extended region. Antibody MN3C5C reacted with eight adjacent overlapping decapeptides in VR2 of MC51 with the common sequence NNT (Fig. 3 *g*). In addition the antibody reacted with a variety of smaller peptides ranging from octamers to trimers all of which contained NNT (fine tuning of this epitope showed that MN3C5C reacted with all peptides ranging in size from decapeptides down to tripeptides which contained NNT [Fig. 4 *g*]). When the additional reactivity of MN3C5C with peptides in the conserved regions was examined in more detail it was found that MN3C5C reacted most strongly with two groups of five adjacent overlapping decapeptides that contained the sequences NST and NTS, respectively. Subsequent reaction with a number of di- and tripeptides revealed that the antibody recognized tripeptides of the form NXT or NXS (where X = N, T, S, or L), suggesting that the additional reactivity of MN3C5C was due to a relatively low specificity and the occurrence of these sequences elsewhere in the molecule.

Discussion

Comparison of the predicted amino acid sequence of the three class 1 protein sequences now available reveals considerable structural homology with major variation confined to only two discrete regions (VR1 and VR2). Strain MC51 differs from the previously published sequence of strain MC50 in only 25 of 374 residues, 13 of these differences occurring in either VR1 or VR2. While the differences between the variable regions involve insertions, deletions, or substitutions of unlike amino acids, the differences seen in the conserved regions generally involve only conservative substitutions of similar amino acids. The high degree of homology between the three sequences results in predicted secondary structures that are almost identical, indicating a highly conserved functional role for the protein in outer membranes of meningococci. The previously noted similarity of class 1 protein from strain MC50 with gonococcal PI (11) indicates that this role is to act as a hydrophilic diffusion pore. This function has been confirmed by recent studies that have demonstrated

that class 1 protein inserted into artificial membranes acts as a slightly cation selective porin (Tommasson, J., and J. T. Poolman, unpublished observations). Like strain MC50 (11), strains MC51 and H44/76 also show alternating domains of hydrophilic and hydrophobic amino acids. Similar patterns are seen with *Escherichia coli* outer membrane porin proteins, and combined with x-ray diffraction data and antigenic analysis this has led to models for porin proteins in which a series of hydrophilic surface exposed regions are separated by membrane spanning β-sheets (24, 25).

The location of antigenic subtype specificity on the class 1 proteins focuses particular attention on differences between the strains, since these must clearly form the basis of antigenic diversity. The existence of only two major variable regions is in accord with the previous studies with mAbs that suggested that the class 1 proteins contain two independent subtype-specific epitopes (15). Comparison of the sequences suggests that these must correspond to VR1 and VR2. This is also in accord with the positions of the two variable regions within the molecule. Examination of the hydropathy profile of the MC50 class 1 protein (11) reveals that both are located in hydrophilic domains that are likely to be surface exposed and hence accessible to antibody binding.

The localization of linear epitopes recognized by mAbs has been greatly facilitated by the development of techniques for rapid, concurrent synthesis of large numbers of peptides for direct testing by ELISA (21, 22). Using this technique has permitted, with the exception of mAb 93H5, precise definition of short peptides that constitute the epitopes recognized by the subtype-specific mAbs. The results confirm the independent influence of sequence variations in VR1 and VR2 in generating two subtype specificities within each class 1 protein molecule. Thus, the P1.7 epitope is associated with a specific sequence in VR1 while the P1.16 epitope lies within VR2. Hence, strain H44/76, which contains the P1.7 sequence ^{31}ASGQ^{34} in VR1 and the P1.16 sequence ^{179}KDTNNN^{184} in VR2, is subtype P1.7, 16 while strain MC50 which contains ^{181}KDTNNN^{186} in VR2 but has an entirely different sequence in VR1 is subtype P1.16. Similarly the particular sequence found in VR2 of strain MC51 is sufficient to define it as subtype P1.15. However, the sequence recognized by the P1.15 antibody MN3C5C shows low specificity, recognizing not only the sequence NNT, which occurs in VR2 of strain MC51, but also NXT or NXS (where X = N, L, S, or T), which occur in the conserved regions at positions 287–289 and 324–326. Although these latter two sequences are also present in the two other subtype strains, antibody MN3C5C is specific for subtyping P1.15 strains (16). Presumably because these regions, unlike VR2, lie in the more hydrophobic regions of the class 1 protein molecule they are not exposed on the surface and hence unavailable for antibody binding to intact cells in the usual subtyping procedure.

Although most of the above data are consistent with a model in which linear sequences in each of the variable regions independently define with two subtypes of any strain, the situation in one case at least must be more complex than this. Both P1.16 specific antibodies recognize the KDTNNN sequence present in VR2 of strains MC50 and H44/76 but they also exhibit weaker reaction with the apparently unrelated sequence ASGQVK in VR1 of H44/76. Since the latter sequence is not present in strain MC50 it is clearly not necessary for subtype specificity. Nevertheless, it suggests that the quaternary structure of the native molecule is such that while the

dominant factor in generation of the P1.16 epitope is the contribution from VR2, there is an additional influence of VR1 in generating the complex three-dimensional structure of the molecule. If this is the case it would seem likely that similar interactions may well occur with other subtype specificities.

The separate influence of VR1 and VR2 on subtype specificity implies that all meningococcal strains would exhibit two distinct subtype specificities. Thus, sequences found within the VR1 regions of strains MC50 and MC51 therefore represent additional, undefined, subtype-specific regions for which the corresponding mAbs have not yet been produced. These data suggest that similar as yet undefined subtype specificities are present on the class 1 proteins of all strains where only one specificity has so far been defined.

A particularly important implication of the current study lies in the fact that the mAbs used are bactericidal (5) and protect infant rats from challenge with heterologous strains of the same subtype (17). Indeed, when compared with antibodies directed against other surface antigens, the anti-class 1 antibodies were found to have the greatest protective effect in the infant rat model (26). Thus we have been able to define linear epitopes on the class 1 protein that are not only responsible for subtype specificity but that represent effective targets for a protective immune response. Any vaccine strategy based on the variable epitopes identified in VR1 and VR2 would limit protection to strains of the same sero-subtype and would therefore necessitate a polyvalent vaccine. However, the techniques used in the current study may ultimately lead to the identification of more conserved, but less immunogenic, epitopes on the class 1 protein that provide alternative targets for vaccination. Indeed solid-phase peptide synthesis has already been used to identify the conserved epitope recognized by a protective mAb on the related gonococcal protein I and immunization with a synthetic peptide containing the epitope was found to induce a bactericidal immune response (27). Such studies suggest that the ultimate goal of an effective vaccine against all meningococcal infection may be a realistic possibility.

Summary

The previously determined nucleotide sequence of the porA gene, encoding the class 1 outer membrane protein of meningococcal strain MC50, has been used to clone and sequence the porA gene from two further strains with differing serosubtype specificities. Comparison of the predicted amino acid sequences of the three class 1 proteins revealed considerable structural homology with major variation confined to two discrete regions (VR1 and VR2). The high degree of structural homology between the sequences gave predicted secondary structures that were almost identical, with the variable domains located in hydrophilic regions that are likely to be surface located and hence accessible to antibody binding.

The predicted amino acid sequences have been used to define the epitopes recognized by mAbs with serosubtype specificity. A series of overlapping decapeptides spanning each of the class 1 protein sequences have been synthesized on solid-phase supports and probed with mAbs. Antibodies with P1.16 and P1.15 subtype specificity reacted with sequences in the VR2 domain, while antibodies with P1.7 subtype specificity reacted with sequences in the VR1 domain. Further peptides have been constructed to define the minimum epitopes recognized by each antibody. Thus we have been able to define linear peptides on each class 1 protein molecule that are

responsible for subtype specificity and that represent targets for a protective immune response.

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