Molecular Epidemiology

Investigation for a more virulent variant among the C:2b:P1.2,5 Spanish meningococcal epidemic strains by molecular epidemiology

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A rise in the incidence of meningococcal disease has occurred in Spain in recent years, especially in some regions in the north-west of the country. Most cases have been caused by meningococci characterised as Neisseria meningitidis C:2b:P1.2,5. A total of 107 C:2b:P1.2,5 meningococcal isolates (60 from patients and 47 from carriers) and 12 isolates showing related antigenic combinations (C:2b:NST, C:2b:P1.2, C:2b:P1.5, C:NT:P1.2,5) was analysed by pulsed-field gel electrophoresis to determine the genetic variability of the epidemic and related strains. Endonucleases BglII and NheI were used to cut chromosomal DNA. When BglII was used, most of the C:2b:P1.2,5 isolates showed the same pulstype regardless of whether they were from clinical cases or carriers. Isolates showing the principal profile after digestion with endonuclease BglII were analysed with NheI. Four pulsotypes were identified, of which two were found in only one isolate each. The major profiles (1 and 2) showed differential distribution among clinical and carrier isolates; pulstype 1 was the most frequent among clinical isolates. However, the proportions of isolates showing profiles 1 and 2 were similar among carrier isolates. This could indicate that there are two variants of the C:2b:P1.2,5 strain with differing pathogenicity.

Introduction

Neisseria meningitidis is one of the main causes of bacterial meningitis world-wide. Asymptomatic nasopharyngeal colonisation in man provides the only known natural reservoir of meningococci. Occasionally, the organism penetrates the mucosal surface and invades the blood, leading to disseminated disease or localisation in the meninges or other body sites. Environmental and host factors, as well as virulence properties of certain strains, are assumed to contribute to bacterial invasion [1]. Typically, the disease progresses rapidly once the mucosal barrier is breached, and death may ensue within hours.

As the human nasopharynx is the only known reservoir of N. meningitidis and most patients with meningococcal disease have not had contact with another person with the disease, asymptomatic carriers are presumed to be the major source of transmission of pathogenic strains. For this reason investigation of the carrier state may contribute significantly to the understanding of the epidemiology and pathogenesis of disease caused by N. meningitidis.

The characterisation scheme for the meningococcus relies on the serological reactivity of cell-surface components. The primary level of characterisation is the serogroup, which is based on the antigenic properties of the capsular polysaccharide. Further immunological characterisation of meningococcal isolates is based on subcapsular antigens. Serotype specificity resides in the outer-membrane porin proteins, which are either class 2 or 3 proteins; subtype specificity is based on antigenic differences in the class 1 outer-membrane protein (OMP).

Classical typing schemes for meningococci, although contributing to the understanding of the epidemiology of meningococcal disease, suffer from a lack of sensitivity in distinguishing between strains [2]. The differentiation between strains of the same species has become a vital part of epidemiological investigations.

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In species with high levels of recombination, such as Neisseria spp., it is important to be careful about using traditional markers such as serogrouping and serosubtyping when trying to determine the relationships among different isolates. Characterisation of the chromosomes of isolates by genotypic methods has demonstrated that identity in surface properties does not necessarily indicate genotypic similarity [2]. Therefore, genotypic techniques such as multilocus sequence typing (MLST) [3] or pulsed-field gel electrophoresis (PFGE) are valuable tools to examine the genetic relatedness of meningococcal isolates.

Meningococcal disease can occur as endemic, hyperendemic or epidemic waves and large-scale epidemics/pandemics [4]. In Europe and the USA, endemic disease is prevalent with occasional hyper-endemic outbreaks or localised disease outbreaks, and most meningococcal cases are usually caused by meningococci of serogroups B and C [4]. In the early 1990s, an increasing number of serogroup C meningococcal strains was observed in Spain, and a rise in the incidence of the disease was found in some regions of the country such as Galicia (a region in north-west Spain with 2.7 million inhabitants). In this region, the incidence in 1996 was three times higher than in the previous year (11.26/10^5 inhabitants versus 3.65/10^5), with serogroup C accounting for c. 80% of isolates, almost all of which were of phenotype C:2b:P1.2,5 [5]. For this reason the local health authorities in Galicia decided to apply a systematic immunisation campaign at the end of 1996. At the same time as the immunisation campaign was implemented, a survey of meningococcal carriers was made in that region to determine the prevalence of the C:2b:P1.2,5 epidemic strain in an asymptomatic population. A year later, a second survey of asymptomatic meningococcal carriers was done with the aim of determining whether the

Fig. 1. Banding patterns (pulsotypes) of genomic DNA from N. meningitidis C:2b:P1.2,5 and related phenotypes (C:2b-NST, C:2b-P1.2, C:2b-P1.5 and C:NT-P1.2,5) obtained with endonuclease BglII (lanes 1–16). Bacteriophage λ-concatemer (lane M) was used as molecular size standard (48.5 kb). Lane 1, pulsotype A1; 2, A5; 3, A6; 4, A16; 5, A8; 6, D; 7, F; 8, A8; 9, A5; 10, A1; 11, E; 12, A1; 13, A5; 14, B; 15, C; 16, G.
polysaccharide C vaccine could alter the meningococcal carrier state.

C:2b:P1.2,5 isolates from patients and carriers, and isolates with related phenotypes (C:2b:NST, C:2b:P1.2, C:2b:P1.5 and C:NT:P1.2,5), were analysed by PFGE with the aim of establishing the genetic relatedness of these isolates.

Materials and methods

Meningococcal strains

Carrier strains. The Dirección Xeral de Saúde Pública (Xunta de Galicia) and the Spanish Reference Laboratory for Meningococcus implemented two meningococcal carriage surveys between 1996 and 1998. In the first survey, the epidemic strain C:2b:P1.2,5 was present in 41 asymptomatic carriers; five strains were characterised as C:2b:NST, one as C:2b:P1.5 and the C:NT:P1.2,5 phenotype was found in one isolate. The second meningococcal carriage study found six C:2b:P1.2,5, one C:2b:NST, two C:2b:P1.2 and two C:NT:P1.2,5 meningococcal isolates.

Clinical strains. From Jan. 1996 to July 1998, this laboratory received 120 meningococcal isolates from Galicia. The isolates were from blood or cerebrospinal fluid, or both, of patients with systemic disease and were sent for characterisation. All isolates were confirmed as N. meningitidis and sero/subtyped [6]; 60 isolates were typed as C:2b:P1.2,5.

Culture, DNA preparation and PFGE assay

Isolates from patients and carriers characterised as C:2b:P1.2,5 and analogous phenotypes (C:2b:NST, C:2b:P1.2, C:2b:P1.5, and C:NT:P1.2,5) were analysed by PFGE.

Isolates were cultured on blood agar for 18–22 h and bacterial growth was scraped into 3 ml of sodium-EDTA buffer (75 mM, NaCl; 25 mM, EDTA; pH 7.4). Suspensions were centrifuged and the pellets were resuspended in sodium-EDTA buffer. The cell suspension was mixed with an equal volume of melted Sea-Plaque agarose (1% w/v in 10 mM Tris-HCl, pH 7.4, 10 mM Cl\(_2\)Mg, 0.1 M EDTA) at 42°C. The mixture was dispensed directly into plastic moulds at 4°C and allowed to solidify. Blocks were treated with Tris-borate-EDTA buffer supplemented with proteinase K 1 mg/ml and Sarkosyl 1% at 56°C for 48 h. They were washed six times for 30 min at room temperature with Tris-EDTA buffer (pH 8) and stored in this buffer at 4°C.

The plugs containing chromosomal DNA were equilibrated in 125 μl of appropriate restriction buffer for 30 min at 4°C, transferred to 125 μl of restriction buffer containing restriction endonuclease and then incubated overnight at the appropriate temperature. Two restriction endonucleases were used – BglII (15 units) and NheI (30 units). The digested DNA plugs were equilibrated in gel-running buffer for 30 min and then loaded in wells of agarose 1% gel prepared in Tris-borate-EDTA (pH 8.2) and sealed with agarose 1% at 42°C. PFGE was performed with a contour-clamped homogeneous electric field apparatus (Chef DR II; BioRad) with initial–final time ranging from 0.1 to 25 s at 200 V for 22 h. Gels were stained with ethidium bromide and photographed under UV light.

The DNA fragment patterns generated by PFGE were analysed by the interpretative criteria proposed by Tenover et al. [7]. Patterns with up to six band differences were considered to be subtypes of a PFGE major pattern. The dendrogram from the different profiles was generated by unweighted pair-group mean average (UPGMA).

Results

After digestion of the genomic DNA with BglII, 16 different patterns were found among 119 isolates analysed (Fig. 1). In all, 96 C:2b:P1.2,5 isolates exhibited a PFGE pattern designated A\(_1\), 42 were carrier isolates and 54 were clinical isolates (Table 1). Eleven C:2b:P1.2,5 isolates produced profiles other than A\(_1\), although nine of these isolates showed a pulsortype related to profile A\(_1\). Two isolates showed profile A\(_2\); profiles A\(_2\), A\(_3\), A\(_4\), A\(_5\), A\(_6\), A\(_8\), A\(_9\), A\(_10\) were found in only one isolate each (Table 1). Two isolates from asymptomatic carriers produced an unrelated pulsortype (pulsortypes D and F) (more than six different bands).

Table 1. Distribution by pulsortype after DNA chromosomal digestion with BglII endonuclease and PFGE; the phenotype and source of isolation of the strains belonging to the different profiles are indicated

<table>
<thead>
<tr>
<th>Pulsortype</th>
<th>Phenotype</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>clinical</td>
<td>carrier</td>
</tr>
<tr>
<td>A(_1)</td>
<td>C:2b:P1.2,5</td>
<td>54</td>
</tr>
<tr>
<td>A(_2)</td>
<td>C:2b:NST</td>
<td>0</td>
</tr>
<tr>
<td>A(_3)</td>
<td>C:2b:P1.2</td>
<td>2</td>
</tr>
<tr>
<td>A(_4)</td>
<td>C:2b:P1.2,5</td>
<td>1</td>
</tr>
<tr>
<td>A(_5)</td>
<td>C:2b:P1.2</td>
<td>0</td>
</tr>
<tr>
<td>A(_6)</td>
<td>C:2b:P1.2,5</td>
<td>0</td>
</tr>
<tr>
<td>A(_7)</td>
<td>C:2b:NST</td>
<td>0</td>
</tr>
<tr>
<td>A(_8)</td>
<td>C:2b:P1.2</td>
<td>0</td>
</tr>
<tr>
<td>A(_9)</td>
<td>C:2b:P1.2,5</td>
<td>0</td>
</tr>
<tr>
<td>A(_10)</td>
<td>C:2b:P1.2</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>C:NT:P1.25</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C:NT:P1.25</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>C:2b:P1.2</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>C:2b:NST</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>C:2b:P1.2</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>C:NT:P1.25</td>
<td>0</td>
</tr>
</tbody>
</table>

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Of the six C:2b:NST isolates analysed, one showed the profile $A_1$, three were defined as pulsotype $A_2$, one as profile $A_3$ and one as profile $A_4$. The two C:2b:P1.2 isolates studied were defined as profile $A_5$ and the only C:2b:P1.5 isolate analysed was defined as pulsotype $A_6$. The three C:NT:P1.2,5 isolates produced three different profiles – pulsotypes B, C and G. Relationships among the different profiles are shown in Fig. 2.

The genomic DNA of 98 strains that produced profile $A_1$ (95 C:2b:P1.2,5, one C:2b:NST and two C:2b:P1.2) with $Bgl$II were analysed by PFGE after digestion with $Nhe$I. Four PFGE types were found, although each was related (less than six different fragments) (Fig. 3). Sixty-two strains produced pulsotype 1 and 34 isolates were characterised as pulsotype 2. The situation differed in clinical and carrier strains. Most

![Dendrogram showing genetic relationships among 119 N. meningitidis strains characterised as C:2b:P1.2,5, C:2b:NST, C:2b:P1.2, C:2b:P1.5 and C:NT:P1.2,5. The number of isolates by pattern is indicated (n).]
(40 of 53) of the clinical isolates were of pulstype 1 (Table 2); in contrast less than half (22 of 45) of the isolates from carriers belonged to this pulstype (p < 0.05).

**Table 2.** Distribution by pulstype of pattern A₃ strains after DNA chromosomal digestion with endonuclease NheI and PFGE

<table>
<thead>
<tr>
<th>Pulstype</th>
<th>clinical</th>
<th>carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

**Discussion**

In the early 1990s, phenotype C:2b:NST represented 55% of all meningococcal serogroup C isolates analysed in the Spanish Reference Laboratory for Meningococci. The prevalence of this phenotype declined to 10.3% in 1996, whilst the prevalence of C:2b:P1.2,5 strains increased from 4.8% to 65% [8]. Berrón et al. suggested that the C:2b:P1.2,5 phenotype could be a genetic variant of the C:2b:NST epidemic strain previously present in Spain. The increase in prevalence of C:2b:P1.2,5 mirrors that of serogroup C strains [9].

Genotypic methods such as PFGE can be used to
discriminate between different meningococcal isolates and determine their genetic relatedness. The present study applied this technique to analysis of disease and carrier isolates that showed related antigenicity (C:2b:P1.2, 5, C:2b:NST, C:2b:P1.2, C:2b:P1.5 and C:NT:P1.2, 5).

Eleven pulsotypes were found among C:2b:P1.2, 5 strains. Nine of these pulsotypes were closely or possibly related; however, two profiles (D, F), obtained from two asymptomatic carrier isolates, showed large separation from the major pattern (A1) (Fig. 2) and for this reason were considered unrelated. On the basis of serological methods these isolates would be defined as members of the epidemic strain; however, molecular characterisation showed that they were very different from the epidemic strain. It is possible that the C:2b:NST isolate belonging to pulsotype A1 might represent a low level of expression of the porA gene, perhaps by variation in the promoter [10], or, as has been described previously [11], integration of an insertion sequence in the porA gene might inactivate it. This can be resolved only by sequence analysis of the porA gene of that strain. The fingerprint patterns are independent of variable antigens, the genes of which may change at a faster rate than the rest of the chromosome, and changes in the expression of class 1 protein would be a possible mechanism to evade the immune defence.

It is possible to increase the discriminatory power of PFGE by the use of several separate endonucleases. By this means it is possible to digest regions of the chromosome that are adversely affected by factors such as site-specific methylation [12]. Therefore, strains that belonged to the principal pattern (A1,) were analysed by PFGE after digestion of chromosomal DNA with the restriction endonuclease NheI.

The NheI fingerprints showed a greater resolution and differentiated four pulsotypes among strains characterised as profile A1 by BglII. Pulsotype 1 was found most frequently among clinical isolates, and carrier strains were distributed between pulsotypes 1 and 2 (Table 2). This result indicates that strains characterised as pulsotype 1 by PFGE after chromosomal digestion with NheI1 might be more virulent strains.

Three band differences were found between pulsotypes 1 and 2. Perhaps these differences could be attributed to a point mutation as described by Tenover et al. [7]. If this genetic event had affected antigenic determinants, such as in class 1 protein, it would have generated a more virulent variant as described by McGuinness et al. [13].

Most infections produced by strains showing profile 2 appeared in children under 2 years of age, so pulsotype 2 strains might be a less virulent variant and might affect only more susceptible individuals.

Based on the above consideration, it is possible to conclude that current serological typing systems for the meningococcus do not necessarily reflect true genetic relationships, and only by genetic studies is it possible to define a ‘true’ epidemic strain. Perhaps the different circulation of both variants within the different regions of Spain might explain the broad differences in the incidence of and mortality from meningococcal disease among them.

Further studies will be necessary to investigate possible microbial virulence factors that might determine the different pathogenicity of these variants.

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References