MOLECULAR EPIDEMIOLOGY

The epidemic wave of meningococcal disease in Spain in 1996–1997: probably a consequence of strain displacement

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During 1996 and 1997 an epidemic wave of meningococcal disease took place in Spain. Initial studies described the antigenic expression of the epidemic strain as C:2b:P1.2,5 and proposed that it was a variant of the previously identified Spanish C:2b:non-subtypable epidemic strain. To clarify this hypothesis, 1036 C:2b:P1.2(5) and 76 C:2b:NST isolates obtained during 1992–1999 were analysed by pulsed-field gel electrophoresis. The majority of the C:2b:P1.2,5 and C:2b:P1.2 isolates showed one of two very closely related profiles. During the epidemic period, 80% of the C:2b:NST strains showed these two pulsotypes. However, before the epidemic wave, most of these C:2b:NST strains (60%) showed a profile that was found infrequently among C:2b:P1.2,5 and C:2b:P1.2 isolates. A similar evolution was observed in C:2b:P1.5 isolates. Thirty-four C:2b:P1.2(5) and 10 C:2b:NST isolates, exhibiting representative pulsotypes, were subjected to multi-locus sequence typing. Isolates belonging to both A4 and ET-37 lineages were identified. These data point to the possibility that the A4 cluster has displaced the ET-37 complex among serogroup C meningococci in Spain.

Introduction

Neisseria meningitidis is a major cause of meningitis and septicaemia [1]. Strains of serogroup A, B and C are responsible for 90% of the cases of meningococcal disease world-wide [2] and almost all the remaining disease is accounted for by organisms of serogroups Y and W-135. Serogroup B and C meningococci are isolated from endemic disease and outbreaks in developed countries. Most serogroup B and C strains isolated from outbreaks are assigned to three genetically distinct lineages: cluster A4, ET-37 and the ET-5 complex, whereas endemic isolates are much more heterogeneous than these epidemic clones or than serogroup A meningococci [3–5].

In Spain, serogroup B strains were the predominant cause of meningococcal disease during epidemic

periods in the 1970s and 1980s [6]. In the 1990s, the prevalence of serogroup C disease increased. The group C disease prevalence was *c.* 3% in the 1970s [7] increasing to 12–14% in the 1980s [8] and escalating to 34% and 42% during 1994 and 1995, respectively [9]. A change in the prevalent sero/subtypes was also observed. C:2b:non-subtypable (NST) strains showed a progressive decrease from 55% of disease-associated group C isolates in 1990–1992 to 10.3% in 1996. In contrast, since 1992, the incidence of disease-associated strains of C:2b:P1.2,5 increased and accounted for 65% of disease-associated isolates in 1996 [10]. A similar increase in serogroup C has been described in other countries, mainly associated with the emergence of a particular clone [11, 12].

To determine the genetic structure of the serogroup C meningococcal population in Spain and examine whether the observed increase in the number of group C isolates over the period 1992–1999 was due to the emergence of a new clone, 1112 serogroup C isolates were analysed. The isolates were sero/subtyped and characterised by pulsed-field gel electrophoresis (PFGE). Selected isolates were further analysed by multi-locus sequence typing (MLST) [13].

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Materials and methods

Bacterial isolates

Disease-associated isolates were cultured from blood or cerebrospinal fluid, or both, of patients with invasive disease by standard methodologies and submitted to this laboratory from all regions of Spain. Isolates included in the present study were isolated during the period 1992–1999. All isolates were confirmed as *N. meningitidis* and then serogrouped and sero/subtyped as described previously [14]. In all, 1112 serogroup C isolates exhibiting various antigenic combinations were included in this study: 897 of type C:2b:P1.2,5; 99 of type C:2b:P1.2; 40 of type C:2b:P1.5; and 76 of C:2b:NST. Details of the year of isolation are shown in Table 1.

Culture, DNA preparation and PFGE assay

Isolates were cultured on blood agar for 18–24 h at 37°C in an atmosphere of CO₂ 5% in air and bacterial growth was scraped into 2.5 ml of sodium-EDTA (SE) buffer (75 mM NaCl, 25 mM EDTA, pH 7.4). Specimens were centrifuged and the pellet was resuspended in SE buffer. The cell suspension was mixed with an equal volume of melted Multipurpose Agarose (Boehringer Mannheim) 1% w/v in Tris-EDTA-MgCl₂ buffer (10 mM Tris-HCl, pH 7.1; 10 mM MgCl₂; 0.1 M EDTA). The mixture was dispensed directly into plastic moulds at 4°C and allowed to solidify. Blocks were treated with a lysis buffer (50 mM Tris-HCl, pH 7.4; 50 mM EDTA) supplemented with proteinase K 1 mg/ml and lauroyl sarcosine 1% at 56°C for 48 h. Subsequently, they were washed eight times at room temperature with Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). Plugs containing chromosomal DNA were equilibrated with appropriate restriction endonuclease buffer and digested with 30 units of *Bgl*II overnight. The digested DNA plugs were placed in wells of agarose 1% gel. PFGE was performed with a contour-clamped homogeneous electric field apparatus (Chef DR II; BioRad, Hercules, CA, USA) in 0.5× Tris-borate-EDTA (10 µM Tris-HCl; 1 µM EDTA, pH 8.1) at 12°C with initial to final time ranging from 0.1 to 10 at 200 V for 22 h. Gels were stained with

ethidium bromide and photographed under UV light. Macrorestriction fragments were compared visually and interpreted according to the criteria of Tenover *et al.* [15].

MLST

To determine whether C:2b:P1.2,5 strains represented a genetic variant of C:2b:NST strains as has been suggested [10], 44 isolates were analysed by MLST: 34 C:2b:P1.2(5) isolates (including C:2b:P1.2,5, C:2b:P1.2 and C:2b:P1.5 antigenic variants) and 10 C:2b:NST strains, according to Maiden *et al.* [13].

Data analysis

The similarity of the PFGE banding patterns was estimated with the Dice coefficient and a similarity matrix was computed and transformed into an agglomerative cluster by the unweighted pair group method for arithmetic averages (UPGMA) [15, 16]. A dendrogram of similarity was constructed from the cluster analysis.

Results

PFGE profiles

The macrorestriction of C:2b:P1.2(5) isolates with *Bgl*II generated 51 different banding profiles or PTs (Fig. 1) with the number of bands ranging from 10 to 19. The majority appeared as closely related PTs with a similarity index that ranged from 0.97 to 0.81. Furthermore, the majority of C:2b:P1.2,5 and C:2b:P1.2 isolates exhibited one of two very closely related profiles (with only one band of difference), designated as PT.7 (34%) and PT.8 (30%). Although most of the isolates grouped in the similarity range 0.97–0.81, a separate cluster of five PTs (29, 48, 49, 50 and 51), with similarity values lower than 0.81 (Fig. 1) was found. Most of the C:2b:P1.5 isolates were represented in this group with two exceptions corresponding to PTs 29 and 48 which were C:2b:P1.2,5 isolates.

The macrorestriction of C:2b:NST isolates generated 24 PFGE profiles with a number of bands ranging from 8 to 15. Eight PTs appeared in both C:2b:NST and C:2b:P1.2(5) strains. The majority of C:2b:NST isolates exhibited one of two PFGE profiles – PT.49 and PT.7. PT.49 was the main profile among C:2b:NST isolates during 1992 and 1993, accounting for 66.7% and 55.6%, respectively, of isolates of this sero/subtype. Nevertheless, from 1994 its frequency decreased, reaching 20% in 1995, decreasing to 10% in 1996 and it was not isolated in 1997. In contrast, PT.7, which appeared in 1994, represented *c.* 30% of the C:2b:NST isolates until 1996, and accounted for 89% and 75% of isolates of this sero/subtype during 1997 and 1998, respectively.

Table 1. Phenotypic expression and year of isolation of the meningococcal strains

Year of isolation	Number of isolates with antigenic expression of			
	C:2b:P1.2,5	C:2b:NST	C:2b:P1.2	C:2b:P1.5
1992	–	9	6	–
1993	3	9	17	4
1994	21	10	4	14
1995	58	8	2	2
1996	204	10	10	5
1997	384	9	27	11
1998	147	12	19	1
1999	80	9	14	3

Fig. 1. Genetic relationships among C:2b:P1.2,5, C:2b:P1.2 and C:2b:P1.5 strains by PFGE with *Bgl*/II. PT, pulsotype number obtained with *Bgl*/II; n, number of isolates in pulsotype (number of isolates analysed by MLST).

MLST

Twenty-four C:2b:P1.2 and C:2b:P1.2,5 isolates were selected from among isolates showing the most frequent PTs (PT.1, PT.7, PT.8, PT.21, PT.12, PT.22) and six from among isolates with other minor PTs,

showing different similarity index values to the main profiles (Fig. 1). Four C:2b:P1.5 isolates grouped in the PT.49 were also included in the MLST analysis. Six C:2b:NST isolates were selected from among isolates showing the most frequent PTs (PT.49 and PT.7) and

four from among isolates showing other minority PFGE profiles.

Among C:2b:P1.2 and C:2b:P1.2,5 isolates, 28 showed an identical sequence type (ST8) which has been found to be associated with the A4 complex (Fig. 1). Only two exceptions were found corresponding with isolates from PT.29 and PT.48 which belonged to the ET-37 complex (Fig. 1).

All C:2b:P1.5 meningococcal isolates showed an allelic profile corresponding to ST.11 and therefore to the ET-37 complex.

Otherwise, MLST results among C:2b:NST isolates pointed to the presence of two principal groups or hypervirulent lineages among these strains: the ET-37 complex and the A4 cluster. Isolates belonging to the ET-37 complex corresponded with those isolated during 1992, 1993 and part of 1994. In contrast, the majority of C:2b:NST isolates obtained after 1994 belonged to the A4 cluster. The allelic profile of A4 isolates differed in four of the seven genes assayed in comparison to ET-37 strains.

Thus, the majority of those isolates showing C:2b:P1.2 and C:2b:P1.2,5 antigenic combinations belonged to the A4 genetic lineage. In contrast, all C:2b:P1.5 and most of the C:2b:NST isolates showed allelic profiles corresponding to the ET37 clonal complex.

Discussion

Serogroup B strains were the predominant cause of meningococcal disease in Spain during epidemic and inter-epidemics periods in the 1970s and 1980s and in the early 1990s [6]. However, since 1992 a progressive increase in serogroup C disease-associated meningococcal strains was noted [14]. The sero/subtype combinations also changed. The principal strain isolated during 1990–1992 among serogroup C isolates (C:2b:NST) declined in frequency of isolation from 1993. At the same time, the incidence of C:2b:P1.2,5 isolates increased [10]. Molecular characterisation of isolates during 1992–1999 was performed to investigate the evolution of the population of serogroup C meningococci in Spain.

PFGE results revealed a homogeneous structure for C:2b:P1.2(5) isolates. Two pulsotypes (PT.7 and PT.8) were identified in 65% of C:2b:P1.2,5 and C:2b:P1.2 isolates. Both pulsotypes were considered identical by the criteria of Tenover *et al.* [15] (only one band difference was found) and it is possible that PT.8 was derived from PT.7 by loss of a restriction site for *Bgl*II. The remaining profiles, with a few exceptions (PTs 29, 49, 50, 51 and 48) (Fig. 1), exhibited a high level of similarity. Analysis of DNA banding patterns indicated that inter-isolate differences in this homogeneous group

(similarity index 0.97–0.81) (Fig. 1) were not higher than six, suggesting that the number of genetic events necessary to produce that variability was not higher than three [15]. The results suggest the possibility that these related PTs may have emerged by processes such as insertion or deletion. In the same way, according with the criteria defined by Tenover *et al.* [15], those isolates grouping in the cluster including PTs 29, 49, 50, 51 and 48 were defined as non-related isolates, showing pattern profiles with more than seven bands of difference from the main group.

The majority of the isolates analysed by MLST presented the same allelic combination for the seven housekeeping genes, identical sequence type (ST) and, therefore, belonged to the same hypervirulent lineage A4. These results indicated that housekeeping genes had not undergone variation. Isolates of PTs 29, 48 and 49 presented a different allelic combination. Four of seven assayed genes differed at sequence level, indicating their origin in the ET-37 complex. The appearance of ET-37 strains in the Spanish population was unusual; previous studies indicated that the 'old epidemic strain' C:2b:NST belonged to this cluster [10]. However, the appearance of C:2b:P1.2,5 isolates belonging to this cluster had not been described previously. These events indicate that different antigenic or phenotypic expressions may be found in a cluster [4].

Otherwise, the analysis of the main antigenic expressions of serogroup C strains in Spain indicated that, in general, C:2b:NST strains were similar to C:2b:P1.5 strains, whereas C:2b:P1.2,5 strains were similar to C:2b:P1.2 strains. These results were similar to those observed previously [10].

Analysis of C:2b:NST isolates by PFGE revealed the existence of two principal PFGE profiles, PT.49 and PT.7, whose appearance over the study period (1992–1999) seemed to indicate an inverse evolution. Thus, whereas PT.49 (associated with the ET-37 complex by MLST) was the principal profile described during 1992–1993, PT.7 (associated with the A4 cluster) was the most frequent profile since 1996. During 1994–1995 a co-existence among PTs was observed.

In conclusion, our previous hypothesis, that the new epidemic strain C:2b:P1.2,5 was a subclone of the 'old epidemic strain' C:2b:NST [10], was not supported by the findings in this large study. The study shows displacement of the ET-37 cluster by strains belonging to the A4 cluster. The first cluster (ET-37) was mainly composed of C:2b:NST and C:2b:P1.5 isolates and the second cluster mainly comprised C:2b:P1.2 and C:2b:P1.2,5 isolates.

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