Opacity proteins of Neisseria meningitides: structure-function relationship and vaccine potential

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Citation for published version (APA):
CHAPTER 1 General introduction
**1 Neisseria meningitidis: a human pathogen**

1.1 From commensal to invasive pathogen

The gram-negative diplococcal genus *Neisseria* is included in the group of β-Proteobacteria (a class that also includes *Bordetella, Burkholderia, Kingella* and *Methylomonas* species) and is closely related to the γ-Proteobacteria, comprising *Vibrio, Haemophilus* and *Escherichia* species. Most neisserial species such as *N. lactamica, N. subflava, N. cinerea* and others are commensals. However, two species are important causes of disease in humans, these are *N. meningitidis* and *N. gonorrhoeae*, which reside at different mucosal sites of the human body (24). Although closely related they cause very different diseases. *N. meningitidis* was first isolated in pure culture from a meningitis patient in 1887 (291).

Asymptomatic nasopharyngeal carriage of *N. meningitidis* is a common phenomenon. Estimations of carriership range from 5 to 10 % in the normal population during non-epidemic periods (38, 88), changing from season to season and depending on the geographical region (44, 96). The median period of carriage is 6 months ranging from 3 to 16 months (5, 59, 206). The estimations of carriership are based on results from cultures of nasopharyngeal swabs on selective media. However, recent studies have shown that this widely used method leads to a substantial underestimation of carriage rates (98, 126, 228).

In a small minority of meningococcal carriers infection progresses to life-threatening invasive disease. Although the causes leading to invasive disease are unknown, a few predisposing or risk factors have been established, such as passive or active smoking (51, 74, 136, 233), viral infections (76, 203) and certain immune defects, especially genetic defects in the complement pathway (72, 77). Crowded living conditions increase the transmission rate of meningococci, thereby leading to higher carrier as well as disease rates (68, 206) especially in individuals visiting various geographic areas (288). Strikingly, the prevalence of meningococcal disease is the highest in the first four years of life and among teenagers (43).

*N. meningitidis* may be described as being either a commensal with pathogenic features or as a pathogen with commensal features. During carriage some meningococci invading the nasopharyngeal epithelium may also gain entrance into the bloodstream. It is thought that in most cases meningococci are then efficiently killed. Although certain meningococcal characteristics determine the ability to invade, host factors play also an important role. It is rather a combination of bacterial properties and host defence factors that disturb the balance of carriage leading to meningococcal bacteraemia, meningitis and septicemia characterised by massive disseminated inflammatory responses.
1.2 Epidemiology

Meningococci have been classified into serogroups based on the immunological reactivity against the polysaccharide capsule constituents (90, 118). In total, 13 different serogroups have been identified, but serogroups A, B, and C account for the most cases of meningococcal disease worldwide. Serogroups B and C are responsible for the majority of cases in Europe and the Americas, while the serogroups A and C predominate throughout Asia and Africa (49, 170, 205). In the U.S.A. and some other western world countries serogroup Y is also a predominant cause of meningococcal disease (200, 207, 238). The first international outbreak of serogroup W135 directly associated with the annual Islamic pilgrimage to Saudi Arabia (the Hajj) occurred in 2000 (2, 288).

Meningococcal disease occurs in western countries year-round, but the majority of cases develop during winter and early spring (211). On the African continent in the area extending from Senegal to Djibouti (the meningitis belt) meningococcal disease occurs mostly during the dry season (40, 229). The pattern of serogroup B disease is typically hyperendemic or sporadic and contrasts with the classical epidemic nature of the serogroup A disease causing 10,000 or more deaths in a single outbreak (49, 104, 211). Within serogroup A several “subgroups” were identified by multilocus enzyme electrophoresis (MLEE) with different epidemiological patterns. Some subgroups were exclusively found in one geographic region (subgroups V and VII in China), whereas others were responsible for repeated pandemics (subgroup III in China to Moscow, Finland, Norway and Brazil). Also for serogroup B and C meningococci MLEE has been used to identify three epidemic lineages consisting of related electrophoretic types (ET), which are specific combinations of different alleles, called the ET-5 complex, the ET-37 complex and the A4 cluster (1) (later confirmed with multilocus sequence typing, MLST) (151).

1.3 Pathogenesis

Meningococci attach to the microvillus surface of non-ciliated columnar mucosal cells of the nasopharynx, where they colonise (237). From there meningococci are transmitted by aerosols or secretions to other persons. Several bacterial cell wall constituents promote the adherence to the nasopharyngeal epithelium. Binding stimulates engulfment of the meningococci by epithelial cells and might lead to intracellular survival within the phagosome (129, 239). Most phagosomes are targeted to fuse with lysosomes, which are acidic and contain microbicidal compounds. Lin et al. (1997) and Hopper et al., (2000) have found, that N. meningitidis and N. gonorrhoeae are able to escape this lysosomal killing. How the bacteria reach the subepithelial space and gain access into the bloodstream is yet to be further established, although disruption of the integrity of the epithelial cell
layer by cytotoxic factors has been suggested as a mechanism (239), but others do not support this hypothesis (282).

1.4 Disease

In most persons, meningococcal carriage is an immunising process, resulting in a systemic protective antibody response (209, 241). In a small number of persons the meningococci gain access to the bloodstream and traverse the blood-brain barrier (23, 178). As a result of released outer membrane vesicles (blebs) and fragmentation of bacterial cell membranes, both containing high amounts of endotoxin or lipopolysaccharide (LPS), meningococcal septicaemia can lead to rather high LPS concentrations in blood and cerebrospinal fluid (8, 31, 32, 263). The sudden onset of headache, fever and stiffness of the neck characterise the clinical manifestations of meningitis, occurring in 75 to 90% of patients with meningococcal disease (201, 283). The bacteria can be isolated from the bloodstream in up to three quarters of patients, but meningococcal septicaemia occurs in only 5 to 20 percent of patients (86, 211). Meningococcal septicaemia is characterised by an abrupt onset of petechial or purpuric rash, which may progress to purpura fulminans and high fever and is often associated with the rapid onset of hypotension, acute adrenal haemorrhage (Waterhouse-Friderichsen syndrome) and multi-organ failure (86).

Despite treatment with appropriate antimicrobial agents and optimal medical care, the overall case fatality rate among patients with meningococcal disease have remained relatively stable over the past 20 years. Among meningitis patients the case fatality rate is 5 – 6% while among patients with meningococcal septicaemia the case fatality rate is around 40% (212).

2 Bacterial surface structures

2.1 Lipopolysaccharide (LPS)

Meningococci typically display a double membrane structure characteristic for gram-negative bacteria, with an inner and outer membrane separated by a peptidoglycan layer and the periplasmic space. The cytoplasmic or inner membrane is a phospholipid bilayer. The outer membrane is asymmetric with phospholipids in the inner leaflet and phospholipids and LPS (also called lipooligosaccharide or LOS) in the outer leaflet. *Neisseria* LPS is anchored to the membrane by a hydrophobic lipid A part, while the hydrophilic oligosaccharide core is surface exposed (83, 137). The biosynthesis of meningococcal lipid A has been explored thoroughly, leading to the discovery of the first LPS-deficient gram-negative bacterium by inactivation of an acyltransferase, LpxA (234, 235, 262). The oligosaccharide part of LPS is highly variable and antigenic variation in this structure is the
basis for immunotyping (99, 125). There is no evidence for the existence of O-side chains in meningococcal LPS.

The terminal lacto-N-neotetraose structure of LPS can be endogenously sialylated. Sialylated LPS has been reported to inhibit entry of both meningococci and gonococci into epithelial cells (102, 266). Asialylated LPS containing lacto-N-neotetraose was found to facilitate the invasion of gonococci into a cervical epidermoid carcinoma cell line (231). The asialoglycoprotein receptor was found to be the receptor for this defined LPS structure (195, 196). Sialylation does also play a role in the serum resistance of gonococci as well as of meningococci due to the resemblance of sialic acid to components present in human serum, leading to binding of factor H (198, 264).

2.2 Polysaccharide capsule

In almost all meningococci isolated from patients, the bacterial cell is enclosed by a polysaccharide capsule. In contrast the closely related bacterium Neisseria gonorrhoeae, causing gonorrhoea, does not synthesise a capsule (149).

The capsular polysaccharide of meningococci is the major component that contributes to the bacterial survival in the blood stream and the cerebrospinal fluid. (64). Serogroup B isolates recovered from blood produce more capsular polysaccharide than isolates from the nasopharynx (52). It has been observed by Jarvis and Vedros (1987) and Frosch et al., (1989) that the capsule mediates resistance to phagocytosis and complement-mediated bacteriolysis. Strong evidence has been found that the presence of a capsule thwarts the process of entry and transcytosis of the nasopharyngeal epithelial cells. After intimate adhesion capsule synthesis is downregulated (103, 240, 274, 276).

2.3 Pili

Pili are filamentous hair-like protrusions extending from the bacterial surface and are found to play an important role in the initial attachment of the bacteria to host cells (214, 215, 176, 177) but are also implicated in DNA uptake (135). Meningococci express two different classes of pili, class I and class II, which are antigenically and structurally distinct (106, 274).

These long-range adhesins are composed of pilin encoded at the pilE locus. Pilin is a single, repeated protein subunit of 145 –160 amino acids. The high-molecular PilQ multimer was found to be required for type IV pilus biogenesis (251). A model of the type IV meningococcal pilus fibre, based on the X-ray crystal structure of the N. gonorrhoeae pilin subunit (184), fitted neatly into the cavity formed by the multimeric PilQ complex. This demonstrated that PilQ may function as a channel for the growing pilus fiber (47). The lipoprotein PilP has been found to facilitate and stabilise PilQ expression in the outer membrane (65). A vital but not yet defined role in type IV pilus biogenesis and function is played by TspA, a newly discovered highly
conserved protein, which is constitutively expressed and surface exposed (6). Furthermore two 110 kDa proteins, PilC1 and PilC2 are essential for piliation. The current model considers PilC1 as a type IV pilus tip-located adhesin (215). The fibre subunit-like protein termed PilV is another pilus protein involved in adhesion, by promoting the functional display of PilC in the context of the pilus fibre. PilT functions as an antagonist of pilus assembly and is critically involved in twitching motility and pilus retraction (160, 161, 292, 293).

The formation of clusters, or plaques of proteins within and immediately subjacent to the epithelial cell surface is elicited by the type IV pili of pathogenic *Neisseriae*. Such cortical plaques are enriched in components of both the cortical cytoskeleton and a subset of integral membrane proteins (159, 160). The membrane cofactor MCP or CD46 has been identified as pilus receptor (127), although an inverse relationship between pilus-mediated gonococcal adherence and surface expression of CD46 has been found by Tobiason *et al.* (2001). The precise role of CD46 in meningococcal and gonococcal adherence remains still to be determined.

### 2.4 Porins, class 1 and class 2/3 proteins

The major outer membrane proteins, expressed at high levels under standard growth conditions, are divided into five classes based on their molecular weight. PorA (class 1, ca. 40 kDa) and PorB (class 2 and 3, 36-38 kDa) are porins, permitting the passage of small molecules across the outer membrane; they show cation- and anion-selectivity, respectively (250). Gene sequencing permitted the construction of a topology model (261). The transmembrane regions are highly conserved, in contrast to the surface exposed loops which are variable. The serotypes are based on antigenic differences of the PorB protein (78). The antigenic heterogeneity of the PorA protein determines the serosubtypes (78).

In addition to facilitating transport of small molecules across the outer membrane, porins can have additional functions. The PorB protein was found to be capable of translocating vectorially into membranes of infected target cells and to function in the infection process (91, 146, 284). The insertion process leads to the formation of a functional channel, which strikingly is regulated by the eukaryotic host cell (216). It also has been found to cause rapid calcium influx in target cells and induce apoptosis by the activation of cysteine proteases (173). However, in contrast to this finding Massari *et al.* (2000) concluded that upon the interaction of PorB with mitochondria the cells are protected from apoptosis. Similar additional functions for PorA have not been established.

### 2.5 RmpM or class 4 protein

The class 4 or RmpM (reduction-modifiable protein M, 33-34 kDa) protein is constitutively expressed and antigenically invariable among
meningococcal strains. This protein consists of two domains, a short N-terminal domain interacting with some outer membrane proteins like PorA (116) and the iron regulated proteins LbpA, TbpA and FrpB (199), and the longer C-terminal domain containing a peptidoglycan-binding motif (133). Therefore, RmpM is thought to be a structural protein playing a role in anchoring of the outer membrane to the peptidoglycan layer (236). In agreement with this function, RmpM homologues are not only present in the two pathogenic Neisseria but also in commensal neisserial species (253). RmpM shows structural homology with Escherichia coli OmpA in the periplasmic domain, although its N-terminal domain is much smaller (132).

2.6 Opacity proteins or class 5 protein

Both meningococcal disease and carrier isolates express on their outer membranes one or more members of a family of closely related proteins, designated Opa proteins (class 5, 26-32 kDa). The prefix ‘Opa’ is derived from the association with colony opacity found in gonococci expressing these proteins (110, 245). Colony opacity was found to correlate with inter-gonococcal adherence (21). Eleven, four and two opa loci scattered over the chromosome have been identified in gonococci, meningococci and in commensal Neisseria species, respectively (163, 242, 243, 244).

Opa is a so-called heat-modifiable protein, meaning that during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), native protein migrates faster than the heat-denatured form (245). This has been found for other outer membrane proteins that possess an amphipathic β-sheet conformation. In the two-dimensional topology model an eight-stranded β-sheet conformation is predicted, with four surface-exposed loops including the hypervariable HV-1 and 2 regions (153, 260). Opa expression correlates with elevated transformation rates in N. gonorrhoeae (108). Also binding of pyruvate kinase has been found to be associated with the expression of Opa proteins (290). The main role for the opacity proteins is to mediate adhesion of the bacteria to epithelial and endothelial cells and to polymorphonuclear leukocytes (17, 277). The majority of gonococcal and meningococcal Opa proteins bind to carcinoembryonic antigen-related cellular adhesion molecules (CEACAM, formerly called CD66) (62). A minority of the Opa proteins target heparan sulphate proteoglycans (HSPG) (45, 266). Opa-receptor-mediated adhesion can lead to invasion of the bacteria into different cell types expressing CEACAM or HSPG (152, 172, 266, 268).

2.7 OpcA or class 5C protein

OpcA (or Opc) is also an integral outer membrane protein with a heat-modifiable character and a basic pI. Despite some functional and structural similarities, only minimal sequence homology with Opa proteins has been found (182). In contrast to opa genes, only one copy of the opcA gene is present
per genome in some meningococci (63), whilst the absence of an opc gene has also been found (224). It was initially thought that no opcA gene was present in gonococci, but Zhu et al. (1999) found an opcA homologue with a low expression. Furthermore, both meningococci and gonococci possess a second paralogous opcA-like pseudogene, \( \Psi \)opcB (297).

OpcA attaches to endothelial cells by binding to the serum glycoprotein vitronectin, which in turn attaches to the integrin \( \alpha_v\beta_3 \) (275, 278). OpcA also binds to the heparan sulphate proteoglycan receptor (HSPG), leading to entry of nonencapsulated meningococci into epithelial cells (58). The protein contains 10 transmembrane strands and five surface exposed loops as predicted in a two-dimensional model (158). The crystal structure of OpcA reveals that the loop regions protrude from the \( \beta \)-barrel well above the predicted surface of the membrane and form a crevice that would form an ideal site for binding to negatively charged proteoglycan polysaccharide.

### 2.8 Neisserial surface protein A

The neisserial surface protein A or NspA is a highly conserved outer membrane protein (39). The crystal structure revealed an eight-stranded \( \beta \)-barrel with short turns at the periplasmic side and longer loops at the extracellular region. NspA shows high homology to the neisserial Opa protein family of adhesins, especially in the transmembrane regions (256). Despite the availability of nspA knockout mutants in \( N. \) gonorrhoeae and \( N. \) meningitidis, no functional role has yet been discovered (28).

### 2.9 The iron regulated proteins

Iron is an essential element for nearly all organisms and microorganisms, facilitating fundamental processes such as nucleotide biosynthesis and electron transport (50). Most gram-negative bacteria including \( E. \) coli produce and secrete small iron-chelating compounds (called siderophores) during iron starvation. \( Neisseria \) is incapable of synthesising these siderophores (285). However, meningococci do produce FrpB (Fe-regulated protein B) (187) (also called FetA), an outer membrane receptor that is capable of binding enterobactin, a siderophore secreted by \( E. \) coli. FrpB has been postulated to allow meningococci to scavenge iron by binding siderophores secreted by other bacteria colonising the same mucosal surfaces. In addition, the pathogenic \( Neisseriae \) are able to directly utilise iron from the host iron-binding proteins transferrin, lactoferrin and haemoglobin, but also haem, ferric citrate and aerobactin can be used as iron sources (11, 164, 165, 295).

In response to iron-limitation additional outer membrane proteins are produced that serve to capture iron-binding proteins and to release and internalise iron into the bacterium. Efficient transferrin-iron acquisition is dependent on the combined action of two proteins, designated TbpA and
TbpB. TbpA is a TonB-dependent outer membrane receptor, whereas TbpB is lipid modified and increases the efficiency of transferrin-iron uptake (128). The lactoferrin receptor complex consists of the two components LbpA and LbpB, which also act in concert, like the TbpA/B complex (22, 188).

Iron uptake through the aforementioned outer membrane receptors requires the TonB complex, a highly conserved macromolecule located in the inner membrane and periplasm of gram-negative bacteria. Proteins in this complex, TonB, ExbB and ExbD cooperate in translating the proton motive force across the inner membrane into conformational changes in TonB-dependent outer membrane receptors (169). However, Biswas et al. (1997) concluded that the pathways for utilisation of Fe bound to haemin or ferric-citrate were not dependent on the TonB system. The periplasmic protein FbpA (Fe-regulated protein) is thought to deliver iron from the receptors to the inner membrane transporter FbpB/C.

2.10 Autotransporters

Members of the autotransporter family share an overall unifying structure comprising three functional domains, an N-terminal signal sequence and a C-terminal translocator domain, which are separated by the secreted passenger domain. The leader sequence directs transport of the protein across the inner membrane, while the carboxy-terminal translocator domain forms a β-barrel pore to allow secretion of the passenger domain across the outer membrane. From there the protein is either released extracellularly or it remains attached to the cell surface (107).

IgA protease, the first autotransporter discovered in Neisseria, cleaves human IgA1 both in serum and at mucosal surfaces of the respiratory and urogenital tracts (33, 190, 192). This autotransporter protein is constitutively produced in the two pathogenic Neisseriae, and is a sequence-specific endopeptidase cleaving single peptide bonds of distinct proline-rich consensus sequences that are found in the hinge region of human IgA1 but not of human IgA2 (190, 193). IgA1 protease has also been found to specifically degrade LAMP1, a major integral membrane glycoprotein of late endosomes and lysosomes, thereby promoting the intracellular survival of the pathogenic Neisseria spp. (142).

A systematic search of the neisserial genome sequences resulted in the identification of eight different ORFs encoding proteins belonging to the family of autotransporters (298). One of these newly identified autotransporter proteins, the neisserial autotransporter lipoprotein or NaIP, was found to negatively influence (auto) proteolytic cleavage of other autotransporter proteins, presumably at the cell surface (270). This is the same protein described as the autotransported serine protease A (AspA), which has significant homology to the secreted serine proteases (subtilases) from several organisms and contains a serine protease catalytic triad (255). The function of
autotransporter A (AutA) and AutB (autB is nmrep3, 186), potent CD4+ T cell and B cell antigens, has not yet been revealed (Ait-Tahar et al., 2000). The adhesion and penetration protein (App) is thought to be involved in adhesion because it shows high homology with the Hap autotransporter from Haemophilus influenzae (100).

2.11 Recently discovered outer membrane proteins

In addition to the major components and classes of proteins present in the outer membrane of Neisseria meningitidis described above, novel outer membrane proteins are still being discovered. Recently, Omp85 was shown to be essential for the viability of N. meningitidis. This highly conserved outer membrane protein was found to be involved in LPS and phospholipid transport to the outer membrane (84), although Voulhoux et al., (2003) concluded that Omp85 is likely to play a role in outer membrane protein assembly. Another newly identified surface protein is NadA (neisserial adhesin A); evidence was found that this protein is involved in the interaction of meningococci with epithelial cells, but its receptor has not yet been identified (12).

The genome sequences of Neisseria meningitidis serogroup B strain MC58 (189) and serogroup A strain Z2491 (185) have revealed new potential outer membrane constituents like the surface exposed lipoproteins GNA33 and GNA1870 (122, 155).

2.12 Phase variable expression and antigenic variability of virulence factors

In order to survive, a pathogen must develop strategies for evasion of the host immune response, establish itself within an appropriate environmental niche, and possess adequate means for transmission. To accomplish these goals, the two pathogenic Neisseriae species have capitalised on the variation of many surface components, such as pili, Opa proteins, porins, iron-binding proteins and LPS.

One mechanism to evade the host response is phase variation, in which expression of individual surface components can be switched on or off randomly and at high frequency. N. meningitidis contains more genes that undergo phase variation than any other pathogen studied to date (248). Sixty-five potentially phase variable genes were identified in the genome (221). Another mechanism, exploited by many pathogens including meningococci and gonococci, is antigenic variation in which many different variants of a single surface component can be expressed. In addition, pathogenic Neisseriae are naturally competent for transformation and can easily exchange DNA during mixed infections (71, 232, 150). This results in an enormous genetic flexibility, with consequences that play a decisive role not only for survival within the infected host but also with regards to the epidemiology and population structure of these organisms.
Neisserial DNA uptake sequences (89) play a role in recognition of homospecific DNA during transformation. In meningococcal B strain MC58 a total of 1910 copies of this uptake sequence distributed throughout the genome have been identified (247). Opa proteins and pili have been found to be involved in sequestration of donor DNA at the cell surface, which results in efficient transformation over time (108, 135). Outer membrane phospholipase A (OMPLA), present in most but not all pathogenic neisserial strains, has a role in autolysis (29). Autolysis and uptake of the released DNA might frequently take place during colonisation of the human nasopharynx. Three major islands of horizontally transferred DNA have been identified from genome analysis, and two of these contain genes encoding proteins potentially involved in pathogenicity (248).

LPS variation is mediated by an alteration through slipped-strand mispairing in the number of guanine residues in the middle of the coding sequences of several key enzymes. This results in altered expression of the encoded glycosyl transferases and as a consequence several distinct LPS isoforms can be formed at the cell surface by a single strain (14, 53, 92) (reviewed by Berrington et al., 2002). Different LPS oligosaccharide chains can influence important functions such as serum resistance and host cell invasion (265). Another mechanism to evade the host immune response, which has been found in commensal Neisseria, is phosphorylcholine decoration of LPS, thereby mimicking the host to avoid clearance from the nasopharynx (225). The meningococcal phospholipids show a diversity of acyl chains, which may result from the different specificities of the acyltransferases (226). However, phase variation of the encoding genes has not been investigated.

Three levels of capsular expression have been observed; colonies that were capsule negative, colonies that were weakly capsule positive and those that were strongly capsule positive, while reversion from weak to strongly positive phenotypes occurred at high frequencies (43, 299). It is not clear yet at which stage of an infection re-expression of the capsular polysaccharide occurs, but evidence has been found that the switching-on already occurs within the epithelial cells at a frequency of $10^{-3}$ (103). The mechanism behind the loss of encapsulation accompanying invasion was explored by searching for mutations within the genes required for biosynthesis of the $\alpha$-2,8-linked polysialic acid. Capsule phase variation results from reversible changes in the number of C residues within the 5' region of the $siaD$ gene encoding the $\alpha$-2,8 polysialyltransferase. The insertion or deletion of one cytidine residue within an oligo-(C) stretch results in a frameshift mutation which leads to termination of translation and expression of a truncated inactive $\alpha$-2,8 polysialyltransferase (67, 81). Another mechanism of capsule variation is reversible inactivation of the $siaA$ gene by a transposable genetic element, termed IS1301, which is present in multiple copies scattered over the meningococcal chromosome (103). Meningococcal serogroup B strain MC58 contains 22 intact and 29
remnants of these insertion sequences (IS) (248). Masson and Holbein (1985) have suggested that bacteria from rapidly growing meningococcal cultures are relatively capsule-deficient compared with bacteria grown in conditions of slower growth, while pH and iron limitation also affect capsule.

Antigenic variation of gonococcal and meningococcal pilin involves a family of variable genes that undergo homologous recombination, resulting in transfer of variant sequences from the pilS silent gene copies into the complete pilE expression locus. Little is known about the specific recombination events that are involved in assembling new variant pilin genes in vivo (162). Only recently a model was presented in which the RecA and RecJ proteins promote pilE deletions through a recombination event that is stabilised by a pilE/pilS interaction (109). Pilin phase variation, considered to be RecA/J-dependent, still occurs frequently in recA-negative bacteria, very probably from frameshift mutations at a poly C stretch in pilE (134). An additional level of pilin variation is caused by posttranslational modifications such as glycosylation, which is also subject to phase variation (121, 197). Finally, PilC1, the pilus-associated adhesin and key element in the initial adhesion to target cells is an antigenic and phase-variable protein (13). Its expression can be switched on and off by slipped-strand mispairing. A promoter element termed the contact regulatory element of Neisseria (CREN) is responsible for the transient induction of this gene upon cell contact. The contact-regulated gene A (crgA) encodes a transcriptional regulator whose expression is also induced upon cell contact from a promoter region similar to CREN of pilC1 (61). This is an example of regulatory adaptation. Strikingly, CrgA appeared to have also a regulatory role in capsule synthesis, causing it to be downregulated during intimate adhesion (61).

Antigenic variation of PorA is concentrated in variable regions (VR), and more than 70 different meningococcal porA sequences were identified (10, 217). PorA expression in Neisseria meningitidis displays phase variation between three expression levels by modulating the length of the homopolymeric tract of guanidine residues between the -35 and -10 regions of the promoter (257). Later additional mechanisms of varying PorA expression were discovered (259). In addition to antigenic and phase variation evidence for naturally occurring deletion and insertional inactivation of the porA gene was found (179, 258).

In the meningococcal PorB proteins regions of sequence variation, which are responsible for serotype specificity have been identified (296). Highly conserved regions flank these VR1 and VR2 regions, located in surface-exposed loops. Later, sequence analysis of porB genes encoding class 3 proteins revealed two additional variable regions (VR3 and VR4) (16).

The different opa genes display a large inter- and intra-strain variability primarily localised within two hypervariable domains and one semivariable domain. In addition to this antigenic variability, evidence for
phase variable Opa expression on the translational level has also been found. Translation of the constitutively transcribed opa genes depends on the intrinsic status of the individual opa loci (223). The basis of this phenomenon was found to be a repetitive sequence, the coding repeat (CR), which is variable in length. The CR codes for the hydrophobic core of the Opa signal sequence and consists of CTCTT pentamer units; depending on the number of CR units present, the translational reading frame of an opa gene is shifted in or out of frame (243). Belland et al., (1997) have found that opa expression is also regulated at the transcriptional level. In addition, variation in the length of a poly-A stretch directly preceding the CTCTT repeat was found to be another mechanism determining expression of some opa genes (111).

Opc phase variation occurs at the transcriptional level. Transcription starts 13 nucleotides after the –10 region of an unusual promoter sequence containing a variable number of contiguous cytidine residues and lacking a standard –35 region. Efficient expression of Opc occurred in strains with 12 to 13 cytidine residues, intermediate expression in strains with 11 or 14 residues, and no expression with < 10 or > 15 residues. Unlike porins and opa genes, only a few sites within the opc coding region are polymorphic and only a few allelic variants have been found (224).

Not much is known about the variation in expression and antigenicity of the autotransporters. IgA1 protease is in contrast to NalP and AutA/B constitutively expressed and antigenic variation is relatively low (143, 144). NalP expression is phase variable due to a poly-G repeat in the coding sequence (269). AutA/B are antigenically conserved but might phase vary through a tetranucleotide repeat upstream of both genes (4, 186). NadA shows limited antigenic variation but evidence for phase variation has been found (48).

3 Vaccine development

3.1 Necessity

Meningococcal meningitis and septicaemia is in most cases an acute infectious disease. Despite adequate antimicrobial therapy and the availability of advanced intensive care, the average case fatality rate of meningococcal disease is still 5 – 10 % in industrialised countries (7, 15, 60, 101, 105). In the developing world case fatality rates are even higher (97, 40). Between 10 % and 20 % of survivors develop permanent sequelae, such as epilepsy, amputations, mental retardation or sensorineural deafness (66, 131, 230, 271). From all the above-mentioned studies it is definitely clear that ‘prevention is better than cure’.

One way to eradicate an infectious disease is to prevent the residence of the pathogen in its particular niche by specific antibodies. From
epidemiological data on meningococcal carriage in relation to outbreak and disease, we know that the potentially invasive meningococci carried by healthy individuals are a constant threat (section 1.1). The human upper respiratory tract is thought to be the only natural reservoir of meningococci. The induction of antibodies by routine infant vaccination could therefore offer the prospect of eradicating this pathogen (171). However, it is also conceivable that vaccination will prevent disease but not asymptomatic carriage.

3.2 Polysaccharide and polysaccharide conjugate vaccines

The polysaccharide capsules of meningococci are important determinants of virulence (64). Mutants without capsular expression are serum sensitive and therefore killed by complement. Capsule-deficient strains are frequently isolated from carriers, but never from meningitis or septicaemia patients. Serum antibody to capsular polysaccharide protects against disease by activating complement-mediated bacteriolysis or opsonisation, or both. Most polysaccharide vaccines however proved to be poor immunogens in infants and fail to induce immunological memory in people of any age (85, 94, 139, 147). Polysaccharide vaccines against groups A and C, or A, C, Y and W135, are licensed and available worldwide. Because the immunological memory does not last long, the approach for control of meningococcal epidemics in the African meningitis-belt is based on early detection of disease and mass vaccination with meningococcal polysaccharide vaccine once a weekly incidence threshold has been crossed (140).

Experiences with Haemophilus influenzae type b (Hib) and pneumococcal conjugate vaccines showed that immunogenicity of polysaccharides can be improved by chemical conjugation to a protein carrier, thereby eliciting a T-cell dependent anti-saccharide antibody response (87, 208). These vaccines were found to be safe, immunogenic in young infants and able to induce long-term protection. In the case of H. influenzae, immunisation also reduced nasopharyngeal carriage and thereby transmission of the organism. Similar approaches are pursued for N. meningitidis. Mass vaccinations with the serogroup C conjugate vaccine in The Netherlands were successfully completed recently. The incidence of meningococcal serogroup C disease in The Netherlands was reduced with 73 % in the three months following the vaccination campaign as compared to the same period in the year before (55). In November 1999, meningococcal serogroup C conjugate vaccine was introduced into routine immunisation in the UK. The incidence of serogroup C disease in the targeted age groups fell by 80 %, and the number of deaths in laboratory confirmed cases in 0-19 year olds decreased from 78 to 8 between 1998-99 and 2000-01 (254). Bivalent A plus C polysaccharide conjugate vaccines have been assessed in clinical trials, and were well tolerated and immunogenic in infants, toddlers and adults (9, 41, 69, 141). Vaccine manufacturers are developing conjugate vaccine combinations incorporating
groups A, C, Y and W135 polysaccharides. Multivalent meningococcal polysaccharide-protein conjugate vaccines will probably be available in the USA and Europe within a few years.

3.3 Constraints on the development of a meningococcal serogroup B vaccine

The polysaccharide B capsule is poorly immunogenic, even when conjugated to a carrier protein (300), probably because of immunotolerance resulting from cross-reactivity between this polysaccharide and polysialic acid expressed on host neural cell adhesion molecules (NCAMs) (73). Although attempts have been undertaken to chemically modify the polysaccharide (119, 120), the safety concern is that group B meningococcal polysaccharide conjugate vaccines might elicit autoreactivity with host polysialic acid (82). For worldwide eradication of meningococcal disease a meningococcal serogroup B vaccine is indispensable. Non-capsular antigens are therefore intensively investigated for their vaccine potential. One of the major hurdles for the development of a non-capsular meningococcal serogroup B vaccine is the extremely variable nature of many meningococcal surface antigens as outlined in 2.12.

Conserved inner-core epitopes of LPS were found to be a target for protective antibodies in mice and possibly also in man (191). Another approach for LPS-based vaccines involves the use of synthetic oligopeptide mimetics to stimulate antibody responses that are cross-reactive with LPS antigens expressed by serogroup B meningococci (36). However, these approaches are still in an early stage and up till now outer membrane protein-based vaccines are the most promising and most advanced alternative.

3.4 Meningococcal serogroup B outer membrane vesicle vaccines

Outer membrane vesicles (OMV) are prepared by detergent extraction from meningococcal cells and have been extensively evaluated in clinical trials. The first OMV-based vaccines were developed by the Walter Reed Army Institute of Research in Washington D.C. (298), the Finlay Institute in Cuba (227) and the National Institute of Public Health in Norway (209), in response to group B meningococcal outbreaks in these countries. Studies with these vaccines have been conducted in several countries and have involved several million adults, older children, and infants (79, 298, 210). The vaccines were found to be safe and induced functional antibodies with bactericidal activity. The efficacies that were reached ranged from 50 to 80%. However, almost no protection was found in children less than 4 years of age, the age group with a high risk for group B meningococcal disease.

PorA was found to play a central role in the induction of bactericidal antibodies in adults (209) and infants (247). Responses to OMV vaccines tend to be directed mainly at the variable regions of PorA, which accounts for the
serosubtype specificity of the bactericidal responses (247). A multivalent PorA vaccine has been developed by the National Institute for Public Health and the Environment (RIVM) in The Netherlands (the vaccine research and production operate since 1-1-2003 independent of the RIVM under the new name: Netherlands Vaccine Institute or NVI). This hexavalent PorA OMV vaccine contains two trivalent vesicles each expressing three different PorA proteins, together covering approximately 80 % of Dutch isolates. Clinical studies have shown this vaccine to be safe and immunogenic in infants, toddlers and school children (56). Since the antigenic variability of PorA is high and can change over time, many different PorA variants would need to be included to confer broad protection. Due to this high variability of the antigen, a hexavalent vaccine would have the potential to prevent less than 50 % of endemic group B meningococcal disease in the USA according to Tondella et al. (2000). However, very recent evidence has been found that this might be an underestimation due to significant cross-protection of the hexavalent PorA vaccine (273).

3.5 Correlates of protection

In all vaccine-related studies, serum bactericidal activity (SBA) has been found to be the best, but not ideal, correlate of protection (reviewed by Vermont et al., 2002). This was experimentally confirmed by Holst et al., (2003). Another assay to measure protection against serogroup B meningococci is the whole blood killing assay (WBA) to assess the total killing capacity of human blood after vaccination or infection (115). The importance of opsonophagocytic activity as a host defence mechanism, especially against serogroup B meningococci, was shown by Ross et al., (1987). Since T-cells play an important role in the regulation of the immune response, including stimulation of B-cells for antibody production and immunological memory, T-cell proliferation studies and T-cell epitope studies have been performed to determine the relation with protection (175, 286). Furthermore antibody avidity and isotype distribution determined by ELISA are promising predictors for protective immunity after vaccination (57, 145, 174, 272). Antibody avidity is a measure of the functional affinity of serum antibody to bind to antigen (204). Isotype determination has been used to predict SBA since human IgG1 and IgG3 were found to be the most effective antibodies for complement-mediated killing of meningococci.

Protection against group B meningococci both at the level of initial colonisation and of bacterial invasion of the blood stream would be much easier to study in a relevant animal-infection model. As humans are the only natural hosts for N. meningitidis, it is difficult to establish such animal-infection models. Early attempts using monkeys (75), rabbits (34) and guinea pigs (35) proved to be unsuccessful. The model most frequently used is the intraperitoneal (IP) injection of meningococcal suspensions in mice, first described by Miller et al., (1933) and later improved by Holbein (1980) and
Schryvers and Gonzalez (1989). This model has been successfully used to study components that contribute to the virulence of meningococci (289) and to study active and passive protection against meningococcal disease (37, 154). IP challenge of infant rats has been used as a model of meningococcal disease (219, 220, 252). Colonisation of mice after intranasal infection has also been described (138, 148, 218). A major impediment is the specificity of many host-pathogen interactions at the molecular level. For instance, Opa proteins and the lactoferrin and transferrin receptors are specific for their human target molecules. As a first step towards overcoming this problem, improved colonisation of human CD46-transgenic mice was recently demonstrated (123). Animal models for meningococcal disease can provide much valuable information for studies of pathogenesis and vaccine development. However, all the available models still have shortcomings in providing relevant data on protection against human meningococcal infections.

3.6 Additional vaccine components

In the year 2000, up to 72 different PorA sequence types of *N. meningitidis* were isolated in the Netherlands (10), underscoring the need for the search of additional vaccine components. The main goal is to identify antigens capable to confer a broad protection. Highly conserved, surface exposed and constitutively expressed antigens present in all strains and at high levels would be ideal for a broad protective response. No single meningococcal antigen will meet all these requirements. It will rather be a combination of antigens that share these requirements. In all approaches undertaken to identify new meningococcal vaccine components, including the reverse vaccinology approach (202), one starts with the analysis of surface exposure since the significant antigens have at least to be accessible for bactericidal or neutralising antibodies. In addition to the main antigen PorA, many other protein antigens have been studied in more or less detail to date.

Relatively well-studied vaccine candidates are the iron-regulated proteins TbpB (54) and FrpB (187), since these proteins are expected to be essential for survival of meningococci in the human host (see also section 2.1). With the highly conserved outer membrane protein NspA promising results were found in SBA assays and in the passive protection mouse model (154). The major disadvantage of NspA appears to be that it is not sufficiently expressed or surface exposed in all meningococcal strains (167). From studies of serum from patients recovering from meningococcal disease, NspA appeared to be a weak immunogen (70).

The reverse vaccinology approach started with data from the recently elucidated genomic sequence of a group B meningococcal strain (202). First, potential vaccine candidates were identified based on prediction methods for surface localisation. These were all expressed in *E. coli*, purified and used for immunisation of mice. In this way 28 novel proteins (genome derived
antigens, GNA) were found which elicited group B specific antibodies. Sequence comparison among a representative panel of meningococcal strains was used to identify the more conserved proteins, but only 7 antigens elicited antibodies with bactericidal activity. The highest bactericidal response was found with GNA33, a highly conserved antigen later found not be surface-exposed but mimicking an epitope on loop 4 of PorA in strains with serosubtype P1.2. Another high-throughput screening method used for the identification of new vaccine candidates is the microarray technology to analyse gene regulation in meningococci grown in contact with either epithelial or endothelial cells (93, 180).

There is epidemiological evidence that N. lactamica, which colonises the nasopharynx of young children, shares common non-capsule antigens with N. meningitidis that may be responsible for natural cross-protective immunity (42, 130). Oliver et al., (2002) reported that immunisation with whole N. lactamica cells, outer membrane vesicles, or outer membrane protein pools, protected mice against lethal challenge by a number of diverse serogroup B and C meningococcal strains. Sequential immunisation with OMV preparations derived from heterologous strains was found to elicit broadly protective responses in animal model (168). The explanation given for this phenomenon is that sequential immunisation with heterologous vesicles results in a portion of antibodies directed at relatively conserved antigenic domains, which normally are relatively poor immunogens. This will not occur when multiple doses of homologous vesicles are given, and the response is mainly detracted to the immunodominant but variable epitopes.

Apart from PorA, not much is known about the use of individual major outer membrane proteins as vaccine components. Purified PorB as well as purified Opc induce moderate bactericidal immune responses (124, 294). Significant contributions of PorB and Opc to the bactericidal response in humans after vaccination with OMV have also been found (210). Structural predictions for RmpM suggest that this protein is not surface exposed (116, 199), making it an unattractive vaccine component in spite of its high immunogenicity and sequence conservation. The opacity proteins were found to contain T-cell epitopes in the constant regions (287), but these proteins were thought to be unsuitable as vaccine components due to their high antigenic variability.

4 Aim and outline of this thesis

4.1 Structure-function relationship of the opacity proteins

The Opa proteins are outer membrane proteins and play a determining role in the pathogenesis of Neisseria meningitidis, since they are involved in
adhesion and subsequent invasion of meningococci into human epithelial, endothelial and phagocytic cells. The use of these proteins as additional components in a protein-based vaccine against meningococcal serogroup B disease is studied in combination with the fundamental features of these proteins. The results of these studies are described in this thesis.

It has been predicted that Opa proteins form an eight-stranded β-barrel in the outer membrane with four extra-cellular loops (153, 260). The use of appropriate conditions for efficient refolding of these proteins enabled us to produce highly pure and native Opa proteins for our structural, functional and immunological investigations. Conformational analysis of the purified, refolded proteins provided experimental evidence for a secondary structure dominated by β-strands, confirming previously proposed topology models (Chapter 2).

The main group of receptors that are targeted belong to the CEACAM family (section 2.6). CEACAM1, CEACAM3, CEA and CEACAM6 serve as receptors for the pathogenic Neisseria species (25, 46, 95, 279). Despite the fact that each receptor is highly glycosylated, binding is a protein-protein interaction (26) with Opa recognising CEACAM residues exposed at the non-glycosylated GFCC' face of the amino-terminal domain (27, 194, 280). Of the many CEACAM members just four members serve as Opa receptors. Virji et al., (1996) found that > 85% of isolates from carriers and patients with meningococcal disease target the CEACAM1 receptor. This raised the question: how can the CEACAM-binding function be so conserved despite the enormous sequence variation of the Opa proteins?

Four different Opa proteins were identified in the meningococcal vaccine strain H44/76. All four proteins bound to CEACAM1, while two out of these four bound also to CEA (OpaB and OpaJ). The expression of the wild-type meningococcal Opa proteins in E.coli resulted in invasion into CEACAM expressing cells. One of the two proteins able to bind to CEACAM1 and CEA, OpaB, was used to study which residues were involved in the interaction. A sequence motif involved in binding to CEACAM1 was identified by alanine scanning mutagenesis of those amino acid residues conserved within the hypervariable (HV) regions of all four Opa proteins. Hybrid Opa variants with different combinations of HV-1 and HV-2 derived from OpaB and OpaJ showed a reduced binding to CEACAM1 and CEA, indicating that particular combinations of HV-1 and HV-2 are required for the Opa binding capacity. Homologue scanning mutagenesis was used to generate more refined hybrids containing novel combinations of OpaB and OpaJ sequences within HV-1 and HV-2. They could be used to identify residues determining the specificity for CEA binding. The combined results obtained with mutants and hybrids strongly suggest the existence of a conserved binding site for CEACAM receptors formed by the interaction of HV-1 and HV-2 regions (Chapter 3).
4.2 Opa proteins, additional vaccine components?

One of the approaches to broaden the protection against meningococcal group B infections is to add vaccine components to the main vaccine component, PorA (section 3.6). Furthermore the OMV vaccines currently under development should provide protection at the level of initial nasopharyngeal colonisation, subsequent invasion and bacteraemia. Since Opa proteins are involved in adhesion and invasion into the nasopharyngeal epithelial tissue (3, 246, 277), blocking this crucial step in the pathogenesis is an attractive possibility which has to be studied for feasibility.

The intrinsic immunological features of the Opa proteins are not well studied, in contrast to PorA. Therefore, an immunisation study was performed in mice to determine the immunogenicity and functional activity of the antibodies raised against OpaJ presented in outer membrane complexes (OMCs) derived from both *E.coli* and meningococci, and as purified denatured or refolded protein. Significant anti-Opa responses were found. Although no opsonophagocytic and only low amounts of bactericidal antibodies were detected, these sera contained blocking antibodies cross-reactively blocking the Opa-CEACAM interaction (Chapter 4).

Since the upper respiratory tract, in particular the nasopharynx, is the specific niche and port of entry for the meningococci, we studied the induction of Opa specific antibodies at the murine nasopharynx. Purified OpaB and OpaJ were reconstituted in liposomes and used for intranasal immunisation after the addition of different adjuvants. The combination of Opa with purified wild-type LPS as adjuvant resulted in high anti-Opa specific titres as measured in nasal lavages and sera. The anti-Opa response was also measured after intranasal immunisation of transgenic mice expressing human CEA, targeted by OpaB. No differences in response were found between CEA-transgenic and non-transgenic mice, showing that the CEA-Opa interaction does not influence the antibody response. (Chapter 5).

It was recently found that CEACAM1-binding Opa proteins have immunosuppressive effects on T- and B-cells (30, 183). In relation to these discoveries we analysed the consequences of CEACAM1- and CEACAM3-mediated binding to neutrophils. Targeting of CEACAM3, which is exclusively expressed by neutrophils, resulted in reduced killing. It could thus be demonstrated that the receptor specificity of individual Opa proteins determines the outcome of the bacterial interaction with neutrophils (Chapter 6).

Finally, the results are discussed and suggestions for the use of opacity proteins incorporated in the meningococcal OMV vaccines are presented (Chapter 7).
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