Variation in the Expression of Pili and Outer Membrane Protein by *Neisseria meningitidis* during the Course of Meningococcal Infection

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The occurrence of antigenic shift during meningococcal infection has been investigated by comparison of paired isolates obtained from the blood, cerebrospinal fluid or nasopharynx of patients. Isolates from any individual produced identical DNA 'fingerprints' and showed stability in expression of both class 2 outer membrane protein and an antigen common to pathogenic *Neisseria*, confirming their origin as a single strain. One of the four strains examined produced variants which differed in the molecular mass of their class 5 outer membrane proteins. Three of the strains produced pili containing the epitope recognized by monoclonal antibody SM1 and two of these gave rise to variants which expressed pili of differing subunit molecular masses. The two variants of the remaining strain produced pilins lacking the common epitope detected by antibody SM1 but radioimmune precipitation with polyclonal anti-pilus antiserum revealed that variation in the molecular mass of the pilin expressed also occurred with this second class of pili. Antigenic variation in expression of both class 5 outer membrane proteins and pili therefore appears to be a common occurrence during meningococcal infection.

INTRODUCTION

Surface proteins of pathogenic species of *Neisseria* participate in host–bacterial interactions such as colonization of mucosal surfaces, uptake by epithelial cells, and interaction with host defences, the results of which determine the course of an infection. Two gonococcal surface proteins, pili and outer membrane protein II (P.II), which have been implicated in such interactions are subject to intra-strain antigenic variation (Heckels, 1986). Colonial variants can be selected during laboratory subculture which express antigenically and functionally distinct pili and/or P.II. Similar variation in antigen expression has subsequently been detected during the course of natural infection (Duckworth *et al.*, 1983; Zak *et al.*, 1984), suggesting that antigenic shift plays an important role in pathogenesis, enabling gonococci to adapt to a changing host environment.

Surface proteins of meningococci show many similarities to those of gonococci and it is likely that antigenic shift may also play an important role in pathogenesis of meningococcal infections. For technical reasons it has proved more difficult on laboratory subculture to isolate meningococcal clones expressing antigenically distinct variant proteins (McGee *et al.*, 1979; Stephens & McGee, 1983). By comparison of different isolates from the same individual, Poolman *et al.* (1980) showed stability in expression of the major outer membrane protein, termed class 2 protein (Tsai *et al.*, 1981), but identified variation in expression of the class 5 outer membrane protein, which corresponds to gonococcal P.II. Subsequently, laboratory-derived variants which differed in expression of both class 5 protein and LPS have been obtained (Poolman *et al.*, 1985). Recent studies have shown that structurally distinct variant pili can also arise during laboratory culture of meningococci (Olafson *et al.*, 1985) but the occurrence of antigenic variation in pilus expression during natural infection has not yet been documented.

Abbreviations: CSF, cerebrospinal fluid; PMSF, phenylmethylsulphonyl fluoride.

This report investigates the occurrence of antigenic shift during meningococcal infection by comparison of isolates obtained from the blood, cerebrospinal fluid or nasopharynx of individual patients.

METHODS

Bacterial strains and growth conditions. All strains of meningococci used in this study were isolated during 1985 from patients with meningococcal meningitis by the Public Health Laboratory Service at Southampton General Hospital. Bacteria were cultured from the patients' cerebrospinal fluid (CSF; strains suffixed '-C'), blood (suffixed '-B') or nasopharynx (suffixed '-N') on chocolate or blood agar at 37 °C in a humid atmosphere containing 5% (v/v) CO₂. Meningococci were subcultured once on chocolate agar plates, then stored frozen as thick suspensions in 10% (v/v) glycerol, 1% (w/v) proteose peptone (Difco) in liquid nitrogen.

For subsequent studies bacteria were grown on proteose peptone (PP) agar containing, per litre, 10 g proteose peptone (Difco), 10 g agar (bacteriological no. 1; Oxoid), 1 g starch, 5.24 g K₂HPO₄·3H₂O, 1 g KH₂PO₄ and 5 g NaCl with a supplement similar in composition to commercial Isovitalax (BRL) apart from the omission of L-cystine and the additions of uracil (8 µg ml⁻¹) and hypoxanthine (3.2 µg ml⁻¹) (Zak *et al.*, 1984).

SDS-PAGE and Western blotting. Bacteria were scraped from plates of PP agar into 1 mM-phenylmethylsulphonyl fluoride (PMSF), 0.05% (w/v) NaN₃ in water. Samples containing 5 to 20 µg protein were heated in dissociating buffer for 5 min at 100 °C, or for 2 h at 37 °C, then subjected to SDS-PAGE on linear gradients of 10% to 25% (w/v) acrylamide (1 × 100 × 200 mm) with a 4% (w/v) acrylamide stacking gel (1 × 30 × 200 mm) using a discontinuous buffer system as previously described (Heckels, 1981). Gels were stained in PAGE blue 83 (BDH).

For immunological detection by Western blotting the separated proteins were electrophoretically transferred to nitrocellulose sheets as described previously (Virji & Heckels, 1983). The sheets were washed thoroughly in 10 mM-Tris/HCl pH 7.4 containing 0.9% (w/v) NaCl and blocked with 3% (w/v) BSA in the same buffer. The sheets were then reacted with the appropriate monoclonal antibody diluted in 50 mM-Tris/HCl pH 7.4 containing 150 mM-NaCl, 5 mM-EDTA, 0.25% (w/v) gelatin (Difco), 0.05% (v/v) Nonidet P-40 (BDH) and 0.05% (w/v) NaN₃. After washing, the sheets were reacted with ¹²⁵I-labelled protein-A, diluted in the same NP-40 buffer. Unbound radioactivity was removed by extensive washing and immunological reactivity was detected by direct autoradiography using Kodak X-Omat AR film for 1 to 5 d.

Antiserum and monoclonal antibodies. The monoclonal antibodies used were SM1, with reactivity against a conserved epitope in gonococcal pili (Virji & Heckels, 1983), and SM70, with reactivity against a conserved epitope on a protein restricted to pathogenic *Neisseria* (Virji *et al.*, 1985). Polyclonal antipilus antibodies were raised by immunization of rabbits with purified α pili from *N. gonorrhoeae* P9 according to previously published schedules (Diaz *et al.*, 1984; Diaz & Heckels, 1982).

Radioiodination of surface-exposed antigens. Bacteria were scraped from PP agar plates into Dulbecco complete phosphate-buffered saline pH 7.4 (Oxoid; PBSB) at 0 °C. The suspensions were centrifuged at 10000 g for 2 min and resuspended to give an OD₅₅₀ of 1.0. Portions (0.6 ml) of each variant were centrifuged and the pellets resuspended in 100 µl PBSB containing 2 × 10⁻⁶ M-KI. To each tube was added one Iodobead (Pierce Chemical Co.) followed by 200 µCi (7.4 MBq) Na¹²⁵I (Amersham). The tubes were incubated at room temperature for 10 min with occasional shaking. The contents of each tube were diluted with 750 µl ice-cold PBSB then centrifuged at 10000 g for 2 min. The labelled meningococci were washed in PBSB and finally resuspended in 0.4 ml PBS containing 1 mM-PMSF and 0.05% (w/v) NaN₃. The specific activity was approximately 50–100 µCi (mg protein)⁻¹. Some strains were also radioiodinated using lactoperoxidase as previously described (Heckels, 1978), which gave a specific activity of approximately 5 µCi mg⁻¹. Samples of the ¹²⁵I-labelled bacteria (containing 5 × 10⁴ c.p.m.) were subjected to SDS-PAGE as described above. The gel was placed in a 50% (v/v) methanol shrinking solution for 1.5 h, dried under vacuum, and radiolabelled proteins were detected by direct autoradiography.

Radioimmune precipitation. Surface-labelled bacteria were incubated at 37 °C for 1 h in 0.3% (w/v) Empigen BB (Albright & Wilson), 0.1% (w/v) SDS, 0.05% (w/v) NaN₃ and 1 mM-PMSF in PBS (RIP buffer). Insoluble material was removed by centrifugation at 100000 g for 20 min. Samples of the soluble antigen preparations containing 5 × 10⁵ c.p.m. in 100 µl RIP buffer were mixed with 50 µl of a 100 mg ml⁻¹ suspension in PBS of protein A-Sepharose CL4B (Sigma) and 40 µl of polyclonal antiserum or ascitic fluid containing monoclonal antibody diluted 1:100 in PBS. The tubes were incubated at 4 °C for 1.5 h with end-over-end rotation and then centrifuged at 10000 g for 2 min. The beads were washed four times with RIP buffer, once with PBS, then transferred to a clean tube, and boiled in SDS-PAGE dissociating buffer for solubilization of precipitated antigens. The beads were removed by centrifugation and a sample of the supernatant solution was subjected to SDS-PAGE. Immunological reactivity was detected by autoradiography.

Restriction endonuclease fingerprinting of chromosomal DNA. Isolates were grown on 7 cm plates of a medium of composition similar to PP-agar in which the agar was replaced by 1.5% (w/v) agarose (Bio-Rad). Bacteria from each plate were harvested into 1 ml of lysis mixture comprising 25 mM-Tris/HCl pH 8.0, 10 mM-EDTA, 50 mM-

glucose and 4 mg lysozyme (Boehringer Mannheim) ml^{-1} . After 20 min at 0 °C, 10 μl 10% (w/v) SDS and 100 μl proteinase K (1 mg ml^{-1} ; Sigma) were added and the mixture was incubated at 37 °C for 30 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) was added and after homogenization the mixture was centrifuged at 10000 g for 5 min to separate the phases. The aqueous phase was removed and, after addition of 10 μl RNAase A (1 mg ml^{-1} ; Sigma) was incubated at 37 °C for 1 h. After two further phenol extractions the resulting DNA solution was extensively dialysed against 10 mM-Tris/HCl pH 7.6, containing 1 mM-EDTA. The solutions obtained contained high-molecular-mass DNA at a concentration of 0.5 to 1.0 mg ml^{-1} . Restriction endonuclease digestion with *Hind*III, *Eco*RI or *Cla*I was done according to the manufacturer's (Boehringer) recommended conditions. The fragments generated were separated on 0.6% agarose gel and detected as described by Maniatis *et al.* (1982).

RESULTS

Isolation of meningococcal variants

Variants of meningococcal strains were obtained as paired isolates from different sites in four patients with meningococcal meningitis. In order to exclude the possibility of concurrent infection by two distinct strains the isolates were subjected to DNA fingerprinting and immunochemical characterization. Chromosomal DNA was digested with restriction enzymes *Hind*III, *Eco*RI or *Cla*I and subjected to agarose gel electrophoresis. With all three enzymes the strains from different patients showed unique patterns but the fingerprint of each isolate was shared by the other isolate from the same patient.

Whole-cell lysates of the isolates were subjected to SDS-PAGE followed by Western blotting with monoclonal antibody SM70 (Fig. 1), which recognizes a conserved epitope on a protein which varies widely in molecular mass between different strains of pathogenic *Neisseria* (Virji *et al.*, 1985). In each case strains from different patients showed a different molecular mass for the 'pathogenic *Neisseria* protein' while no differences could be seen between the isolates from any individual. The identical molecular mass of the protein (Table 1) combined with the unique DNA fingerprints obtained confirmed that isolates from the same patient were indeed the same strain.

Surface protein expression in paired isolates

Whole-cell lysates of the paired isolates were subjected to SDS-PAGE (Fig. 2). Numerous bands were common to all strains but each pair showed characteristic mobility of major bands in the 40 kDa region of the gel. In addition, the isolate from the CSF of one patient showed a major band at 27.0 kDa which could not be detected in the nasopharyngeal isolate (Fig. 2, 51-C vs 51-N). In general, however, the large number of bands present prevented detailed comparisons within pairs of isolates.

In order to simplify the pattern and to identify surface-exposed proteins, isolates were subject to surface labelling with ^{125}I . Preliminary experiments comparing the effect of lactoperoxidase or Iodobeads as the oxidizing agent revealed little difference in the pattern of labelling but the latter showed considerably greater efficiency, and was therefore used routinely. Autoradiographs of SDS-PAGE gels of labelled isolates revealed that differences between strains which could be detected in the 40 kDa and 28 kDa regions of gels of whole-cell lysates were due to surface-exposed proteins (Fig. 3). The 40 kDa band, corresponding to the major outer membrane or class 2 protein (Tsai *et al.*, 1981), showed inter- but not intra-patient variation in molecular mass. In contrast, the class 5 protein, which could be identified by virtue of its characteristic change in mobility on derivitization at 37 °C rather than 100 °C (Frasch *et al.*, 1985), showed a difference between the isolates in one of the four pairs (Fig. 3, 51-C and 51-N). The isolate from the CSF expressed a single class 5 protein of 27 kDa whereas the isolate from the nasopharynx expressed proteins of 28.7 kDa and 28.0 kDa. The remaining three pairs showed no difference in expression of class 5 protein between the isolates from an individual patient (Table 1). Outer membranes prepared from some of the pairs of isolates showed a pattern of protein staining after SDS-PAGE which was identical with the pattern of surface labelling with the exception that surface-labelled peptides in the 18 kDa region (Fig. 1) could not be detected in outer membrane preparations (data not shown).

Fig. 1. Molecular mass differences in a 'pathogenic *Neisseria* antigen' expressed by meningococcal isolates, detected following SDS-PAGE and Western blotting with monoclonal antibody SM70. The arrow shows the position and molecular mass (kDa) of antigen from isolate MC50-C.

Table 1. *Molecular masses (kDa) of surface antigens expressed by paired meningococcal isolates from individual patients*

Patient	Site	Reaction with antibody SM1	Pathogenic <i>Neisseria</i> protein*	Class 2 OMP	Class 5 OMP†	Pili‡
50	CSF	+	19.8	40.5	27.0	18.5
	Blood	+	19.8	40.5	27.0	17.9
51	CSF	+	20.9	40.5	27.0	18.5
	Nasopharynx	+	20.9	40.5	28.7, 28.0	19.3, 18.2
52	CSF	+	21.0	38.4	27.5	18.9
	Blood	+	21.0	38.4	27.5	18.9
54	CSF	—	23.0	40.2	28.1	14.1
	Blood	—	23.0	40.2	28.1	14.1, 16.0

* Detected by reactivity on Western blots using monoclonal antibody SM70 (Virji *et al.*, 1985).

† Detected by change in mobility on SDS-PAGE according to temperature of derivatization (Frasch *et al.*, 1985).

‡ Detected by reactivity on Western blots using monoclonal antibody SM1 (strains 50, 51, 52) or following radioimmune precipitation with polyclonal anti-pilus antiserum (strain 54) (Diaz *et al.*, 1984).

Detection of variation in pilus expression

The location of the variable 18 kDa bands on the meningococcal surface but not in outer membranes, combined with their molecular mass range, suggested that they probably represented pili (Diaz *et al.*, 1984). Pilus expression was therefore investigated further by use of a monoclonal antibody, SM1, initially raised against a common epitope present on gonococcal pili (Virji & Heckels, 1983). Initial screening by 'dot blotting' showed that three of the four groups of isolates reacted with the antibody. Western blotting of SDS-PAGE gels of the whole-cell lysates

Fig. 2. SDS-PAGE of whole-cell lysates of paired meningococcal isolates. The arrows show the position and molecular mass (kDa) of class 2 and class 5 outer membrane proteins in isolate MC50-C.

Fig. 3. Radioiodination of surface-exposed proteins in paired meningococcal isolates. Bacteria were labelled using ^{125}I and Iodobeads then subjected to SDS-PAGE and autoradiography. The arrows show the position and molecular mass (kDa) of class 2 and class 5 outer membrane proteins and pilin in isolate MC50-C.

Fig. 4. Immunological detection of pilus expression in paired meningococcal isolates. Pili expressed by isolates MC50, MC51 and MC52 were detected following SDS-PAGE by Western blotting with monoclonal antibody SM1. Pili expressed by isolate MC54 were detected following radioimmune precipitation with polyclonal anti-pilus antiserum.

revealed heterogeneity in pilus expression (Fig. 4). The isolate from the CSF of patient 50 produced pilin of 18.5 kDa while that from the blood produced pilin of 17.9 kDa. The isolate from the CSF of patient 51 produced a single pilin band of 18.5 kDa whereas that from the nasopharynx produced two pilin bands of 19.3 kDa and 18.2 kDa. The isolates from the third patient showed no difference, both producing pilin of 18.9 kDa.

Previous studies (Diaz *et al.*, 1984) have shown that while the majority of fresh meningococcal isolates produce pili of approximately 18 kDa, containing a common epitope recognized by antibody SM1, a second group express pili of lower molecular mass which lack the epitope but which can be detected by radioimmune precipitation with polyclonal antisera. On radioimmune precipitation with anti-pilus antiserum lower molecular mass pili were detected in both isolates from patient 54 and again differences were seen between the two sites (Fig. 4, 54-C and 54-B). The variant from the blood showed two diffuse but discrete pilin bands of approximately 14.1 kDa and 16.0 kDa while the variant from the CSF produced a single strong band at 14.1 kDa.

DISCUSSION

Despite the ability of pathogenic *Neisseria* readily to undergo antigenic shift *in vitro*, stable markers exist which are not subject to intrastrain variation. DNA fingerprinting (Bjorvatn *et al.*, 1984) and the molecular mass of the class 2 outer membrane protein (Frasch *et al.*, 1985) have been used to identify strains for epidemiological purposes. The pathogenic *Neisseria* antigen detected by antibody SM70 (Virji *et al.*, 1985), corresponding to the 'H8 reactive antigen' described by Cannon *et al.* (1984), also varies between, but not within, strains. The stability of these markers between two isolates from any individual in the present study confirms their origin as a single strain. In contrast, the class 5 outer membrane protein varied, although less than might have been anticipated from the studies of Poolman and colleagues (Poolman *et al.*, 1980) or by analogy with gonococci (Zak *et al.*, 1984).

In the current study greater variation was seen in pilus expression. Three of the four strains produced pili containing the epitope recognized by monoclonal antibody SM1 and of these two

gave rise to variants which expressed pili of different subunit molecular mass. Structural (Schoolnik *et al.*, 1984), immunochemical (Virji & Heckels, 1983; Rothbard *et al.*, 1984) and genetic studies (Hagblom *et al.*, 1985) have suggested the basis for antigenic diversity of gonococcal pili. Pilin molecules contain a conserved region at the N-terminus and a variable region at the C-terminus. The variable determinants are immunodominant and so antibodies induced by one pilus type show little cross-reactivity with variant pili produced by the same strain. Monoclonal antibody SM1 recognizes a determinant contained within amino acid residues 48–60 (Heckels & Virji, 1986) which is present on all gonococci so far tested. Clearly a large proportion of meningococci also produce pili of similar structure and potential for variation. Previous studies have shown that some strains of meningococci express pili of a different type which have a lower subunit molecular mass and lack the epitope recognized by antibody SM1 (Diaz *et al.*, 1984). Such a strain, isolated from patient 54, has revealed that variation in expression also occurs with this second class of pili.

The common occurrence of antigenic shift in pilus expression suggests that it must play an important role in the pathogenesis of meningococcal infection, although the role of pili in meningococcal disease is far from clear. Pili mediate adhesion of meningococci to nasopharyngeal epithelial cells and may thus be important factors in establishing the carrier state (Stephens & McGee, 1981) but isolates cultured from the CSF and blood of patients with meningococcal infection are also invariably piliated (Stephens & McGee, 1981; Diaz *et al.*, 1984; Stephens *et al.*, 1985). In addition, pili have been directly demonstrated in the CSF of a child with meningococcal meningitis (Stephens *et al.*, 1982). Antigenic variation in gonococcal pilus expression has been associated with the ability to colonize different anatomical sites and with a mechanism to evade the consequences of the host immune response (Zak *et al.*, 1984). Similar factors may well operate during meningococcal disease. Indeed, differences between strains in their ability to attach to human buccal epithelial cells and erythrocytes have been associated with differences in the mechanism of attachment of the pili expressed by those strains (Trust *et al.*, 1983). Such differences may well arise as a result of the ability of individual strains to produce structurally and antigenically distinct pili with altered receptor specificity. The ability to interact specifically with different cell types may be important in the pathogenesis of meningococcal disease since the varied symptoms of infection result as the consequence of a complex and poorly understood series of interactions between the bacteria and a variety of host cells.

Alternatively, the ability to undergo antigenic shift in surface antigen expression may enable meningococci to evade the host defences and could, for example, explain the persistence of nasopharyngeal carriage despite presence of the host immune response (Goldschneider *et al.*, 1969). Indeed one previous report of a recurrent meningococcal infection caused by the original invading strain, despite the induction of an apparently adequate immune response, suggested that some *in vivo* alterations in the meningococcus must have occurred (Griffiss *et al.*, 1974). Certainly the occurrence of antigenic shift in the expression of major surface antigens is likely to have important consequences for our understanding of the pathogenesis of meningococcal infections.

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