

Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease

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A B S T R A C T

The human species is the only natural host of *Neisseria meningitidis*, an important cause of bacterial meningitis globally, and, despite its association with devastating diseases, *N. meningitidis* is a commensal organism found frequently in the respiratory tract of healthy individuals. To date, antibiotic resistance is relatively uncommon in *N. meningitidis* isolates but, due to the rapid onset of disease in susceptible hosts, the mortality rate remains approx. 10%. Additionally, patients who survive meningococcal disease often endure numerous debilitating sequelae. *N. meningitidis* strains are classified primarily into serogroups based on the type of polysaccharide capsule expressed. In total, 13 serogroups have been described; however, the majority of disease is caused by strains belonging to one of only five serogroups. Although vaccines have been developed against some of these, a universal meningococcal vaccine remains a challenge due to successful immune evasion strategies of the organism, including mimicry of host structures as well as frequent antigenic variation. *N. meningitidis* express a range of virulence factors including capsular polysaccharide, lipopolysaccharide and a number of surface-expressed adhesive proteins. Variation of these surface structures is necessary for meningococci to evade killing by host defence mechanisms. Nonetheless, adhesion to host cells and tissues needs to be maintained to enable colonization and ensure bacterial survival in the niche. The aims of the present review are to provide a brief outline of meningococcal carriage, disease and burden to society. With this background, we discuss several bacterial strategies that may enable its survival in the human respiratory tract during colonization and in the blood during infection. We also examine several known meningococcal adhesion mechanisms and conclude with a section on the potential processes that may operate *in vivo* as meningococci progress from the respiratory niche through the blood to reach the central nervous system.

INTRODUCTION

Neisseria meningitidis is a human-specific Gram-negative organism, often diplococcal in form, and is recognized as the leading cause of bacterial meningitis

globally. The genus *Neisseria* also includes another pathogenic species *N. gonorrhoeae*, the cause of gonorrhoea, which shares numerous common features with *N. meningitidis*. However, the niche preference (nasopharyngeal compared with urogenital tracts) as well

Key words: bacterial meningitis, blood–brain barrier, colonization, *Neisseria meningitidis*, outer-membrane protein, pilus, polysaccharide.

Abbreviations: App, adhesion and penetration protein; BBB, blood–brain barrier; C4bp, C4-binding protein; CEACAM, carcinoembryonic antigen-related cell-adhesion molecule; ChoP, phosphorylcholine; CNS, central nervous system; GPI, glycosylphosphatidylinositol; Hep, heptose; HSPG, heparan sulphate proteoglycan; IL, interleukin; KDO, 2-keto-3-deoxy-D-manno-2-octulosonic acid; LNT, lacto-N-neotetraose; LPS, lipopolysaccharide; MLST, multi-locus sequence typing; MspA, meningococcal serine protease A; NadA, Neisserial adhesin A; NANA, 5-N-acetyl-neuramic acid; NhhA, *Neisseria* hia homologue A; NspA, Neisserial surface protein A; OCA, oligomeric coiled-coil adhesin; OMV, outer-membrane vesicle; PEA: phosphoethanolamine; SIGLEC, sialic acid-binding, immunoglobulin-like lectin; SSM, slipped strand mispairing; ST, sequence type; TLR, Toll-like receptor; TNF- α , tumour necrosis factor- α .

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as the nature of diseases caused suggests significant differences also exist between these pathogens, borne out of the identification of variations at the genetic level [1]. One major difference between the two organisms is the expression of surface polysaccharide capsule which is absent from *N. gonorrhoeae*, whereas *N. meningitidis* strains commonly express one of several capsule types, which form the basis of their primary classification by serogroup [2]. Further classification of *N. meningitidis* is based on major outer-membrane porins into serotypes and serosubtypes as well as LPS (lipopolysaccharide) into immunotypes [3]. In addition, MLST (multi-locus sequence typing) classifies strains into STs (sequence types) based on variations among seven housekeeping genes [4].

CARRIAGE AND DISEASE

Globally, meningococcal carriage rates of generally between 10–35% have been reported for healthy adults [5,6]. Estimation of carriage is, however, limited by the swabbing techniques employed. Nonetheless, in populations with individuals in close contact, such as university students or military recruits, carriage rates approaching 100% have been found [2,7]. Compared with the carriage rate, meningococcal disease is rare, and disease rates vary in different geographic regions of the world. What changes the colonization state of the organism into a disease state is not entirely clear. It appears that a combination of bacterial virulence factors and host susceptibility, including age, prior viral infection, smoking [2] and genetic polymorphisms (reviewed in [8]), may ultimately lead to meningococcal disease.

Although 13 meningococcal serogroups have been described (A, B, C, D, 29E, H, I, K, L, Y, W-135, X and Z), the majority of disease is caused by organisms expressing one of five capsule types namely A, B, C, Y and W-135 (Table 1). Meningococcal disease in Europe and the Americas is predominantly caused by serogroups B and C, whereas in Africa the principal causes are serogroups A and C [9]. Serogroup W-135 causes outbreaks around the world, with serogroup Y generally associated with disease in the U.S.A. and Canada [9]. The factors that determine such geographic variation are also incompletely understood [10]. Through MLST, many meningococcal STs have been identified which are independent of serogroup. Of these, a few are disproportionately associated with disease relative to their carriage levels and so are termed hyperinvasive lineages [11].

In keeping with carriage, rates of meningococcal disease are also variable and range from the sporadic outbreaks observed across Europe to the epidemics observed across the African meningitis belt (1 per 100000 to 1000 per 100000 population [9]). In general, mortality occurs in up to 10% of patients with invasive meningococcal

disease [9]. Mortality rates are dependent on the type and severity of invasive disease, and are greatest for fulminant septicaemia (up to 55%) followed by meningitis with associated septicaemia (up to 25%), and lowest for meningitis without sepsis (generally <5%) [12]. However, patients who survive invasive meningococcal disease often live with a number of physical and mental sequelae, including amputation of limbs and digits, scarring of skin, deafness, speech impairment and seizures [13].

PREVENTION AND TREATMENT

Treatment of choice for meningococcal disease is parenteral administration of β -lactam antibiotics, such as cephalosporins and penicillins, to which resistance is rarely reported [14]. Meningococcal disease can progress rapidly from its onset and first symptoms, necessitating pre-hospital administration of antibiotics which has been shown to reduce mortality rates in patients with invasive disease [15].

Preventative measures against several serogroups have been available for a considerable period in the form of capsule-based vaccines [16]. However, although purified capsular antigens elicit protective antibody responses, they do not induce long-term memory. To overcome this deficiency, conjugate vaccines have been introduced. In the U.K., a conjugate vaccine against serogroup C polysaccharide was first licensed in 1999 and has resulted in a significant reduction in disease due to this serogroup (reviewed by [17]). In the U.S.A., tetravalent conjugate vaccines covering serogroups A, C, W-135 and Y are undergoing randomized trials for use in the 11–55-year-old age group [18]. However, where serogroup B prevails, polysaccharide-based vaccines have proved more difficult to generate as this polysaccharide is a poor immunogen for reasons discussed below. In this case, locally effective vaccines have been developed, for instance in Cuba, Norway and New Zealand based on strain-specific OMV (outer-membrane vesicle) preparations and have proved to be successful in reducing the incidence of local group B meningitis outbreaks [19–21]. At present, several approaches are being adopted to produce a universal serogroup B vaccine, including genome-based identification of potential novel vaccine antigens [22] and more traditional approaches to identify the key meningococcal components required for colonization and disease [23]. These, either individually or in combination, could form a robust means to control meningococcal infections.

MODELS TO STUDY HOST–PATHOGEN INTERACTIONS

Animal models have been used in a number of investigations [24,25] and have provided some insights into disease and vaccine efficacy. However, as meningococcal

Table 1 Important meningococcal serogroups, capsular structures, operon compositions and geographical prevalence

Serogroup	Capsule Structure		Operon	Geographic prevalence	
A	Non-sialic acid capsule	Homopolymers of N-acetyl-D-mannosamine-1-P	(α 1–6)-linked-N-acetyl-D-mannosamine-1-phosphate	Gene operon: <i>mynA-mynD</i>	Africa Asia
B	Sialic acid capsule (5-N-acetyl-neuraminic acid)	Homopolymers of sialic acid	(α 2–8)-linked-N-acetyl-neuraminic acid <i>Mimics human structures – Poorly immunogenic</i>	Gene operon: <i>siaA-siaD</i>	Worldwide - Covering all 5 continents
C			(α 2–9)-linked-N-acetyl-neuraminic acid <i>Highly immunogenic</i>		Worldwide - Covering all 5 continents
W135		Heteropolymers of sialic acid-containing disaccharides	(α 2–6)-linked-6-D-Gal(α 1–4)-N-acetyl-neuraminic acid		Africa
Y			(α 2–6)-linked-6-D-Glc(α 1–4)-N-acetyl-neuraminic acid		North America

specificity for its host involves a number of key host-specific events during colonization, including cellular interactions, iron acquisition and immune evasion, the relevance of animal studies is limited [9,23]. To overcome this, some studies, especially on cellular interactions, have been conducted using human organ cultures which were established to examine the adherence of *N. meningitidis* to nasopharyngeal epithelium, and the bacterium was found to adhere specifically to non-ciliated cells [26]. However, the majority of the studies on *N. meningitidis* have been carried out using cultured human cell lines to identify the molecular components of the bacteria required for the adhesion, invasion and traversal of human cellular barriers.

The remainder of the current review is presented in two sections. The first combines some of the main strategies of immune evasion and mechanisms of bacterial colonization of the human nasopharynx. The second section presents an overview of the interactions of the pathogen with host components described in sequence of the perceived bacterial progression from its colonization site, the nasopharynx, through the blood stream and the BBB (blood–brain barrier) to reach the meninges.

STRATEGIES OF *N. MENINGITIDIS* SURVIVAL, COLONIZATION AND INFECTION

Immune evasion by surface modulation

In order to overcome immune detection, meningococci have evolved several mechanisms to change their surface components. Structural/antigenic variation of these molecules is one strategy and can involve allelic exchange of genes or gene fragments from imported neisserial DNA. This can occur frequently in *N. meningitidis* as it is naturally competent and readily takes up DNA from its environment. In addition, as its genome contains multiple copies of certain genes, for example *opa* and *pil*, discussed below, intragenomic recombination also results in frequent surface structural variation [27,28].

Another surface modulation occurs via phase variation, a process involving on/off expression of genes, for which several mechanisms have been reported [27–29]. A detailed review of the mechanisms can be found in [30], and include SSM (slipped strand mispairing) and reversible insertion of mobile elements. The former involves tracts of repetitive DNA sequences that occur either upstream of a gene or within an open reading frame, and, through SSM,

a loss or gain of individual nucleotides or repeat units can occur at high frequency. Such changes upstream of a gene determine its transcriptional efficiency whereby a protein may be synthesized at various levels or may be totally absent, as in the case of *Opc* [31]. However, changes within a gene may introduce stop codons resulting in a lack of full translation of the gene. Such a situation occurs in *opa* genes, multiple copies of which occur in pathogenic *Neisseriae* [28]. The *opa* genes code for related, but not identical, proteins. Switching on and off of distinct genes independently of each other is therefore equivalent to antigenic variation in *Opa* proteins. Antigenic variation of LPS on the other hand may arise from phase variation of one or more enzymes involved in the synthesis of the oligosaccharide chain (Figure 1) by SSM, or by modification of LPS, for example by the addition of sialic acid [32].

Key surface structures involved in host interactions

Surface glycans

N. meningitidis, when isolated from carriers, may be capsulate or acapsulate, whereas blood and CSF (cerebrospinal fluid) isolates are invariably capsulate, as the capsule aids survival in the blood by rendering bacteria resistant to antibody/complement-mediated killing and inhibiting opsonic and non-opsonic phagocytosis [5,33–35]. Similarly, certain LPS structures (L3, L7 and L9) may also help immune evasion and are found more frequently in blood isolates compared with carriage isolates; the latter tend to express L1, L8 and L10 LPS immunotypes [36]. In addition, both capsule and certain immunotypes of LPS can influence the bacterial adhesion and invasion events which are discussed below.

Capsule

In meningococci, capsular genes are clustered within a single chromosomal locus, *cps*, divided into three regions. Region A encodes enzymes for the biosynthesis and polymerization of the polysaccharide, and regions B and C carry the genes responsible for its translocation from the cytoplasm to the cell surface [37].

The capsular polysaccharides of the serogroups B, C, W-135 and Y contain sialic acid [NANA (5-*N*-acetylneuramic acid); Table 1], and *cps* region A of these serogroups harbours a set of conserved genes *siaA*, *siaB* and *siaC*. These are responsible for the synthesis of sialic acid in the form of CMP-NANA, required for incorporation into the capsular polysaccharide. The fourth gene in this region, *siaD*, encodes a serogroup-specific polysialyltransferase involved in capsule polymerization [38,39]. In serogroup A, the locus contains four mannosamine biosynthesis genes designated *mynA–D* [40].

The incorporation of sialic acids into the capsule and LPS enables bacteria to become less visible to the immune system, as sialic acids are also commonly present on host

cell surfaces. The most striking mimicry, however, occurs in serogroup B capsule as this $\alpha(2-8)$ -linked sialic acid homopolymer is structurally identical with a component of human NCAM (neural cell-adhesion molecule), crucial for functional plasticity of the central and peripheral nervous systems. Such identity is responsible for the particularly poor immune response generated against serogroup B capsule by humans [41].

Variation of capsule expression

Genetic similarities in the structures of the capsule loci of serogroups B, C, W and Y (but not serogroup A) apparently favour horizontal exchange of portions of the capsule biosynthetic operon between different serogroups resulting in the phenomenon described as capsule switching [42]. As a consequence, any naturally acquired and vaccine-induced anti-capsular antibodies become ineffective in controlling the spread of the pathogen [43].

Capsule switching between serogroups B and C has reportedly arisen in several geographic areas through *in vivo* recombination during co-carriage and, overall, such events cannot be regarded as uncommon [9]. Capsule gene transfers from Y to B and C to W serogroups have also been observed [44,45]. This phenomenon has raised concerns about the immune pressure that serogroup C vaccination programmes may apply, leading to potential switching of hyperinvasive serogroup C strains to B, against which no vaccines are currently available. Recent studies from Spain and Portugal following serogroup C vaccination programmes have reported some capsule switching, but it is unclear whether the incidence is enhanced by vaccination [46]. It is noteworthy that immunization-associated capsule switching has not been observed in the U.K. [47].

On/off expression of capsule also occurs and is controlled via a number of genetic events including SSM of a poly-cytosine tract present within the *siaD* gene, resulting in premature termination of its translation [29]. Another mechanism involves reversible disruption of the sialic acid biosynthesis gene *siaA* by the precise integration and excision of an insertion sequence element, *IS1301* [29]. Phase variation of capsule influences bacterial interactions with target cells as its absence fully exposes subcapsular adhesins allowing manifestation of their full functional efficacy.

LPS

N. meningitidis LPS [also referred to as LOS (lipo-oligosaccharide)] comprises an inner and outer oligosaccharide core attached to lipid A. The inner core of meningococcal LPS consists of the diheptose (HepI and HepII) attached to lipid A, via one of the two KDOs (2-keto-3-deoxy-D-manno-2-octulosonic acids). The outer core is heterogeneous, composed of variable numbers of sugars extending from HepI, added by glycosyltransferases encoded by *lgt* genes (Figure 1) [48].

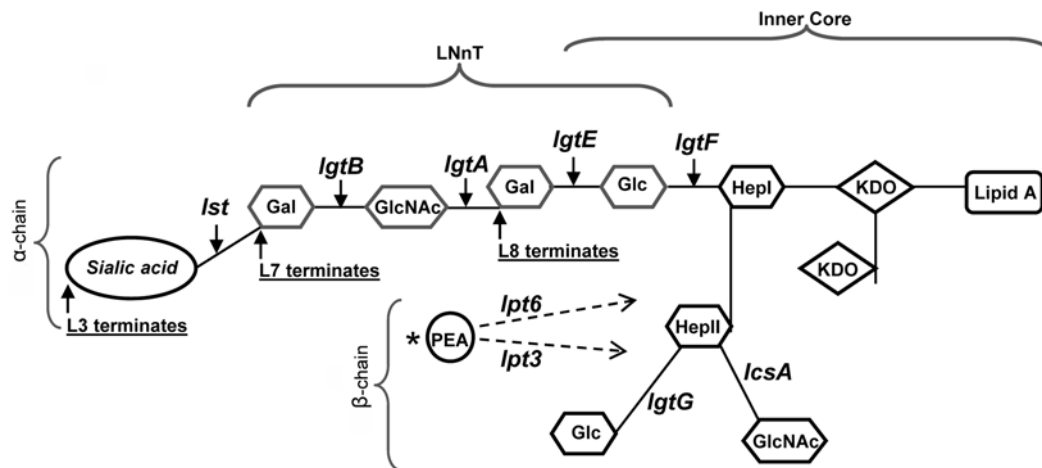


Figure 1 Schematic diagram showing structural organization of *N. meningitidis* LPS and some important determinants of immunotypes

The membrane-located part of meningococcal LPS comprises lipid A bound to two KDO and two heptose (HepI and HepII) moieties. Extended from the membrane are the structurally variable α - and β -chains of LPS. The different immunotypes of LPS are determined by variations both in the HepI α -chain extensions (Glc, Gal, GlcNAc, Gal and sialic acid) and the HepII β -chain extensions (GlcNAc, PEA and Glc). These arise as a result of phase variation in a number of genes shown and account for the antigenic variation of meningococcal LPS (see [48] for details). PEA may be added at position 3 or 6 on HepII by two distinct enzymes encoded by *ipt3* and *ipt6* [143]. The α -chain structures of the L3, L7 and L8 immunotypes are indicated. LNNt and silylation: the immunotypes L7/L9, L2/L4/L5 contain identical α -chains terminating in LNNt (Gal-GlcNAc-Gal-Glc structure bound to HepI) which can be sialylated. The L7 immunotype, found in serogroup B and C isolates, refers to the immunotype lacking sialic acid, whereas L3 is sialylated. Phase variation of the *igtA* gene gives rise to the immunotype L8, which cannot be sialylated [48].

Phase and antigenic variation of LPS

LPS antigenic variation is largely linked to phase variation of the genes that encode enzymes involved in the extension of saccharide chains linked to HepI. Variations in the chain composition dramatically alter antigenic properties of LPS and form the basis of its classification into different immunotypes (Figure 1) [48]. In addition, SSM of a poly-cytosine tract in the *igtG* gene determines the presence or absence of a glucose or PEA (phosphoethanolamine) at position 3 of HepII, which also affects its antigenicity [49]. Phase variation of one or more of the various LPS biosynthesis genes enables individual meningococci to display a repertoire of different LPS structures simultaneously [48].

Lactoneotetraose and LPS sialylation

Several LPS immunotypes (L3, L7 and L9) contain LNNt (lacto-*N*-neotetraose), comprised of galactose, *N*-acetylglucosamine, galactose and glucose linked to a HepI [50] (Figure 1). LNNt is found in virulent strains of meningococci and is an acceptor for sialic acid, which can be added to its terminal galactose residue by the product of the *lst* gene, the α -2,3-sialyltransferase [51]. In serogroups B, C, W-135 and Y that contain genes for sialic acid synthesis, endogenously produced CMP-NANA is used for incorporation into LPS. Other serogroups acquire sialic acid from exogenous sources, such as human serum and serous secretions, for this purpose [52]. Both LNNt and sialylated LPS mimic host-

cell-surface structures [53]. Besides its role in immune evasion [54], LPS sialylation may allow interaction with SIGLECs (sialic acid-binding, immunoglobulin-like lectins) located on myeloid cells [55].

Adhesins and invasins of *N. meningitidis*

N. meningitidis strains express a number of surface and secreted proteins that bind to human molecules. Such proteins include, among others, lactoferrin- and transferrin-binding proteins that enable meningococci to acquire iron, a crucial growth factor during colonization and disease (reviewed in [56]). Neisserial porins, whilst not considered adhesins, interact with numerous human cells and proteins. The *in vitro*-defined properties of porins could have implications in pathogenesis and generation of an effective immune response (reviewed in [57]). *N. meningitidis* expresses two distinct porins, PorA (formerly class 1 protein) and PorB (formerly class 2/3 protein based on molecular mass). Both porins are β -barrel proteins, which associate into trimers in the bacterial outer membrane through which small hydrophilic nutrients diffuse into the cell. Individual porins vary in molecular mass with PorA (~46 kDa) being expressed in all strains of meningococci, but may vary in its level of expression via an SSM mechanism, and PorB being expressed as one of two mutually exclusive forms: PorB2 (~41 kDa) or PorB3 (~38 kDa) [3]. The potential functions of these proteins in bacterial

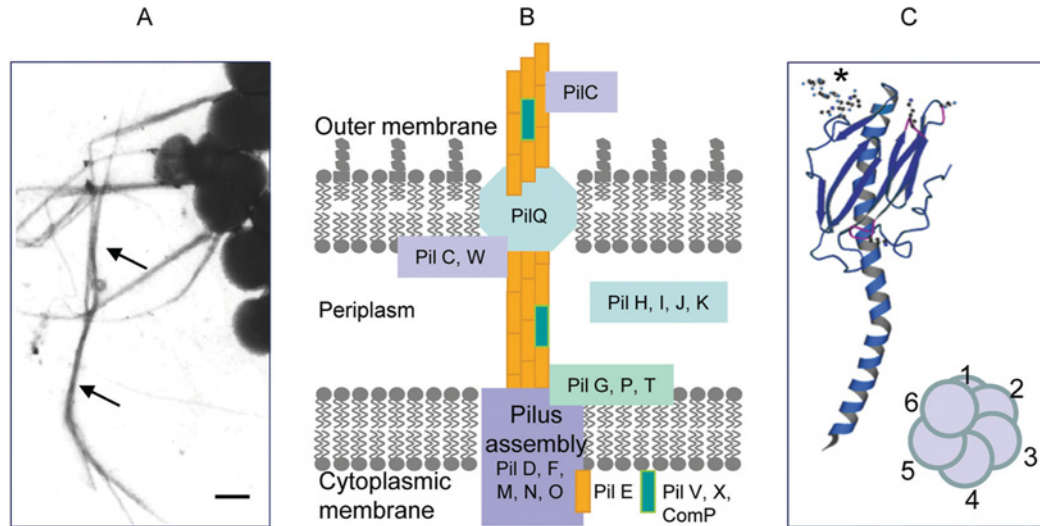


Figure 2 Pili of *N. meningitidis*

(A) Transmission electron micrograph of negatively stained preparations of a meningococcal strain (M. Virji and D.J.P. Ferguson, unpublished work). Bundles of hair-like pilus filaments stretch several micrometres from bacterial surfaces (arrows). The scale bar represents $0.5 \mu\text{m}$. (B) Schematic diagram indicating the relative cellular location of the gene products involved in pilus biogenesis. Several of these proteins (PilD, F, M, N, O and P) are implicated in the early stages of pilus synthesis, others (PilC, G, H, I, J, K, and W) may be necessary for functional maturation of the pilus. The pilus is extruded through the outer-membrane pore formed by PilQ. The remainder of the proteins play roles in pilus function. For instance, PilF and PilT (both inner-membrane-associated ATPases) appear to have opposing roles in pilus extension and retraction, and control pilus-associated functions, including twitching motility. PilX has been reported to be involved in bacterial aggregation and could have a role in colonization through promotion of microcolony formation by *N. meningitidis* (based on [60]). (C) Ribbon diagram of the three-dimensional structure of a pilin monomer of strain C311 (M. Virji and A. Hadfield, unpublished work) based on that of *N. gonorrhoeae* pilin [144]. The asterisk shows the structure and position of the unusual glycan modification (digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose) of the pilin [69]. Pili are made up of multiple pilin monomers stacked in a helical array, such that each helical turn is made up of five monomers (shown in a schematic representation of the fibre cross-section, bottom right).

dissemination are discussed in the latter half of the present review.

Meningococcal adhesins that enable bacteria to localize on specific host cells can be divided into major and minor groups. The major adhesins, pili and the opacity proteins, are expressed in abundance on the bacterial surface and have been studied for a considerable period. Genome sequencing has led to the discovery of several other adhesins, which are expressed normally at low levels *in vitro*; these may be up-regulated *in vivo*, but their potential roles in pathogenesis remain to be fully defined. *In vitro*, several meningococcal adhesive structures have also been shown to lead to cellular entry and can therefore also act as invasins. The molecular architecture and, where known, the binding properties of meningococcal adhesins are described below.

The major adhesins

Pili

Pili are hair-like projections and are considered primary adhesion factors utilized by Gram-negative and Gram-positive bacterial species [58]. Meningococcal pili belong to the type 4 pilus family, members of which undergo rapid extension and retraction and thus impart twitching

motility to bacteria expressing the fibres [59]. Besides these functions, they are involved in facilitating the uptake of DNA by bacterial cells [58]. Neisserial pili are $\sim 6 \text{ nm}$ in diameter and can extend several micrometres from the bacterial surface. They may also aggregate laterally to form bundles of pili (Figure 2A). Numerous genes (Pil C-X and ComP) have been implicated in the biosynthesis and various functions of meningococcal pili (Figure 2B) [60]. Although no structure is available for the meningococcal pilus, a high-resolution structure of the related gonococcal pilus filament has been reported, employing three-dimensional cryo-EM (electron microscopy) reconstruction based on the crystal structure of the pilin subunit ([61]; Figure 2C).

The pilus shaft is composed of identical pilin (PilE) subunits arranged in a helical array [61]. Pilin encoded by the *pilE* gene undergoes sequence variation by inter- and intra-genomic recombination with one of several truncated silent pilin genes (*pilS*) [27]. The resultant variations in pilin primary structure influence cellular interactions via the fibre [62,63]. A distinct minor pilus-associated protein, PilC, may also promote neisserial adhesion to host cells [64]. Recently, PilQ has been shown to act as an adhesin to target laminin receptor [65].

Besides PilE and PilC, potential functions of other pilin-like proteins in pilus biogenesis have been described (Figure 2) [60].

Post-translational modifications of pili

Neisserial pili have been shown to undergo distinct and unusual post-translational modifications. Different PilE modifications have been reported at several serine residues in meningococcal strains, including glycosylation at position 63 and an α -glycerophosphate at position 93. However, at position 68, the residue has been reported to be replaced by phosphate, PEA or ChoP (phosphorylcholine) [23,66–68]. Pilus glycosylation was the first protein glycosylation reported for a bacterial protein [69,70]. Although the roles of these various modifications remain unclear, several studies have implicated O-linked glycans in influencing cellular interactions, perhaps via effects on pilus/bacterial agglutination [69–71]. It has also been suggested that pilin glycosylation may promote secretion of the soluble (S) pilin subunits [71]. Secreted pilin, by competing for both anti-pilus antibodies and host cell receptors, could assist in the protection of bacteria against immune challenge, as well as promote spread by preventing further bacterial adhesion at the original site of colonization.

ChoP occurs as a surface component in many mucosal pathogens. When present on the bacterial surface it may be targeted by CRP (C-reactive protein) and anti-ChoP antibodies leading to bacterial clearance. As such, this component is of potential importance as a vaccine candidate to boost natural immunity. The presence of ChoP on pathogenic neisserial pili is somewhat unusual in that, in related bacteria, for example in commensal *Neisseria* and *Haemophilus influenzae*, ChoP is added to LPS [72,73]. Although the functional significance of neisserial pilus modification with ChoP remains unknown, it may promote bacterial adhesion through binding to the PAF (platelet-activating factor) receptor [74,75]. In addition, ChoP may play a role in niche-specific immune evasion, i.e. when expressed, in helping to resist antimicrobial peptides in the nasopharynx, whereas, when absent, in decreasing complement activation in the blood [75].

Receptors for pili

Direct interactions of piliated organisms with recombinant CD46, also known as membrane co-factor protein, have been reported [76]. Additionally, CD46-transgenic mice have increased meningococcal traversal of the nasopharyngeal epithelium, which results in increased septicaemia and meningitis compared with controls [25]. However, other studies using gonococci have suggested that pilus-mediated binding was not dependent on cellular CD46 expression levels [64,77]. The related gonococcal pili have been shown to bind to $\alpha 1$ and $\alpha 2$ integrins on urethral epithelial cells [78]. It is, therefore, possible that other factors besides CD46 are involved in

mediating meningococcal pilus interactions with human cells and tissues.

Opa and Opc

N. meningitidis strains commonly express two types of outer-membrane proteins, Opa and Opc, which impart an opaque phenotype to agar-grown colonies. Whilst Opc is only expressed by *N. meningitidis*, Opa proteins are expressed by both meningococci and gonococci. Opa and Opc are similar in size (27–31 kDa) and were initially known as Class 5 proteins. Meningococcal strains possess three to four *opa* loci, whereas gonococci possess in excess of ten loci, all of which can be expressed independently of each other [79,80]. Structurally, the Opa proteins are made up of eight transmembrane domains, arranged in a β -barrel, presenting four surface-exposed loops. The β -barrel constitutes a highly conserved region of these proteins, whereas three of the four surface loops are variable between different Opa proteins [81]. No crystal structure has been determined for the Opa proteins, but structurally they resemble Neisserial surface protein A (NspA; Figure 3A) [82].

The genes encoding Opa proteins undergo frequent phase variation of expression ($\sim 10^{-3}$). Such high-phase variability is due to an SSM of tandem CTCTT repeats present within all *opa* genes. Although the major antigenic variation results from the shift of expression from one *opa* gene to the next, antigenic differences between the Opa proteins can also arise through a variety of genetic events, for example point mutation, deletion, translocation and import from other members of the *Neisseriaceae*. Thus the expressed Opa type can alter randomly; however, certain Opa types may predominate in clinical isolates apparently due to their adhesion/virulence properties [83].

The Opc protein is encoded by a single gene, *opcA*, which is only expressed in *N. meningitidis*. The structure of Opc was solved in 2002 and, like Opa proteins, it is a β -barrel protein but with five surface-exposed loops (Figure 3B) [84]. The transcriptional control of Opc expression occurs through an SSM of a poly-cytosine tract in the promoter region of *opcA* [31].

Receptors for the opacity proteins

The majority of meningococcal Opa proteins recognize one or more members of the CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) family, a branch of the immunoglobulin superfamily [85,86]. Within this family of receptors, CEACAM1 is the most widely expressed, and is found on epithelial and endothelial cells, as well as cells of the immune system [87]. Other members, such as CEA and CEACAM3 are restricted to epithelial cells and neutrophils respectively, whereas CEACAM6 is expressed by both epithelial cells and granulocytes [87]. Meningococcal Opa proteins have been shown to bind to all these receptors [88]. However, the affinity and tropism of Opa proteins for distinct

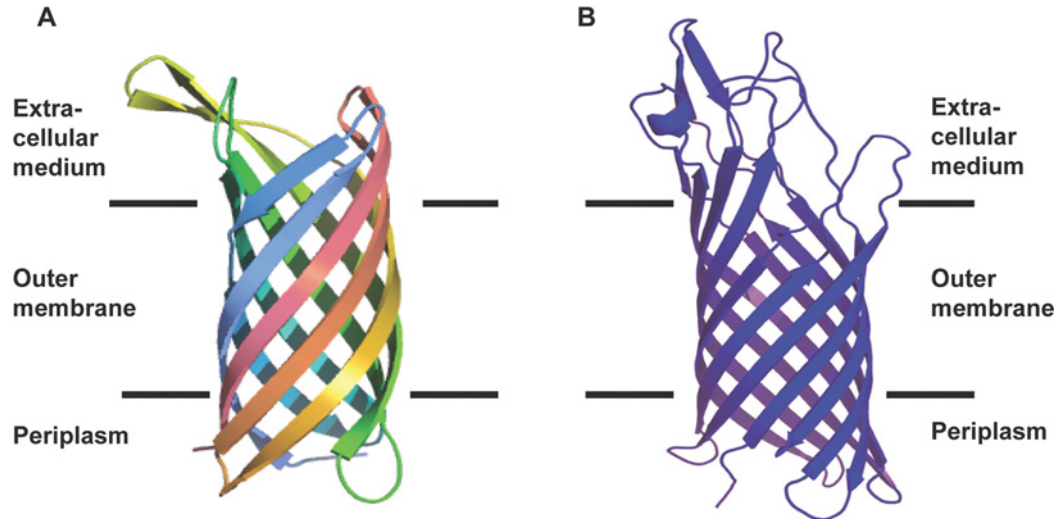


Figure 3 Structures of the β -barrel outer-membrane proteins NspA and Opc

(A) The ribbon diagram shown represents an Opa-like eight-stranded β -barrel with four surface-exposed loops. The structure is derived from the co-ordinates of an Opa-like molecule, NspA [82], and was kindly provided by Professor Leo Brady (Department of Biochemistry, University of Bristol, Bristol, U.K.). The transmembrane domains are highly conserved between NspA and Opa proteins. However, the flexible surface loops are dissimilar and, in Opa proteins, the first loop is semivariable (SV), whereas the second and third loops are more extensively variable (designated hypervariable: HVI and HV2). (B) Structure of *N. meningitidis* Opc protein presented as a ribbon diagram. Opc is a ten-stranded β -barrel presenting five largely invariant surface-exposed loops. This Figure was kindly provided by Professor Jeremy Derrick (Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology, Manchester, U.K.) and is reprinted by permission from MacMillan Publishers Ltd: *Nature Reviews Microbiology* [23], copyright (2009) (<http://www.nature.com/nrmicro/index.html>).

CEACAMs may vary, influenced by the sequence variation within the Opa variable loops. Minor variations in the receptor structure also influence bacterial interactions with the receptor [88–90]. CEACAMs are involved in a range of cellular processes and the signalling outcomes following Opa binding depends on the repertoire of CEACAM family members present on target cells and the type of Opa protein expressed. In addition, the level of CEACAM expression may influence the outcome of bacterial interaction. For example, CEACAMs may be expressed at low levels on target tissues, but are subject to up-regulation under the influence of inflammatory cytokines [91,92]. Such increased receptor density has been shown to increase the strength of bacterial interaction and leads to increased cellular invasion by virulent forms of meningococci [93]. Hence increased CEACAM density resulting from inflammation could be a factor that increases host susceptibility to meningococcal infection and, thereby, responsible for the temporal association reported between influenza A viral infection and meningococcal disease [2].

In addition to CEACAMs, some meningococcal Opa proteins may also interact with cell-surface-associated HSPGs (heparan sulfate proteoglycans) [88]. There are two major groups of HSPGs, the GPI (glycosylphosphatidylinositol)-linked and the transmembrane syndecan family. The latter are present on most epithelial cells, and different Opa-expressing

isolates derived from a single strain have been shown to bind to human conjunctival epithelial cells in a heparin-sensitive manner, presumably via syndecans [88]. Tyrosine residues in HV2 (hypervariable 2) of Opa proteins have been implicated in binding to saccharides, with differing binding specificities observed between distinct Opa types [94].

Opc–host interactions

Initially meningococcal Opc was shown to mediate adhesion and invasion of human endothelial cells by the formation of a trimolecular complex primarily involving serum vitronectin and, to a lesser extent, fibronectin and their corresponding integrin receptors [95,96]. More recently similar observations primarily involving fibronectin and human brain endothelial cell integrins have also been reported [97]. Opc is also able to mediate adhesion to and invasion of epithelial cells in the absence of serum proteins by binding to HSPGs [98]. Binding to HSPGs may involve a basic cleft formed by the surface loops of Opc [84,94]. It has been suggested that Opc requires heparin to target vitronectin [99]. In addition, recently, a novel mechanism of vitronectin targeting by Opc has been identified requiring the activated unfolded form of vitronectin, which reveals its otherwise cryptic tyrosine-sulfated moieties required for Opc interactions (C.S. Cunha, N.J. Griffiths and M. Virji, unpublished work). This form of vitronectin may be conceivably generated

during meningococcal sepsis [100]. Availability of an increased supply of activated vitronectin to meningococci in the blood stream could enhance cellular interactions at the brain and vascular endothelial interfaces, and increase the bacterial potential to traverse these cellular barriers. Whether this occurs *in vivo* remains to be shown.

Minor adhesins

Several of the novel adhesins more recently identified belong to the autotransporter family of molecules (reviewed in [101]). Of these, NadA (Neisserial adhesin A), an OCA (oligomeric coiled-coil adhesin), was first identified as a novel vaccine candidate by genome mining and later shown also to possess adhesive properties. NadA interacts with human epithelial cells through protein–protein interactions, but the nature of the receptor is unknown [102]. Although phase variable, NadA may contribute to bacterial virulence as it is expressed by ~50% of disease isolates compared with ~5% of strains isolated from healthy individuals [103]. Two other proteins, NhhA (*Neisseria hia* homologue A) and App (adhesion and penetration protein) are widely expressed in virulent *N. meningitidis* strains. NhhA mediates low levels of adhesion to epithelial cells, HSPGs and laminin. App, which may be processed and released, may aid bacterial colonization as well as spread [104]. MspA (meningococcal serine protease A) is homologous to App and may also be cleaved and secreted. It is expressed by several, but not all, virulent meningococcal lineages and may support binding to both epithelial and endothelial cells [105]. No receptors have been identified for either App or MspA to date.

MENINGOCOCCAL PROGRESSION FROM THE NASOPHARYNX TO THE MENINGES

An overview of meningococcal and host factors that may be involved at distinct stages of meningococcal–host interactions are shown in a schematic form in Figures 4–6.

Colonization and penetration of the respiratory mucosa

During transmission, *N. meningitidis* is believed to be encapsulated which may enhance survival of the organism outside the host [41]. Although one study has demonstrated that encapsulated *N. meningitidis* has the potential to survive for several days *ex vivo* [106], it is also possible that acapsulate organisms can pass from person to person over short distances and by direct contact. Meningococcal strains carried by asymptomatic individuals in most non-epidemic situations may be capsulate or acapsulate, whereas, in epidemic situations, such as those that occur regularly in the sub-Saharan meningitis belt, carriage of capsulate phenotypes is more common [107,108].

From *in vitro* observations, the following potential interactions may be surmized, but *in vivo* evidence is

lacking for most presumed events. In considering the primary events, it would appear reasonable to assume that a firm and fast adhesion to mucosal epithelial cells is essential for the pathogen to avoid being flushed away by the flow of mucus. The adhesive properties of capsulate *N. meningitidis* are primarily mediated by pili which extend beyond the capsule and initiate binding to non-ciliated epithelial cells [26,58,63].

Although the capsule promotes bacterial survival by resisting the environmental and host factors discussed above, it may adversely affect bacterial ability to colonize as it can sterically hinder the surface-expressed adhesins and thus prevent more intimate cellular interactions. Thus phase variation in capsule expression that may occur via the genetic mechanisms described above could be beneficial following initial attachment by pili. Acapsulate phenotypes arising at this stage can engage more intimately with cells via the outer-membrane proteins, including Opa and Opc, aiding barrier penetration. However, although pili are considered to be primary adhesins in capsulate phenotypes, under some circumstances, outer-membrane proteins may also come into play in fully capsulated phenotypes. As mentioned above, at high levels of CEACAM expression induced during inflammation, cellular invasion can occur in an Opa–CEACAM-dependent manner even in capsulate organisms, a process that is synergized by pili [93,109]. Thus, in some circumstances (such as inflammation induced by a prior viral infection), encapsulated meningococci may penetrate the epithelium and enter the blood without the need for capsule down-modulation. Opa–CEACAM1 interactions also promote epithelial cell attachment through up-regulation of endoglin (CD105) and co-operation with β 1 integrins, thus overcoming potential innate epithelial shedding mechanisms to remove infected cells [110].

As for Opa proteins, in unencapsulated meningococci, Opc is able to facilitate adhesion to and invasion of epithelial and endothelial cells independently of other adhesins [111]. Following Opc engagement with endothelial integrins, a number of signalling events result in the internalization of *N. meningitidis* [95,96] and release of the cytokines IL (interleukin)-6 and IL-8 [112].

In recent studies, Opc was shown to bind to the cytoskeletal protein α -actinin of both epithelial and endothelial cells following cellular invasion [113]. Up-regulation of α -actinin in the late stages of endothelial cell infection has also been observed *in vitro* [114] and raises the question as to which role this cytoskeletal protein might play in the course of meningococcal disease.

Besides the possible interactions described above, a number of the minor adhesins are also likely to support bacterial colonization and invasion of the mucosal barriers. However, whether this occurs in concert or independently of the major adhesins *in vivo* remains to be determined.

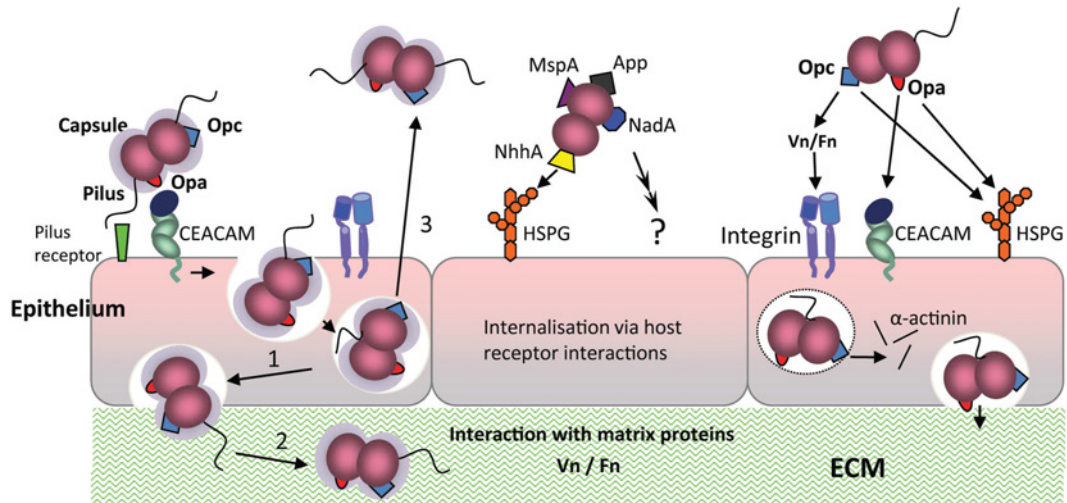


Figure 4 Schematic overview of meningococcal interactions at the epithelial barrier of the nasopharynx and the mode of barrier penetration

Encapsulated (and possibly acapsulate) meningococci inhaled via respiratory droplets must first adhere to the epithelium within the nasopharynx to avoid removal by innate immune mechanisms such as mucus clearance. Pili extending beyond the capsule are considered to mediate the primary interaction with epithelial cells. Capsule down-modulation (or up-regulation of host receptors during inflammatory condition) allows interactions between outer-membrane proteins and their cognate host receptors. For example, Opa proteins may bind to CEACAMs and HSPGs, and Opc proteins can interact with HSPGs and, via vitronectin and fibronectin, to their integrin receptors. Although some minor adhesins such as NhhA have been shown to interact with HSPGs, the receptors targeted by MspA, App and NadA remain to be determined. Engagement of CEACAMs, integrins and HSPGs can result in meningococcal internalization by epithelial cells (1) by triggering a variety of host cell signalling mechanisms. Meningococci can be found in subepithelial tissue (2) in healthy individuals thus cellular entry or otherwise traversal across the epithelium may not be an unusual event. In addition, the fact that meningococci can interact with subcellular proteins such as α -actinin may also lend some support to this notion, although the role of this interaction *in vivo* remains unclear. On crossing the epithelial barrier, meningococci are able to interact further with proteins of the extracellular matrix including fibronectin (Fn) and vitronectin (Vn). Internalized bacteria may also migrate back to the apical surface for transmission to a new host (3). An animated version of the Figure is available at <http://www.ClinSci.org/cs/118/0547/cs1180547add.htm>.

N. meningitidis is subject to constant selective pressures and its ability to adapt rapidly to environmental challenges is essential for its survival [115]. Phase and antigenic variation of a number of surface components permits immune evasion during infection. This also has the potential to generate variants with an altered ability to colonize and heightened ability to penetrate the mucosal barriers [86,116]. In addition, the invasive ability of meningococci could also enable the bacteria to avoid host immune mechanisms by entering epithelial cells. Indeed, *N. meningitidis* has been found in tissues underlying the mucosal surface in healthy individuals [117]. Whereas, in an immune host, further dissemination from such a site would be prevented by active serum bactericidal and other defences, in a susceptible host any meningococci traversing the epithelial barrier could survive and spread via the vasculature.

Haematogenous spread

Within the blood stream, meningococci produce a strong inflammatory response and activate the complement and the coagulation cascades. A key inducer of cellular inflammatory responses, LPS, is pivotal in causing meningococcal sepsis [118]. LPS-induced secretion of

various cytokines within the vasculature ultimately leads to endothelial damage and capillary leakage, leading to necrosis of peripheral tissues and multiple organ failure [119]. A relationship between circulating levels of LPS and mortality rates in meningococcal disease has been demonstrated [120].

The lipid A moiety of LPS is the active component responsible for eliciting the inflammatory response associated with meningococcal sepsis. LPS induces the release of several cytokines, including IL-6 and TNF- α (tumour necrosis factor- α), as well as chemokines, ROS (reactive oxygen species) and NO, acting in part through TLR (Toll-like receptor) 4 [121,122]. Natural LPS variants lacking a single acyl chain engage less well with TLR4, yet can cause clinical disease and so may be better placed to evade the innate immune system [123].

In the blood, *N. meningitidis* encounters numerous host killing mechanisms, including antibody/complement-mediated lysis, as well as opsonophagocytic killing. Disruption of genes associated with capsule and LPS synthesis results in an increase in meningococcal sensitivity to serum killing, indicating the importance of these polysaccharides for survival in the blood [124]. The amount of polysaccharide capsule expressed also

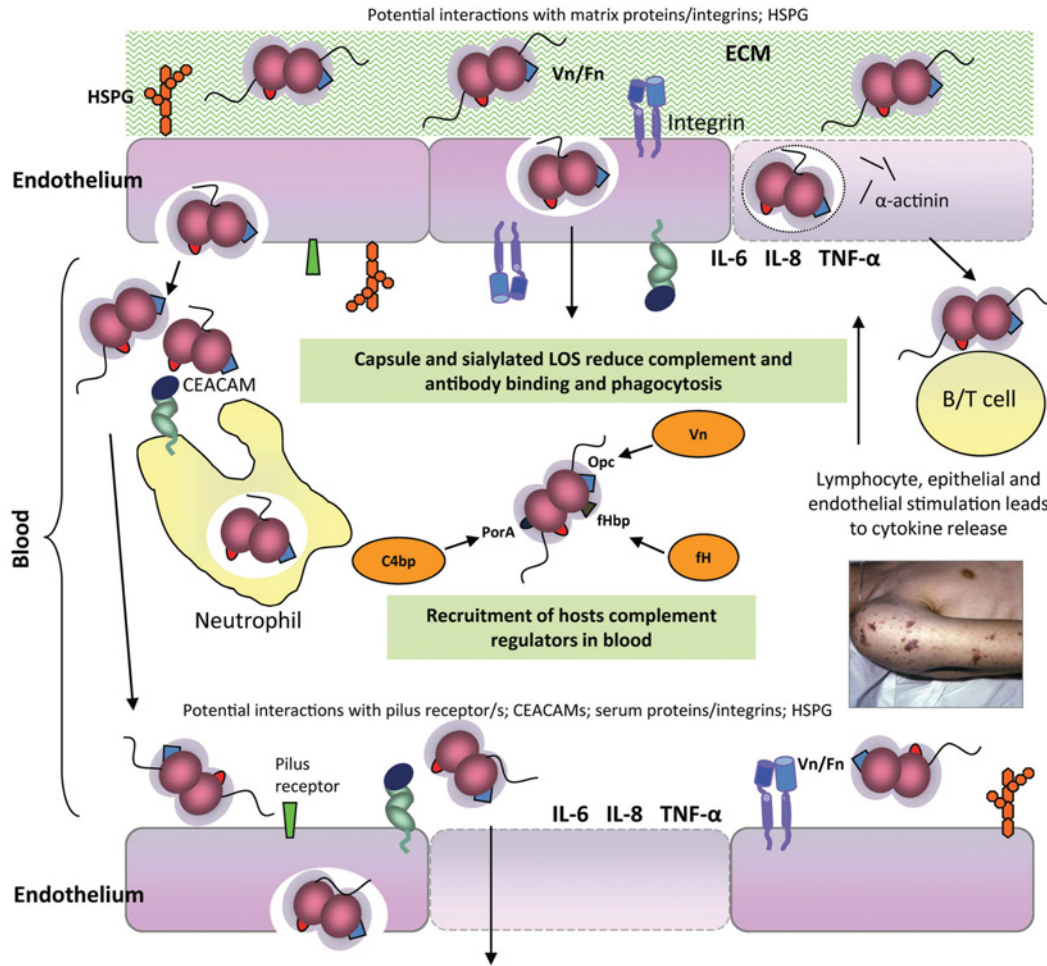


Figure 5 Meningococcal entry into and survival within the vasculature

Capillaries in close proximity to mucosal epithelial tissues are a possible point of entry into the blood for *N. meningitidis*. *In vivo*, meningococci initially encounter the basolateral surface of endothelial cells and need to traverse in a basal to apical direction to enter the vasculature. The *in vitro* studies, however, do not allow easy examination of basal interactions as cultured cells present their apical surfaces to the media. Both integrins and HSPGs are known to be expressed on the basolateral surface of endothelial cells and, hence, are likely targets for vascular penetration. However, it should be noted that these receptors are also expressed apically and are also probably involved in the exit from the bloodstream. Once in the blood, only capsulate meningococci appear to survive; whether acapsulate bacteria arising naturally can survive in any microenvironment is not known. In addition, meningococci are able to bind to a number of negative regulators of complement such as C4bp, factor H and vitronectin. Acquisition of such factors could lead to decreased complement-mediated killing *in vivo*. Interactions with vascular cells via protein adhesins and their cognate receptors and via LPS–TLR4 provoke an inflammatory response leading to cytokine release and cellular damage. This could increase further cell barrier penetration and leakage, which accounts for the damage and clinical symptoms observed during meningococcal sepsis, typified in latter stages by a petechial rash (see inset; from meningitis.org). LPS has also been shown to be toxic for human endothelial cells *in vitro* [40].

appears to influence meningococcal resistance to host killing [125].

Negative regulators of complement can be recruited by meningococci to promote their survival. Factor H is recruited by fHbp (factor H-binding protein; also named GNA1870), a 27 kDa lipoprotein which is expressed by all meningococcal strains and which promotes serum resistance [126]. The porin PorA of meningococci can also bind a complement regulator, C4bp (C4-binding protein), and influence serum resistance. However, capsule may inhibit C4bp binding to PorA [33].

It has been suggested that both PorA and PorB may be involved in bacterial uptake via re-arrangement of the cytoskeleton [127]. Porins may also act via TLR2 as an adjuvant leading to the stimulation of B-cells [128]. It has also been demonstrated that PorB has an anti-apoptotic effect on epithelial cells, by localizing in the mitochondrial compartment, enhancing survival of the cell upon apoptotic stimuli [129]. Porins therefore appear to have multiple roles in meningococci from aiding not only colonization, but also survival in the blood.

Besides facilitating entry into the vasculature, some bacterial adhesins may also function directly in resisting

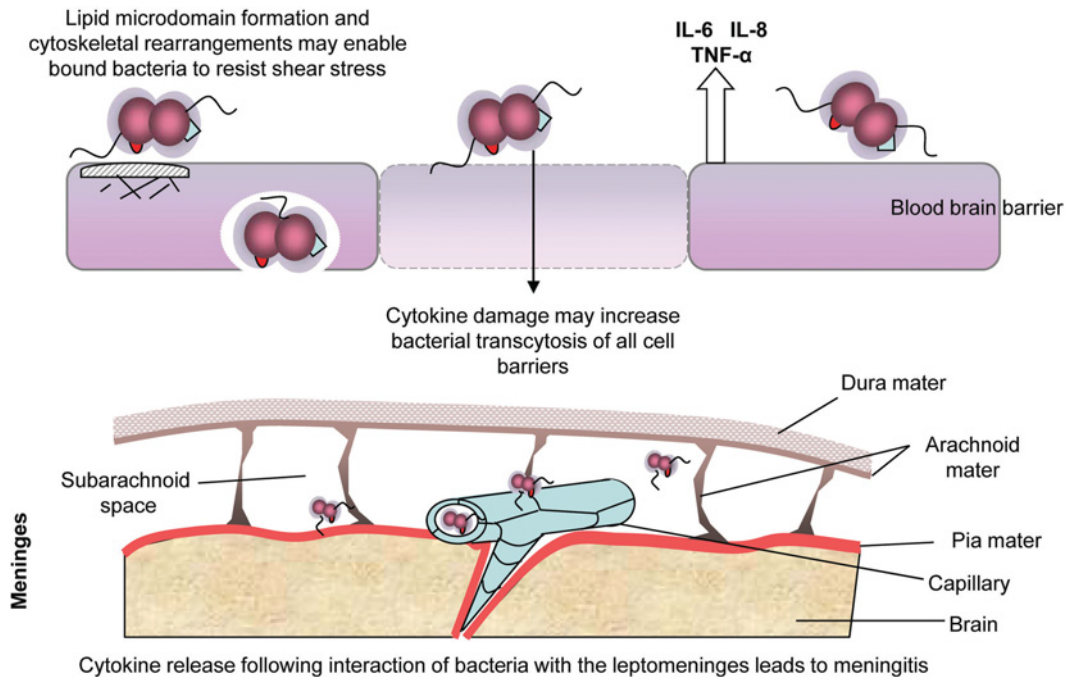


Figure 6 Meningococcal penetration of the BBB and interaction with the meninges leading to meningitis

In areas of low blood flow and, hence, low shear stress, meningococci have been shown to adhere to the vasculature within the brain [139]. In addition, cytoskeletal re-arrangements leading to lipid microdomain formation could facilitate resistance to shearing forces once bacteria are bound [145]. Whether bacterial transmigration in this niche is predominantly by transcytosis across intact cell barriers or requires damage to the barrier (for example, due to the action of pro-inflammatory cytokines) for ease of passage is unknown, although more than one mechanism may operate. Once the BBB is breached, meningococcal interaction with cells lining the leptomeninges leads to the release of pro-inflammatory cytokines [9,108,142], provoking an inflammatory reaction resulting in meningitis.

complement-mediated killing. Vitronectin inhibits the formation and insertion of a MAC (membrane attack complex) into bacterial membranes. In binding directly to vitronectin, Opc-expressing bacteria are able to resist serum-mediated killing [130]. Thus meningococci have the means to interact with several regulators of the complement pathways which could lead to increased bacterial survival in the blood.

N. meningitidis can bind to and influence cells within the vasculature. Peripheral blood mononuclear cells from individuals immunized with a range of outer-membrane proteins of *N. meningitidis* have a higher proliferative response to Opa than to other neisserial proteins [131]. Another study demonstrated a suppressive effect on T-cell activation and proliferation in response to Opa-containing meningococcal OMV preparations [132]. However, no such deleterious effects of Opa-containing OMVs used as vaccines have been reported [133]. Recent studies have also shown Opa-independent proliferation of T-cells in the presence of *N. meningitidis* [134]. Thus the influence of Opa proteins on immune cells is unclear and whether the Opa receptor CEACAM1, which is expressed on stimulated T-cells is involved, remains to be clarified.

Engagement of CEACAM3 by Opa-expressing *N. gonorrhoeae* has been postulated to result in increased

cell death of neutrophils during infection [135]. Such interactions of *N. meningitidis* could also lead to evasion of killing by promotion of neutrophil cell death, but this remains to be investigated.

Of the minor adhesins, NadA-expressing *Escherichia coli* adhere to and activate human monocytes and macrophages. In addition, purified NadA induced high levels of TNF- α and IL-8 production by these cells [136]. Recent work has demonstrated that OMVs containing NadA possessed enhanced immune stimulation compared with controls, suggesting an additional role for this adhesin in septic shock ([137] and references therein).

In conclusion, it is widely believed that the key players in meningococcal survival in the blood include capsule and LPS. In addition, proteinaceous adhesins also play important roles in entry to and exit from the vasculature and may also modulate immune responses. Notably, however, perceptible bacteraemia is not required for meningitis to follow, although the vasculature is considered the primary route to the brain [138].

Reaching the meninges

Two structures make up the BBB: first, the choroid plexus, located in the ventricles and formed by cuboidal epithelial cells with tight junctions; and, secondly, the capillary endothelia also having tight junctions. Adhesion

in the vasculature is greatly influenced by the flow rate and shear stresses imposed on meningococci. On a post-mortem histological examination of one individual, meningococci were observed adherent to capillaries with low rates of cerebral blood flow [139]. Using an *in vitro* model in which endothelial cells were subjected to various shear stresses, the investigators concluded that pili play a major role in maintaining adherence to endothelial cells under high flow conditions (although low shear rates are needed for initial attachment) [139]. Following attachment, a small number of piliated bacteria are internalized by endothelial cells. These may transcytose further to enter the meninges. Alternately, it is possible that signalling in endothelial cells induced by pili may lead to disruption of the intercellular junctions enabling meningococcal passage [60]. An alternate route involves endothelial damage by LPS-mediated cytopathic effects, a process that has been shown *in vitro* to be enhanced by the presence of pili [140].

In addition to pili, other adhesins discussed above may also function in bacterial adhesion to and penetration of the BBB. Indeed, *in vitro* experiments have shown that meningococci lacking the Opc protein were unable to traverse human brain microvascular endothelial cell monolayers [97].

To examine meningococcal interactions within the CNS (central nervous system), a meningioma model has been established, representative of cell layers covering the pia mater and arachnoid membranes (the leptomeninges) [141]. In capsulate bacteria, pilus interactions dominate, yet, in the presence of certain pilus structures with lowered adhesion capacity, Opa proteins also mediate adherence of capsulate meningococci to the meningioma cells [141]. In comparison with *N. lactamica*, a commensal species rarely associated with meningitis, meningococcal adherence to meningioma cells was higher and meningococci caused greater damage to the cell monolayers [142]. Increased production of the cytokines IL-6 and IL-8 and decreased expression of the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted; CCL5) were also observed in meningioma cells infected with *N. meningitidis* compared with those infected with *N. lactamica* [142]. Species-specific responses, in terms of cytokine production and cell damage, point to specific bacterial factors and possible host receptors in the inflammation of the meninges. Thus, in common with colonization and survival in the blood, CNS events resulting in meningitis are likely to involve dynamic interactions of several bacterial factors acting in a co-operative manner.

CONCLUSIONS

N. meningitidis encounters a number of challenges during transmission, colonization and disease development in

humans. These organisms have evolved to colonize humans specifically and, in doing so, have acquired a range of virulence factors to enable survival within their chosen niche. Normally, meningococci are transient visitors of the human nasopharynx, but on occasion they can cause devastating disseminated diseases such as septicaemia and meningitis in susceptible individuals. The present review has described a number of surface structures expressed by *N. meningitidis* during colonization and the course of pathogenesis. Several of these structures are likely to come into play repeatedly during mucosal colonization haematogenous spread and the infiltration of the meninges. Although considerable advances have been made in our understanding of meningococcal disease, the majority of studies (that have been, of necessity, conducted *in vitro*) have examined the impact of individual bacterial components on cultured host cells in isolation; far fewer studies have either examined the co-ordinate action of multiple meningococcal components in cell adhesion and invasion or employed whole tissue models. Host factors, including genetic determinants as well as lifestyle, influence an individual's susceptibility to meningococcal disease. The full dynamic spectrum of the bacterial components that may facilitate different stages of infection, and their interplay and orchestration during the course of pathogenesis are likely to be considerably more complicated than we currently understand. There is still much to unravel about the organism: in particular, what precisely determines whether it will establish a commensal or a pathogenic relationship with its only host.

ACKNOWLEDGEMENTS

We would like to thank Professor Leo Brady, Dr Andrea Hadfield (Department of Biochemistry, University of Bristol, Bristol, U.K.), Professor David Ferguson (Nuffield Department of Clinical Laboratory Science, Oxford University, Oxford, U.K.) and Professor Jeremy Derrick for their contribution to data and images included in the present review.

FUNDING

The authors' work cited in the present review has been funded by the Wellcome Trust, the Medical Research Council, the Meningitis Research Foundation, Meningitis UK and GlaxoSmithKline.

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Received 12 October 2009/3 December 2009; accepted 7 December 2009
Published on the Internet 9 February 2010, doi:10.1042/CS20090513