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Molecular variation of meningococcal serotype 4 antigen genes

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SUMMARY

Changes in the frequency of serogroup B non serotypable (B:NT) meningococci isolated in England and Wales were investigated by T-track fingerprint analysis, DNA nucleotide sequence determination, and serotyping by whole cell ELISA and dot blot assay. Seventy-three per cent of the isolates designated as B:NT by the Meningococcal Reference Unit (MRU) dot blot assay during 1993–4, expressed variants of the serotyping antigen, PorB, that were serotype 4 by whole cell ELISA. T-track fingerprint patterns of these and other 'serotype 4' isolates revealed five distinct *porB* alleles which were shown by nucleotide sequence determination to encode different peptide sequences. Differential binding of the 'serotype 4' mAbs MN14G21 and 5DC4C8G8 in whole cell ELISA and dot blot assays was the result, (i) of differences in the peptide sequence of predicted surface loop I and (ii) an amino acid deletion in predicted loop VI of the PorB protein.

INTRODUCTION

Neisseria meningitidis is a major cause of bacterial meningitis and severe septicaemia world wide [1]. For routine epidemiology, meningococcal isolates are characterized using antibodies against surface antigens into serogroups (capsular polysaccharide), serotypes (PorB protein), and serosubtypes (PorA protein) [2]. While most disease in western Europe and North America is caused by serogroup B and C meningococci, changes in the prevalent serotypes and serosubtypes of disease-causing isolates within these serogroups, particularly within serogroup B, have been observed in a number of European countries over the past 25 years [3–5].

In England and Wales, serogroup B case isolates submitted to the Meningococcal Reference Unit (MRU) throughout the 1970s were predominantly serotype 2a (B:2a) (6). The number of B:2a isolates received by MRU declined in the 1980s with the emergence of serogroup B, serotype 15 strains (B:15). The B:15 strains were themselves superseded in the late 1980s and early 1990s by serogroup B, non-serotypable (B:NT) isolates. Antigenic change was also observed in the Netherlands (5) in the same time period, and when such change results in an increase in the proportion of NT isolates, the epidemiological information available from serotyping is compromised. In 1989–94, the percentage of B:NT strains submitted to MRU rose from 25% to over 60%.

As serological reagents are frequently developed against the antigenic types prevalent in a particular country at a particular time, the development of new reagents is often necessary. In the Netherlands, the

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inclusion of the serotype 4 monoclonal antibody (mAb) MN14G21 [7] in the serotyping reagent panel decreased the number of B:NT isolates. In England and Wales, however, the number of B:NT isolates continued to increase despite use of this mAb in the MRU dot blot serotyping assay. The addition of a second serotype 4 mAb (5DC4C8G8) in September 1995 to the MRU serotyping panel, did result in an increase in serotype 4 identification and a decline in NT isolates, although a proportion of isolates remained NT.

As a number of serotype 4 mAbs with differences in their specificities have been described [8, 9], the present study used gene-fingerprinting techniques and nucleotide sequence determination to characterize the *porB* genes of B:4 and B:NT isolates from England and Wales during 1993–5. The aim of this work was to establish whether the increased proportion of NT strains submitted to the MRU were the result of minor variation in existing genes, emergence of new serotypes, or inconsistency in the performance of serotyping assays. In addition, the reaction patterns of the two serotype 4 mAbs used at the MRU were compared in both whole cell ELISA and dot blot assay, and correlated with deduced protein sequences.

MATERIALS AND METHODS

Propagation of meningococcal strains and DNA preparation

The meningococci used in this study were chosen from case isolates submitted to the MRU in the period 1993–5. Isolates were propagated on heated blood agar plates in an atmosphere of 5% CO_2 for 8–16 h. Approximately 10^7–10^9 colony forming units were used to prepare DNA with an 'Isoquick Nucleic Acid Extraction Kit' (Orca Research Inc.), according to the manufacturer's protocol.

Amplification of *porB* by PCR and template preparation

For amplification of meningococcal *porB* genes from extracted DNA samples, reaction components were as follows: reaction buffer (10 mM Tris-HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin); 200 μM each of dATP, dCTP, dGTP, dTTP; 1 μM of PCR primers 27 and 28 [10]; 0·5 units of *Taq* polymerase (Amplitaq; Perkin–Elmer Corp.); 1 μl template DNA (approximately 50 ng/μl). Reaction conditions were 30 cycles of 94 °C for 2 min, 60 °C for 2 min and 72 °C for 3 min, followed by incubation at 72 °C for a further 3 min. The PCR products were precipitated by incubation at 37 °C for 15 min with 20% PEG_{8000}, 2·5 M NaCl. After centrifugation for 10 min at 12000 ***g***, the precipitates were washed in 70% ethanol, dried and resuspended in 10–20 μl of sterile distilled water [11].

T-Track fingerprint analysis and direct nucleotide sequence determination of the *porB* gene

Isotopic end-labelling of sequencing primers was carried out at 37 °C using [γ-^{32}P]dATP and T4 polynucleotide kinase. The primers used for T-track fingerprinting and nucleotide sequence determination were 27, 28, 8U and 8L, described previously [10], and primer 260 (5′-AGT GAG TTT GGA GAA GTC GT-3′). Sequencing reactions were carried out with the ΔTaq Cycle Sequencing Kit (United States Biochemical), used in accordance with the manufacturer's instructions. The T-Track fingerprint of the *porB* amplicons were obtained by termination with dideoxythymidine mix only. All nucleotide sequences and T-Track fingerprint patterns were determined at least once on each DNA strand and resolved on 4–6% w/v polyacrylamide gels. Dried gels were exposed to Hyperfilm β-Max (Amersham) for 12–48 h. Sequence data are identified by the appropriate EMBL databank accession number.

Monoclonal antibodies (mAbs)

The serotype 4 mAbs used in this study were MN14G21 (7) and 5DC4C8G8 (from Dr W. D. Zollinger, Walter Reed Army Institute of Research, Washington, DC, USA). Ascitic fluid containing these mAbs, and the serotype 2a mAb MN2D3F [12] which was used as a negative control, were produced at the National Institute for Biological Standards and Control (NIBSC).

Meningococcal serotyping using dot blot assay

Samples (2 μl) of pasteurized meningococcal suspensions were applied to nitrocellulose membranes (Bio-rad), which were air dried and cut into strips. The strips were blocked in skimmed milk, 4% (w/v) in phosphate buffered saline (blocking buffer) and incubated for 30 min at room temperature with an

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appropriate dilution of ascitic fluid. After washing with saline containing Tween 20, 0·05% (v/v) the membranes were incubated for 30 min at room temperature with protein A peroxidase conjugate diluted 1:1000 in Dulbecco/0·01% Tween 20. After the final saline wash the membranes were incubated for 5 min in freshly made 4-chloro-1-naphthol substrate (0·05 mg/ml in 17% ethanol (v/v); 0·05% H_2O_2 (v/v)) [13].

Meningococcal serotyping using a whole cell ELISA

The method used was adapted from the published method [12] by using anti-mouse IgG (whole molecule) peroxidase conjugate and *O*-phenylenediamine dihydrochloride substrate (Sigma Chemical Corp.). Absorbances were read at 492 nm.

RESULTS

T-track fingerprint analysis of *porB* genes in B:NT isolates

A total of 93 meningococcal B:NT isolates were chosen from those submitted to the MRU in the period January 1993 to September 1994. These represented approximately every 11th such isolate received. The *porB* gene T-track fingerprint patterns were determined for each of these isolates and compared to the *porB* T-track patterns obtained from meningococci of known serotypes [10] and to the deduced T-track patterns of published *porB* sequences in the EMBL nucleotide sequence database. A total of 13 T-track patterns were obtained and arbitrarily named T1–T13 (Table 1). The most common pattern, T1 (68/93; 73%), corresponded to the deduced T-track pattern of the *porB* nucleotide sequence U07193, from serotype 4 strain BB1350 (14). T-track patterns T3 (1 isolate), T2 (8 isolates), and T4 (3 isolates) corresponded to the deduced T-track patterns of sequences X65530 (strain M1080, serotype 1) [10], U07190 (strain 6557, serotype 17), and U07189 (strain M978, serotype 8) [14]. T-track patterns T5–T13, which accounted for 13 B:NT isolates, did not correspond to known *porB* sequences (Table 1).

Table 1. *The* porB *T-track patterns obtained from 93 B:NT meningococcal isolates and their inferred serotypes. The T-track patterns obtained were compared with those of 12 serotype reference strains and with other published* porB *sequences from isolates of known serotype. T5–T13 were novel T-track patterns that did not correspond to any available porB sequences*

T-track pattern	No. of isolates	EMBL database accession no. of corresponding *porB* sequence	Inferred serotype
T1	68	U07193	4
T2	8	U07190	17
T3	1	X65530	1
T4	3	U07189	8
T5	3	Novel T-track pattern	NT
T6	1	Novel T-track pattern	NT
T7	1	Novel T-track pattern	NT
T8	3	Novel T-track pattern	NT
T9	1	Novel T-track pattern	NT
T10	1	Novel T-track pattern	NT
T11	1	Novel T-track pattern	NT
T12	1	Novel T-track pattern	NT
T13	1	Novel T-track pattern	NT

T-track fingerprint analysis of *porB* genes in B:4 isolates

Sixteen serogroup B meningococci, isolated in England and Wales during 1995, were chosen to be representative of the different reactions obtained with the two serotype 4 mAbs used in the MRU dot blot serotype assay. Twelve isolates reacted with 5DC4-C8G8 only and four isolates with MN14G21 only. The *porB* T-track fingerprint was determined for each of these isolates. The T1 fingerprint was obtained for nine of these strains (isolates 129–137), while new T-track patterns were obtained from the remaining seven. The new T-track patterns were assigned names: T14 (isolates 138–140), T15 (isolate 141), T16 (isolate 142), and T17 (isolates 143 and 144) (Table 2).

Nucleotide sequence determination

The *porB* nucleotide sequence of two strains with the T1 T-Track fingerprint pattern were determined: isolate 85 was one of the 93 B:NT isolates; and isolate 129 represented the 1995 B:4 isolates. The *porB* genes in both of these isolates were identical to nucleotide sequence U07193. The nucleotide sequences of the *porB* genes of isolates 138–144 were also determined, giving four novel *porB* sequences, corresponding to each of the new T-track patterns. These sequences have been submitted to the EMBL database with accession numbers AJ003083, AJ003084, AJ003085, AJ003082 (Table 2). The amino acid sequences

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Table 2. *The* porB *T-track patterns,* porB *nucleotide sequences, and serological assignment* (+*positive assignment, or* −, *negative assignment*) *by dot blot assay or whole cell ELISA with two* '*serotype 4*' *monoclonal antibodies. The serological reaction patterns of the 16 meningococcal isolates are summarized as 1–3*

Isolate name	T-track pattern	*porB* sequence	Dot blot assay		Whole cell ELISA assay		Serological reaction pattern
			MN14G21	5DC4C8G8	MN14G21	5DC4C8G8	
129	T1	U07193	−	+	+	+	1
130	T1	U07193	−	+	+	+	1
131	T1	U07193	−	+	+	+	1
132	T1	U07193	−	+	+	+	1
133	T1	U07193	−	+	+	+	1
134	T1	U07193	−	+	+	+	1
135	T1	U07193	−	+	+	+	1
136	T1	U07193	−	+	+	+	1
137	T1	U07193	−	+	+	+	1
138	T14	AJ003083	−	+	+	+	1
139	T14	AJ003083	−	+	+	+	1
140	T14	AJ003083	−	+	+	+	1
141	T15	AJ003084	+	−	+	+	2
142	T16	AJ003085	+	−	+	+	2
143	T17	AJ003082	+	−	+	−	3
144	T17	AJ003082	+	−	+	−	3

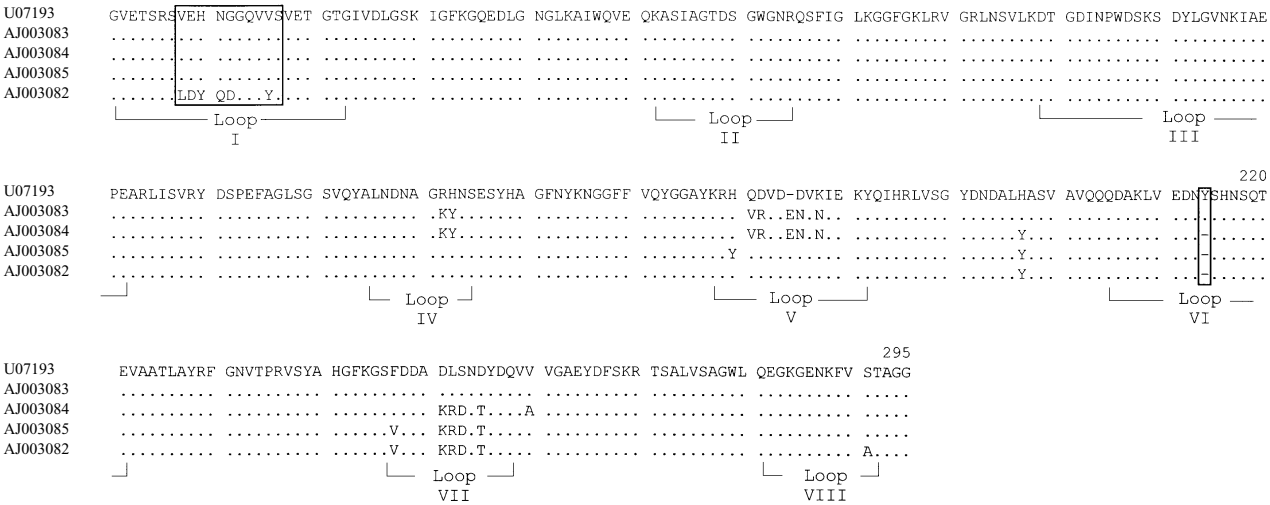

Fig. 1. Alignment of the five meningococcal PorB protein sequences obtained by translation of the nucleotide sequences of each of the *porB* alleles identified in this study. The locations of the putative surface loops (I–VIII) of the porin are indicated. Boxed areas define the regions of putative surface loops I and VI that are likely to be important for recognition of the PorB proteins by serotype 4 mAbs.

deduced from these new nucleotide sequences, aligned with the deduced amino acid sequence of U07193, are shown in Figure 1.

Comparison of serotyping results obtained with whole cell ELISA and dot blot

The 16 B:4 isolates were re-serotyped using mAbs MN14G21 and 5DC4C8G8 by whole cell ELISA and dot-blot assay. The 12 isolates that were positive with 5DC4C8G8 and negative with MN14G21 in the dot blot assay (isolates 129–140) gave a positive result with both mAbs in the whole cell ELISA (reaction pattern 1). Isolates 141 and 142, which gave a positive dot blot result only with MN14G21 were also positive with MN14G21 and 5DC4C8G8 in the whole cell ELISA (reaction pattern 2). Isolates 143 and 144 gave positive results only with MN14G21 in both the whole

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cell ELISA and the dot blot assay (reaction pattern 3) (Table 2).

Seven of the 1993/4 B:NT isolates with T1 T-track patterns were tested in the serotype dot blot assay and whole cell ELISA using both MN14G21 and 5DC4-C8G8 mAbs. These isolates gave reaction pattern 1, positive reactions with MN14G21 and 5DC4C8G8 in whole cell ELISA and with 5DC4C8G8 in the dot blot assay and negative reactions with MN14G21 in the dot blot assay.

DISCUSSION

This study establishes that 73% of B:NT isolates submitted to the MRU in the period 1993–4 contained *porB* genes that encoded proteins which could be categorized in the current scheme as serotype 4. The inconsistency of assignment of isolates to serotype 4 is explained by the fact that there are at least five PorB protein variants which can give a positive reaction with one or other or both serotype 4 mAbs, depending on the assay used. In this respect the rise of NT isolates during this period can be regarded as reagent and assay failure. These data further highlight the problems of using serotype, based on mAbs binding to PorB, for characterizing meningococcal isolates [15]. While meningococcal serotyping remains a commonly used epidemiological tool for the meningococcus, it is important that these shortcomings are appreciated, particularly when efforts to compare and compile the results obtained in different laboratories using diverse serotyping reagents and methodologies are made.

Although the whole cell ELISA using mAb MN14G21 could be regarded as the most 'sensitive' assay, in that it assigned each of the five PorB variants discussed here to serotype 4, this property can also be regarded as a lack of discrimination. The ability of this antibody to react with the PorB proteins encoded by five distinct alleles in whole cell ELISA assays explains why fewer B:NT isolates were seen in the Netherlands [16] compared with England and Wales, as the Dutch reference laboratory used an ELISA assay, while the MRU used a dot blot assay. However, as serotypes were originally developed to distinguish meningococcal isolates, the categorization of isolates with differences in their PorB proteins as belonging to the same serotype is questionable, particularly in outbreak management. While the dot blot technique was rather better in distinguishing differences among the related PorB proteins, this assay did not distinguish between the PorB proteins encoded by alleles U07193 and AJ003083 or between those encoded by alleles AJ003084, AJ003085 and AJ003082.

The molecular basis of meningococcal serotyping is the interaction of mouse mAbs with regions of the PorB porin protein [2], which are thought to form surface-exposed loops of protein structure [17]. In this respect, there is some similarity with the serosubtyping scheme for meningococci, where mAbs react with linear epitopes present in the variable surface-exposed loops I and IV (designated VR1 and VR2 respectively) of the proposed secondary structure of the serosubtyping antigen, PorA [18, 19]. These linear epitopes made the redefinition of subtypes by peptide sequence, rather than antibody reactivity, straightforward once large numbers of nucleotide sequences of *porA* genes became available [13, 20]. In the case of PorB, however, mAbs frequently recognize discontinuous epitopes comprising sequences from several surface loops [21], making such a transition difficult and probably inappropriate. A further complication is that there are two mutually exclusive PorB protein families found in meningococci, class 2 and class 3 outer membrane proteins [22]: the 'serotype 4' PorB proteins are class 3 OMPs.

By comparison of the deduced peptide sequences in Figure 1, and reference to Table 2 and previously published data, it is possible to identify the putative loop sequences most likely to be involved in the interaction of mAbs MN14G21 and 5DC4C8G8 with PorB proteins. Nucleotide sequence studies of various serotype reference strains have shown that putative loops II, III and VIII are largely invariant in class 3 PorB proteins and are unlikely to be important in the specific reactions of serotyping mAbs. The peptide sequences of PorB proteins that react with both serotype 4 and serotype 21 mAbs are known to differ from serotype 4 PorBs in loop VII only [10], so it is also unlikely that this loop is involved in the specific interactions of the serotype 4 mAbs. Figure 1 shows no consistent pattern of sequence variation in putative loops IV and V with serological reaction pattern (Table 2), indicating that these putative loops are also probably not important in determining the specific reactions with these antibodies. Of the remaining loops, loop I is conserved in all five PorBs except that encoded by nucleotide sequence AJ003082, and as this is the only strain that fails to react with mAb DC4C8G8 in whole cell ELISA assays, it is probable that this loop is important in recognition of PorB proteins by this antibody: the binding of mAb

MN14G21 is unaffected by this change. The remaining putative loop, VI, appears to be important in determining the behaviour of these two antibodies in the two assays. The presence of a deletion in this loop, relative to sequence encoded by U07193, promotes the interaction of MN14G21 in the dot blot assay but has the opposite effect in the case of mAb 5DC4C8G8.

Faced with the high degree of variation in meningococcal serotypes, together with assay variability, it is not possible to envisage a comprehensive mAb-based serotyping system. Indeed, the validity of serotypes as a means of detecting related bacteria and identifying trends in meningococcal epidemiology is questionable on the basis of these data. The issue is further complicated by the population biology of the meningococcus. Meningococci frequently exchange DNA [23, 24] and this means that *porB* genes, or parts of them, can be shared among genetically unrelated bacteria [25]. Figure 1 shows some evidence that the *porB* genes present in the isolates included in this study are mosaic genes, similar to those that have been observed previously in other *porB* genes [10, 14], various other antigen genes [13, 26–29], and genes encoding antibiotic resistance determinants [30].

Genetic analysis, using techniques such as multilocus enzyme electrophoresis [31], which can reliably identify the genetic relationships among bacterial isolates [32–34] have yet to be routinely applied to bacterial typing. It is to be hoped that the recent advances in DNA technology will permit the development of techniques for the reliable routine genetic characterisation of bacterial isolates in the near future. Where such genetic studies have been done, it is clear that the spread of novel antigenic types of meningococci is often linked to the spread of new clones [35]. For example, the rise in prevalence of the serotype 4 *porB* alleles seen in the Netherlands and the UK was due to the spread of a particular clonal lineage of meningococci, lineage 3 [36]. However, the frequent horizontal genetic exchange seen in the meningococcus means that while changes in frequencies of serotypes observed nationally may reflect changes in the meningococcal clones circulating, serological characteristics themselves do not reliably identify individual members of such clones. This is accentuated by the fact that serological characteristics are defined by very small parts of the genome which are potentially under strong immune selection.

In conclusion, the rise in NT isolates observed by the MRU was the consequence of changes in the *porB* alleles prevalent in the population of invasive meningococci circulating in England and Wales, combined with the particular assay reagents and techniques used. However, these problems are inherent in the serotyping system for meningococci which relies on antibody reactions which are poorly defined. This work shows that while serotype 4 may be defined as the presence of a particular peptide sequence in loop VI of the proposed protein structure, PorB proteins containing this sequence may be highly diverse at the other variable parts of the PorB protein. Thus the character serotype 4 is a poor indicator that two isolates are related.

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