Neisseria meningitidis: an overview of the carriage state

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During periods of endemic disease, about 10% of the general population harbour Neisseria meningitidis in the nasopharynx. Since N. meningitidis is a strict human pathogen and most patients have not been in contact with other cases, asymptomatic carriers are presumably the major source of the pathogenic strains. Most carrier isolates are shown to lack capsule production. The capsule deficient state of meningococcal strains in the nasopharynx may aid evasion of the human immune defence and hence be selected to survive nasopharyngeal colonization. Carriage itself can be an immunizing process resulting in systemic protective antibody responses. Frequent nasopharyngeal colonization with related bacteria like Neisseria lactamica improves natural immunity to meningococci by the formation of cross-reacting antibodies. While most meningococcal strains recovered from patients belong to a limited number of clonal groups worldwide, strains isolated from carriers comprise numerous genotypes, with only a small proportion of the strains representing invasive clones. During the carriage state, co-colonization with other pathogenic and non-pathogenic bacteria may lead to genetic exchange, which may result in the emergence of new meningococcal clones. The high diversity of meningococcal carrier strains, compared with hypervirulent strains, supports the idea that transmissibility, not invasion, is essential in the life cycle of N. meningitidis.

Introduction

Neisseria meningitidis, the meningococcus, is a Gram-negative bacterium with a coccoid shape, and a pathogenic member of the Neisseriae family. Anton Weichselbaum first isolated the bacterium from the cerebrospinal fluid (CSF) of a patient and identified it as the cause of meningitis in 1887; he called the organism Diplococcus intracellularis (Weichselbaum, 1887). Meningococci are obligate commensals in man and colonize the nasopharyngeal mucosa without affecting the host, a phenomenon known as carriage. The asymptomatic carrier state was already recognized by 1890 (Broome, 1986). In non-epidemic settings, approximately 10% of healthy individuals at any time carry N. meningitidis in the upper airway (Cartwright et al., 1987; Stephens, 1999). Rates of transmission and carriage increase in closed and semi-closed populations, such as military recruits, university students and in the household contacts of a case of meningococcal disease (Olcén et al., 1981). The duration of the carrier state varies; it may be chronic, lasting for several months, intermittent or transient (Broome, 1986). Colonization of the oropharynx by meningococci produces an antibody response from the three major immunoglobulin classes within a few weeks after acquisition of the bacterium and may act as an immunizing event (Kremastinou et al., 1999). Occasionally, shortly after the onset of colonization, N. meningitidis strains will penetrate the mucosal membrane and enter the bloodstream. Then, various forms of disease may develop. The meningococcus may cause meningitis, severe sepsis with an often fatal outcome, and more rarely, other diseases such as septic arthritis, pneumonia, purulent pericarditis, conjunctivitis, otitis, sinusitis and urethritis (Tzeng & Stephens, 2000). The overall incidence of meningococcal disease in Europe and North America is of 1–3/100 000 population, but incidence rates of 1000/100 000 may be reached during severe epidemics in sub-Saharan Africa. The factors that lead to invasive disease are still poorly understood. However, non-carriers are considered as a high-risk group for meningococcal disease since their capability to maintain a commensal relationship with an acquired strain is not known (Griffiss, 1995). Studies on meningococcal carriage provide insights into the epidemiology and pathogenesis of meningococcal disease. Further, knowledge about meningococcal

Abbreviations: LOS, lipooligosaccharides; LPS, lipopolysaccharides; OMP, outer-membrane protein.
carriage is important for outbreak management and possibly for individual patient management. In addition, carriage studies can be very useful in enhancing our understanding of the population biology of N. meningitidis.

Methodology

Culturing of pharyngeal swabs for isolation of N. meningitidis is an essential clinical and epidemiological tool that has proved to be of interest for outbreak management. Several factors potentially influence the estimation of the carriage rate, including factors related to swabbing technique and to laboratory methods. The sensitivity of nasopharyngeal cultures is affected by the number of swabs taken and the site swabbed (Broome, 1986). Addition of selective antibiotics to media inhibits the growth of non-meningococcal bacteria in the sample, and thus, significantly increases the yield of meningococci from nasopharyngeal specimens. Direct plating of swabs onto solid medium on site has been shown to yield a significantly higher carriage rate than use of a transport medium (Cunningham et al., 2001).

A recent study showed that the carriage rate might be underestimated when using conventional nasopharyngeal swabbing. By using immunohistochemistry for detection of N. meningitidis in patients undergoing tonsillectomy, it was found that meningococci were present in 45% of the samples, while only 10% were positive by culture of nasopharyngeal swabs (Sim et al., 2000). Comparison of culture of throat swabs and PCR for specific detection of N. meningitidis in carriers demonstrated that the sensitivity of throat swab culture was higher than a PCR assay based on ctrA, a gene involved in export of the meningococcal capsule. PCR was shown to be a useful adjunct to culture for detecting nasopharyngeal carriage, but it failed to detect some non-capsulated strains (Jordens et al., 2002). In addition, the failure of PCR to directly detect N. meningitidis in throat swabs may be caused by the presence of inhibitory factors in the samples.

Cell envelope and cell membrane structure

Traditionally, meningococcal strains have been classified by serological typing based on antigenic variation of the capsular polysaccharide, identifying the serogroup; the PorB outer-membrane protein (OMP), identifying the serotype; the PorA OMP, identifying the serosubtype and the lipopolysaccharide (LPS), giving the immunotype (Frasch et al., 1985). A meningococcus antigenic type is designated, for example, as: B:2b:P1.5,12::I3,7,9, indicating the serogroup: serotype: serosubtype: immunotype (Tsai et al., 2000). Each of these characteristics can be determined using specific antisera and monoclonal antibodies.

Capsular polysaccharides are the outermost antigens on the meningococcal surface and the target primary for mucosal and humoral immunity. N. meningitidis have been divided into 13 serogroups: A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z. Patient strains are encapsulated and five of these serogroups (A, B, C, W135 and Y) cause more than 90% of the invasive disease worldwide. In contrast, approximately 50% of the strains isolated from carriers lack capsule and are therefore serologically not serogroupable (Claus et al., 2002b). Non-serogroupable meningococci were assumed to be non-pathogenic, but it was found that capsule production in meningococcal strains can switch on and off at a high frequency (Swartley et al., 1997). There is evidence that the loss of capsule enhances the capability of meningococci to colonize the human nasopharynx and to avoid human defence systems (Hammerschmidt et al., 1996). Mechanisms such as slipped-strand mispairing (Kahler & Stephens 1998) and acquisition of an insertion sequence (Claus et al., 2004) in genes responsible for synthesis or transport of the polysaccharides are involved in loss of capsule expression.

The meningococcal capsule synthesis (cps) locus consists of seven different regions (Frosch et al., 1989; Vogel et al., 2001). Region A encodes the enzymes responsible for polysaccharide synthesis (Edwards et al., 1994; Swartley et al., 1998), while region B participates in lipid modification (Frosch & Muller, 1993). Region C consists of the ctr genes enabling polysaccharide transport across the bacterial membranes (Frosch et al., 1991, 1992) and region D is involved in LPS synthesis (Hammerschmidt et al., 1994). Region D which probably resulted from duplication of region D is followed by genes homologous to methyltransferase genes. The function of region E is not known. Of 830 N. meningitidis strains obtained from asymptomatic carriers in Germany, 136 lacked region A (polysaccharide synthesis), region B (lipid modification) and region C (polysaccharide translocation) (Claus et al., 2002b). A non-coding intergenic region of either 113 or 114 bp replaced these operons. However, the authors did not indicate how many strains were non-groupable with serological methods. These findings partly explain why a majority of meningococcal carrier strains are non-groupable by traditional serogrouping systems with antisera. The PorA and PorB OMPs are the two major porins in N. meningitidis. PorB constitutes anion-selective pores, whereas PorA makes cation-selective pores (Tommassen et al., 1990). All meningococci express PorB, either as a class 2 or class 3 OMP and most strains express PorA, as a class 1 OMP (Tsai et al., 1981). PorA consists of two variable regions (VRs), referred to as VR1 and VR2, which are located on loops I and IV of the molecule, respectively (Maiden et al., 1991, 1992; McGuinness et al., 1990; van der Ende et al., 2000). The class 2 and 3 proteins are mutually exclusive, as they are products of two alleles of the porB gene locus (Hitchcock, 1989). PorB carries four variable VRs which are involved in making the serotype epitopes (Urwin et al., 2002). The available monoclonal antibodies directed against PorA and PorB do not cover the complete range of antigenic variations on these major OMPs. Further, the high variability of PorB and PorA antigens makes it difficult to rely on serological markers to characterize the fate of individual meningococcal isolates or the clonal relation of disseminated strains. Thus, some meningococcal strains are classified as non-serotypable and non-serosubtypable. Molecular genetic methods, such as PCR-restriction fragment length poly-
morphism (PCR-RFLP) and sequencing of the genes encoding the PorA and PorB OMP, have been demonstrated to be more reliable than serological methods for typing of meningococcal strains (van der Ende et al., 2000; Zhu et al., 1995).

PorA protein can be stable during chronic carriage of *N. meningitidis* (Jones et al., 1998), but other researchers have found that serotype and serosubtype of the meningococcal strains changed during the carriage period (Ala’Aldeen et al., 2000). In the latter study which aimed to identify the sequential isolates of meningococci in persistent carriers, only 9 of 98 carriers exhibited strains with the same serotype and serosubtype from spring to autumn. However, the stability or change of PorA or PorB molecules during carriage can only be confirmed when individuals harbour the same meningococcal clones over time. Neither the former nor the latter study investigated this issue. However, strains of the same clone within individual carriers may vary from being (sub)typable to non-(sub)typable, suggesting that changes in the level of expression of the PorA and PorB OMPs occur during carriage (Caugant, 1998). Many of the meningococcal surface antigens like PorA and PorB are phase-variable proteins. Tandem-repeat DNA motifs in the genes involved in these proteins are known to mediate phase variation (Snyder et al., 2001). Gene switching and expression of phase-variable proteins is a central aspect of the population response to changing conditions. DNA sequence analysis of the promoter region of genes and the coding region of meningococcal outer-membrane proteins among carrier and disease-associated isolates may reveal the length, composition and instability of repeats associated with phase variation, and provide additional information on the mechanisms generating diversity in *N. meningitidis*.

*N. meningitidis* LPS (endotoxin) is a major virulence factor which induces the production of pro-inflammatory mediators leading to septic shock in meningococcal disease (Brandtzæg et al., 2001). Contrary to the LPS of enteric bacteria, meningococcal LPS lack repeating polysaccharide O-antigens, and are thus often referred to as lipooligosaccharides (LOS). The LOS molecule consists of three short oligosaccharides, termed α, β and γ chains (Griffiss et al., 1988) which are used to divide *N. meningitidis* into 12 immunotypes (L1–L12) based on specific antibody reactions (Zollinger & Mandrell, 1977, 1980). Heterogeneity of immunotypes is more common in meningococcal carrier strains than in invasive strains. A greater proportion of meningococcal carrier strains express the L1 and L8 immunotypes (Poolman et al., 1995). Three genetic loci, lgt-1, 2 and 3, encoding glycoacyltransferases, are involved in the biosynthesis of the LOS chains. Pathogenic and commensal *N. meningitidis* share a common lgt gene pool and horizontal gene transfer seems to contribute to the genetic diversity of lgt loci in *Neisseria* (Zhu et al., 2002). Immunotypes are subject to phase variation, which is due to high-frequency frameshift mutations within the homopolymeric tracts situated in the coding regions of the lgt genes, and may permit the organism to evade the host immunity system. The diversity of the lgt loci responsible for the biosynthesis of the LOS should be studied further, especially in relation to disease versus carriage isolates of the same clone.

In recent years, determination of the type of meningococcal capsules, OMPs and LOS has switched from immunological methods to molecular technologies based on PCR and subsequent DNA sequencing (Dolan-Livengood et al., 2003; Sadler et al., 2003). This evolution is likely to continue and especially needs to include carrier studies where the phenotypic methods have been deficient.

**Adhesion and colonization of human nasopharynx**

For colonization of the human nasopharynx, the microorganism must adhere to the mucosal surface, utilize locally available nutrients and evade the human immune system. *N. meningitidis* produces a number of structures and molecules that are important in its relation to the human host:

1) Adhesion molecules: pili facilitate primary adherence to the epithelial cell surfaces (Pujol et al., 1999). During the last 4 years, a number of works have focused on the mechanisms of attachment and movement in *N. meningitidis* and the closely related species *Neisseria gonorrhoeae* (Koomen 2001; Merz et al., 2000; Merz & So 2000; Wolfgang et al., 2000). *N. meningitidis* initiates infection by attaching to host cells by surface-associated filaments called type IV pili (Merz et al., 1996; Pujol et al., 1999), which are long hair-like structures that extend from bacterial surface. A membrane cofactor protein (CD46), which is expressed on all human cells except erythrocytes, has been identified as a receptor for neisserial type IV pili (Johansson et al., 2003; Källström et al., 1997). At multiple stages of colonization, type IV pili are involved in complex host cell responses. Piliated *Neisseria* trigger a cytosolic Ca$^{2+}$ flux in human epithelial cells (Källström et al., 1998). Further, the pilus-mediated adhesion includes a number of rearrangements in the cortical cytoskeleton and plasma membrane. The rearrangements comprise high accumulations of phosphotyrosine, actin, ezrin and a subset of transmembrane glycoproteins at the neisserial attachment sites on the epithelial cells (Merz et al., 1999). Adhesion of meningococci to host cells leads to a transient up-regulation of PiC production and down-regulation of capsule synthesis (Deghmane et al., 2000; Taha et al., 1998).

A more intimate association is established by the colony opacity-associated (Opa and Opc) proteins (Dehio et al., 1998). Meningococcal LOS can be bound by host-cell-expressed lectins and may also contribute to bacterial adhesion (Porat et al., 1995). Down-regulation of capsule expression and removal of sialic acid from LOS seem to be essential for meningococcal interaction with host cells. The Opa and Opc do not seem to affect the interaction with eukaryotic cells when meningococcal strains are capsulated (Virji et al., 1993, 1994). The regulatory protein CrgA also appears to play a central role in meningococcal adhesion (Deghmane et al., 2000, 2002). Inactivation of the crgA gene, which encodes a transcriptional regulator belonging to the
LysR family, decreases meningococcal adhesion to epithelial cells and abolishes intimate adhesion. Genetic study of the crgA gene and structural study of CrgA protein among meningococcal carrier and disease-associated isolates may help to understand the adaptive response of meningococci upon cell contact and provide insight into Neisseria–host interactions at the carriage level.

2) Receptors capable of binding to human transferrin and lactoferrin: human transferrin and lactoferrin are assumed to be sources of iron, a basic component essential for growth of meningococci (Larson et al., 2002). The iron-acquisition system in meningococci includes the following OMP receptors: transferrin-binding proteins (TbpA, TbpB), lactoferrin-binding protein (LpB, LpA), haemoglobin-binding protein (Hbp) and haemoglobin-haptoglobin-binding protein (Hbp-Hpp) (Schryvers & Stojilkovic, 1999). These proteins bind to human iron-carrying protein, release and internalize iron into the bacterium. Presumably, at the stage of colonization, LpB and LpA are more important than the other iron-binding proteins, since lactoferrin is the major iron source at the mucosal surface (Gray-Owen & Schryvers, 1996). The level of expression of LpB and LpA might thus be higher in carrier isolates than in invasive isolates, and this should be studied further.

3) An extracellular IgA1 protease with the capability to cleave human IgA1, the dominant immunoglobulin in secretions (Lomholt et al., 1992). IgA1 has an essential role in mucosal tissues, such as in the nasopharynx, by preventing adherence and colonization of bacteria. IgA1 protease, encoded by the iga gene, is a sequence-specific endopeptidase with the capability to cleave single peptide bonds in the proline-rich sequence that exists in the hinge region of human IgA1 (Pohlner et al., 1992). The three most common causative agents of bacterial meningitis, *N. meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, which all first colonize the nasopharynx (Tunkel & Scheld, 1993), all produce IgA1 protease (Kilian et al., 1996). Levels of IgA1 protease activity have been shown to be significantly higher in invasive meningococcal strains than in strains isolated from carriers (Vitovski et al., 1999).

No genetic risk factors for colonization or invasion of the host by *N. meningitidis* have been established (Stuart et al., 1989), and the mechanism of adhesion and colonization of the human nasopharynx is likely to be similar in invasive as well as in carrier strains. However, OMPs involved in colonization and adhesion are likely to be expressed at different levels at the colonization and adhesion stage, compared to those in isolates that have penetrated the blood or the CSF.

Development of new molecular techniques, like DNA-microarrays, for identification and differentiation of meningococcal isolates may permit us to understand the link between meningococcal carriage and disease. The technology may allow the monitoring of the transcription levels of tens of thousands of genes and to identify the factors that are involved in pathogenesis of the invasive meningococcal strains in comparison with non-invasive strains from carriers.

**Epidemiology of carriage**

Investigation of the carrier state may contribute significantly to our understanding of the epidemiology and pathogenesis of disease caused by *N. meningitidis*. In addition, it is essential to gain a better grasp of the population biology of the meningococcus. Meningococcal bacteria spread from person to person by contact with upper respiratory secretions of nasopharyngeal carriers, such as by kissing, and less efficiently by aerosolised droplets.

When approximately 10 % of individuals from the general population at any time were carrying *N. meningitidis* in the nasopharynx (Cartwright et al., 1987), the carriage rate was shown to be < 3 % in children younger than 4 years and increased to 24–37 % in the age-group 15–24 years (Blackwell et al., 1990; Cartwright et al., 1987; Caugant et al., 1988, 1994). Then carriage rates decrease to less than 10 % in older age-groups. High rates of meningococcal carriage have also been reported among military recruits in many countries (Caugant et al., 1992; Djupesland et al., 1990; Tyski et al., 2001). In a study performed in Norway, the carriage rate among a troop of military recruits was higher than 70 % (Caugant et al., 1992). A surveillance study performed in the spring and autumn of 1998 in Poland showed that meningococcal carriage among military recruits was dynamic, with overall carrier rates ranging between 36 and 61 % within 2-month periods (Tyski et al., 2001).

Meningococcal carriage increases rapidly among university students in the first month of the academic year and much of this increase probably occurred during the first week (Neal et al., 2000). The study, performed at the University of Nottingham, UK, showed the carriage rate increased in the first week of term from 6-9 % on day 1, to 11-2 % on day 2, to 19-0 % on day 3 and to 23-1 % on day 4. High social mixing probably caused this increase.

The annual pilgrimage to Mecca (Hajj), which attracts more than two million pilgrims from all over the world, is also a situation providing ideal conditions for transmission of meningococci as a consequence of overcrowding (Wilder-Smith et al., 2002). Returning pilgrims may spread the bacteria to their household contacts or to the community at large.

Carriage prevalence of *N. meningitidis* is generally higher among household contacts of meningococcal patients (Cardenosa et al., 2001; Cooke et al., 1989; Olcén et al., 1981) than in the general population. The overall carriage rate of *N. meningitidis* among household contacts in New Zealand was 20-5 % (Simmons et al., 2001). The study demonstrated that age-specific carriage in children under 5 years of age was low at 5-8 % but increased to 35-1 % in 15–19-year-olds. Using phenotypic characteristics, 50 % of household carriers harboured the same strain as the patient.
In a population-based study in Spain, the strains were phenotypically identical in 14 of 22 (64%) confirmed meningococcal cases with carrier contacts, and in four cases, only a minor change was observed. During an epidemic period of meningococcal disease in Africa, the carriage rate was found to be high (15%) in the healthy population (Hassan-King et al., 1988). The carriage rate remained at the same level for 1 year after the epidemic and then decreased to < 1%.

In addition to age and having contact with a case or with another carrier, a number of risk factors have been shown to be associated with meningococcal carriage (Cartwright, 1995). Individuals with respiratory tract infections, of viral or bacterial origin, may be at high risk of becoming carriers (Stephens, 1999). Smoking, passive as well as active, is a risk factor that increases the possibility of individuals to become carriers. Socio-economic conditions also appear to influence the carriage rate of meningococci (Davies et al., 1996): people with low socio-economic status, whatever their ethnic origin, are more likely to be carriers and develop meningococcal disease (Stuart et al., 1988). There are slightly more male carriers than females.

Several studies have provided information on factors which are assumed to be related to carriage rate (Blackwell et al., 1990, 1992; Olcén et al., 1981; Stuart et al., 1989; Young et al., 1972). The studies have usually been performed in selected population groups like personnel in the armed forces, or in relation to outbreaks. The role of these factors was confirmed by a study that estimated the extent of meningococcal carriage in a randomly sampled population in Norway (Caugant et al., 1994). It was found that age between 15 and 24, male sex and active and passive smoking were associated with meningococcal carriage. The risk for carriage increased when individuals, older than 17, worked outside home and had an occupation in transportation or industry.

Knowledge about the duration of carriage is limited because most studies of carrier strains have only used phenotypic techniques for strain characterization. This information needs to be re-evaluated, especially the duration of carriage for different meningococcal clonal groups. Further, interspecies horizontal gene transfer requires carriage of multiple meningococcal strains in the throat of healthy individuals. There are still no reports on how common multiple carriage is in the human population.

**Molecular epidemiology**

Serological typing methods have been useful for rapid public health decisions and vaccine development, but they have a number of weaknesses, which make them inappropriate for epidemiological studies (Hobbs et al., 1994). These methods are based on variation in cell surface antigens, which are likely to be under selective pressure (Caugant, 1998). Especially, strains recovered from carriers often lack expression of the capsular antigen and the serotype and/or serosubtype cannot be identified either because of lack of relevant monoclonal antibodies or because of low level of expression of the genes.

During the last 20 years a number of methods, like multilocus enzyme electrophoresis (MLEE), pulsed field gel electrophoresis, random amplification of polymorphic DNA (RAPD), repetitive element-based PCR, insertion–sequence analysis, ribotyping, RFLP-analysis of PCR products, sequencing of individual genes that may be related to virulence (Caugant, 1998) and multilocus sequence typing (MLST) (Maiden et al., 1998) have been developed for epidemiological studies of meningococci. Among these epidemiological methods, MLEE and MLST have been considered as ‘gold standards’ for study of the epidemiology of meningococci: MLEE has been shown to be a powerful technique to distinguish various meningococcal clones (Caugant, 1998). The technique allows the characterization of each meningococcal strain by identification of the alleles of 10–20 housekeeping genes indirectly from the electrophoretic mobilities of their products, the enzymes. MLEE analyses of meningococcal strains isolated from carriers showed that they were more diverse than those isolated from patients. In Norway, approximately 9% of the carriage isolates belonged to two of the known hypervirulent clonal complexes, the electrophoretic type-5 (ET-5) and ET-37 complex (Caugant et al., 1994). Members of ET-37 complex were serogroup C, but the isolates belonging to ET-5 complex were serogroup B or non-groupable. These two clonal complexes included about 91% of the strains from patients affected by invasive meningococcal disease, at the time when the carriage study was performed. A major problem with MLEE is that the results from different laboratories are difficult to compare.

During the last 5 years, MLST has been used to identify the alleles of seven meningococcal housekeeping genes by the nucleotide sequence of 450–500 bp from PCR fragments of the genes (Maiden et al., 1998). Variation within housekeeping genes, either caused by point mutation (single mutation) or recombination (multiple nucleotide changes) is likely to be neutral. The ratio of recombinational exchanges to point mutations in the diversification of meningococcal lineage was analysed using nucleotide sequence data of housekeeping genes included in the MLST scheme (Feil et al., 1999). Analysis of various alleles of these genes indicated that a single nucleotide change in a meningococcal housekeeping gene is at least 80-fold more likely to be caused by a recombination event than as a result of a point mutation. Availability of nucleotide sequence data using the MLST website (http://pubmlst.org/neisseria), makes it possible to analyse clonal diversification, as well as to determine the ratio of recombination to point mutations. In a study in meningococcal strains from carriers in the Czech Republic, high diversity of meningococcal strains was reported. The strains comprised 71 different sequence types (STs), which were assigned to 34 different complexes or lineages (Jolley et al., 2000). Three STs represented among hyperinvasive lineages were present among the carriage isolates. These three STs included ST-41 (lineage 3), ST-11 (ET-37) and ST-32 (ET-5)
complex, and constituted 17-0, 2-6 and 0-6 % of the carriage population, respectively. The high diversity of the carrier strains identified by the MLST study in the Czech Republic is in agreement with previous findings from our laboratory using MLEE (Caugant et al., 1988, 1994). Only a small proportion of strains isolated from carriers represent the hyperinvasive clones. It is still not clear which factors and mechanisms determine the hyperinvasive properties of meningococcal strains. However, despite many advantages, MLST does not necessarily provide the discrimination desired for fine typing of some clonal groups of N. meningitidis.

Among high-throughput analytical methods for exploiting the information of genome sequences, DNA microarray technology has been utilized for typing of single nucleotide polymorphisms (Yoshino et al., 2003). This can be used to establish a microarray-based sequence typing system of meningococci (Claus et al., 2002a) and may become the new generation of molecular typing technique for invasive as well as carrier meningococcal strains.

**Horizontal gene transfer**

Foreign DNA with particular uptake signal sequences will preferentially be incorporated in the genome of N. meningitidis by transformation. N. meningitidis contains approximately 2000 copies of the uptake signal sequence 5′-GCCGTCTGAA-3′ (Kroll et al., 1998; Smith et al., 1999). It has been suggested that unidentified bacterial surface components facilitate binding and transfer of uptake-signal-sequence-containing exogenous DNA in the initial phase of the transformation process (Davidsen et al., 2004).

Because of mixed colonization with other bacteria and because of its duration, the carrier state is an ideal condition for horizontal gene transfer between different strains of meningococci, and between meningococci and other commensal bacterial species. The human nasopharynx may harbour diverse pathogenic bacteria, like N. meningitidis, H. influenzae, S. pneumoniae, as well as non-pathogenic bacteria, such as N. lactamica and the moraxellae. Transformation with DNA released from other bacteria during mixed colonization of the nasopharynx can result in transfer of foreign DNA to the chromosome of N. meningitidis (Linz et al., 2000). The PilQ complex in N. meningitidis is an antigenically conserved and highly abundant OMP (Bitter et al., 1998; Tonjum et al., 1998), which has been implicated in both production and retraction of the type IV pili and DNA transformation. After uptake, the DNA may be integrated into N. meningitidis by recombination, which may lead to rapid evolution of the meningococcal genome. Three independent domains of Haemophilus-like DNA have been reported in the meningococcal chromosome, providing evidence that horizontal gene transfer occurs between phylogenetically distant species. These domains were associated with a virulence gene (superoxide dismutase sodC), the bio gene cluster and an unidentified open reading frame (orf) (Kroll et al., 1998). DNA sequence analysis in N. meningitidis serogroup A clone complex subgroup IV-1, isolated from patients and carriers in The Gambia, revealed high frequencies of DNA exchange (Linz et al., 2000) and the size of imported DNA was shown to be up to 5 kb.

**Chemotherapy and antibiotic resistance**

Chemotheprophylaxis may eradicate nasopharyngeal carriage of meningococci. It is used to eliminate transmission to close contacts. Until 1960, sulfonamides were successful in eradicating meningococcal carriage. Sulfonamide-resistant meningococcal strains have been identified from patients with meningitis and from carriers that have not been treated with sulfonamides. Because these bacteria can survive in competition with other bacteria in humans in the absence of sulfonamide selective pressure, they can be considered as well-adapted strains (Fermer et al., 1995; Fermer & Swedberg, 1997).

The antibacterial agent rifampicin has been shown to be effective in reducing the carriage rate by up to 90 % (Deal & Sanders, 1969). Resistance against rifampicin is rare, but resistant strains have been identified from recipients of the drug (Yagupsky et al., 1993). Resistance to rifampicin in meningococci is caused by point mutations in a region of the rpoB gene, the gene encoding the β-subunit of the RNA-polymerase (Nolte, 1997). The point mutations affect two amino acids in the RpoB protein and causes Asp542—Val substitution or His552—Tyr or Asn substitution (Stefanelli et al., 2001). Recently, the efficacy of ceftriaxone in eliminating nasopharyngeal carriage of N. meningitidis was compared with rifampicin during an epidemic of serogroup B meningococcal disease (Simmons et al., 2000). The authors reported a similar efficacy of both drugs in eliminating nasopharyngeal carriage of N. meningitidis.

N. meningitidis is basically susceptible to β-lactam antibiotics, therefore, penicillin is the major antibacterial agent used for treatment of meningococcal disease. However, treatment with penicillin-G may fail to eradicate carriage (Abramson & Spika, 1985). Reduced susceptibility to penicillin has been reported from many countries (Oppenheim, 1997). High levels of resistance to penicillin caused by plasmid-encoded β-lactamases (Bäckman et al., 2000) are rarely found in meningococcal strains and most penicillin resistance is chromosomally encoded. This is linked to the changes in the structure of one of the penicillin-binding proteins, PBP2, which is encoded by the penA gene (Mendelman et al., 1988).

A plasmid containing the resistance gene to tetracycline, tetM, identified in N. gonorrhoeae, has shown the capability to be transferred in vitro to N. meningitidis (Roberts & Knapp, 1988). Since both species can occasionally co-exist in the genitourinary tract or in the nasopharynx, it has been speculated that transfer of resistance plasmids may also occur in vivo (Dillon et al., 1983). Evolution of resistance to antimicrobial agents in N. meningitidis has been shown to result from horizontal DNA transfer from commensal Neisseria species (Arreaza et al., 2002). Furthermore, throat
commensals such as *N. lactamica*, *Neisseria flavescens* and *Neisseria mucosa* that are intrinsically more resistant to penicillin than *N. meningitidis* have been suggested as sources of DNA in the emergence of *N. meningitidis* strains with intermediate resistance to penicillin (Saez-Nieto et al., 1990).

A recent study of meningococcal carriage in Greece showed that no strains were resistant to rifampicin, cefaclor, chloramphenicol, ceftriaxone or ciprofloxacin (Kremastinou et al., 2003). The percentage of strains with reduced sensitivity to penicillin was over 20% in some regions however, and was especially high in military personnel. Despite the low occurrence of antibiotic resistance among meningococci, eradication of meningococcal carriage using chemotherapy leads to development of resistance (Jackson et al., 1996), which should be monitored closely and continually.

**Immune response elicited by carriage**

Immunity to invasive meningococcal disease is dependent upon the presence of serum immunoglobulin G (IgG) which elicits bactericidal activity toward the infecting organism. Infants may be protected from meningococcal disease by the presence of maternal IgG which is obtained passively during gestation and lactation. Maternal immunity will be replaced by acquired immunity. Exposure to non-pathogenic *Neisseria* and other cross-reacting species in the nasopharynx increases the level of specific antibodies during childhood (Sanchez et al., 2001, 2002; Troncoso et al., 2000).

Carriage of commensal *Neisseria*, especially *N. lactamica*, is associated with a high titre of antibodies against *N. meningitidis*. Highly homologous structures are present in *N. lactamica* and *N. meningitidis* (Troncoso et al., 2000, 2001). Sera from mice immunized with *N. lactamica* and boosted with *N. meningitidis* have been shown to kill meningococci. The absence of capsule in *N. lactamica* indicates that OMPs and LOS are the major sources of this immunological activity (Sanchez et al., 2002). Immunization studies of mice with *N. lactamica* should be used to provide information about the immune response developed in response to meningococcal mucosal carriage.

Later on in life, exposure to meningococci and asymptomatic carriage will result in further immunity. Little is known, however, regarding the immune responses elicited by meningococcal carriage. Mucosal immunity in carriers can be detected by increasing concentrations of IgA in saliva (Robinson et al., 2002). Small quantities of complement proteins have been identified in saliva of carriers in confrontation with *N. meningitidis* (Andoh et al., 1997). Mucosal immunity is not able to prevent the colonization of the nasopharynx by meningococci, but it plays an important role in preventing the invasion of epithelial cell (Griffiss, 1995).

Humoral immunity has an essential role in protection against meningococcal infection, and carriage of *Neisseria* causes an increased bactericidal antibody response (Jones et al., 1998; Reller et al., 1973). Antibody studies indicate that carriage of some strains of *N. meningitidis* leads to the development of a response directed against the OMPs, particularly PorA, PorB and Opa, and against the LPS (Jones et al., 1998). The response is strain-specific, but some degree of cross-reactivity with heterologous strains is developed. The response may last several months after the carried strains have been lost. The anti-meningococcal antibodies have protective properties through binding of meningococcal surface antigens, activation of the complement system that causes phagocytosis or direct bactericidal killing (Pollard & Frasch, 2001). However, it is not clear whether natural immunization leads to immunological memory. Over half of the individuals with a late complement deficiency factor (C5-C9) are at risk of developing meningococcal disease and approximately 50% of them will have recurrent attacks (D’Amelio et al., 1992). These cases have a different age distribution than meningococcal patients in the general population, with most attacks occurring in late childhood or adulthood when carriage is higher (D’Amelio et al., 1992; Ross & Densen, 1984). The infection often involves an unusual serogroup of *N. meningitidis*, like W135 or Y, and has less severe clinical features (Fijen et al., 1989).

Cellular immunity and cytokine production in relation to meningococcal disease and carriage are poorly understood. *In vitro* study of T-cell responses were investigated by stimulating peripheral blood mononuclear cells from healthy individuals with a whole-cell lysate of a meningococcal strain (Robinson et al., 2002). Using intracellular staining and flow cytometry, the expression of the cytokines among activated CD4 cells was measured. The authors concluded that an unbiased T-helper cell subset response was elicited by meningococcal carriage.

Further studies of the immune response elicited by meningococcal carriage might be very valuable for the development of mucosal immunization strategies, which could be an effective approach to control meningococcal disease.

**Effect of vaccination on carriage**

Several investigations have been carried out to study the effect of vaccination on meningococcal carriage, using the polysaccharide vaccines, the polysaccharide conjugate vaccines, and, to a lesser extent, the serogroup B outer membrane vesicle vaccine.

In general, most meningococcal polysaccharide vaccines are poor immunogens in infants and fail to induce immunological memory in people of any age. Further, the effect of the polysaccharide vaccines on colonization and transmission of the organisms are transient or negligible (Jodar et al., 2002). Local immunity in the nasopharynx against meningococci was observed, after vaccination with serogroup C polysaccharide vaccine (Gotschlich et al., 1969). These observations were confirmed by others after vaccination with the A/C polysaccharides (Nurkka et al., 2000; Zhang et al., 2000). However, the level of antibodies declined rapidly to near pre-vaccination levels after 6–12 months, which indicated that the local protection against *N. meningitidis* was of short

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duration. The effect of vaccination with meningococcal serogroup A and C polysaccharide vaccine on carriage was studied in Spain. The study demonstrated a decrease of carriage rate of serogroup C 1 year after vaccination. The decline in prevalence of *N. meningitidis* serogroup C was especially considerable in 10–14- and 15–19-year-olds, but not in the 5–9-year-olds (Fernandez et al., 2003). Culturing of nasopharyngeal swabs, 3 weeks after vaccination with the polysaccharide A/C vaccine in the training centre for army medical officers in Italy, revealed that the percentage of isolates belonging to serogroup C was significantly reduced from 6 to 0–6 % (Di Martino et al., 1990). In another study in Italian army recruits, the vaccination of recruits with meningococcal A/C polysaccharides was shown to influence the carriage rate of *N. meningitidis* in these serogroups, but an increased prevalence of meningococci belonging to serogroup Y was observed (Di Martino et al., 1990).

Development of a vaccine against serogroup B meningococci has proved to be difficult because the serogroup B polysaccharide is poorly immunogenic in humans (Pollard & Frasch, 2001). Outer membrane vesicle vaccines are an alternative and the effect of such a serogroup B vaccine on carriage of meningococci was studied in Norway. The results indicated a slight reduction in carriage rate among the vaccinated individuals (Rosenqvist et al., 1994).

Polysaccharides may be conjugated to protein antigens to stimulate T-helper cells. These new conjugate vaccines against *N. meningitidis* induce an immune response, which is better than that achieved by the polysaccharide vaccine. They are immunogenic in infants and induce immunological memory (Fairley et al., 1996). In a study comparing the meningococcal group C conjugate and the A/C polysaccharide vaccine in adolescents, higher levels of IgA and IgG were observed in mucosal immune response (Zhang et al., 2000). Maiden et al. (2002) studied the effect of the introduction of the C conjugate polysaccharide vaccine on carriage in the UK. Carriage of serogroup C meningococci was reduced by 66% 1 year after vaccination. There were no significant changes in carriage of other disease-associated serogroups. The authors suggested that meningococcal serogroup C conjugate vaccines induced sufficient mucosal immunity to inhibit serogroup C polysaccharide.

The studies suggested that reduction of carriage might decrease the incidence of meningococcal disease and contribute to herd immunity. It is not yet known, however, whether this is a result of down-regulation of the capsule expression in serogroup C strains or whether these virulent serogroup C strains are eliminated from the population. Thus, the introduction of new meningococcal vaccines and assessment of their effectiveness must consider the dynamics of meningococcal carriage.

Genomic technologies like bioinformatics, proteomics and DNA microarrays offer exciting new opportunities for vaccine research. The opportunities emerging from genomic and post-genomic studies could shorten the time to vaccine discovery. Genes encoding potential protective antigens can now be identified using computer prediction and transcriptome and proteome analyses (Zagursky et al., 2003).

**Concluding remarks**

Many problems remain in understanding the meningococcal carriage state. These include the main mechanisms involved in the establishment of a commensal relationship between the bacterium and the host, and the link between meningococcal carriage and disease.

Additional molecular genetic studies on meningococcal carrier isolates are necessary to provide information on the mechanisms generating the high genetic diversity in *N. meningitidis*. Analysis of gene switching and mechanisms of regulation of expression of phase-variable proteins in both meningococcal carrier and disease-associated isolates will provide insights into the population’s response to changing conditions. Studies on meningococcal carrier and disease isolates employing DNA microarray technology should make it possible to monitor the whole genome on a single chip, providing a picture of the simultaneous interactions between thousands of genes. The technology can give valuable information on gene regulation at the transcriptional level during different stages of meningococcal infection. Understanding natural immunity as a consequence of carriage may allow the identification of individual antigens capable of inducing a protective immune response and thus facilitate the development of a vaccine against meningococci. Consequently, studies of the meningococcal carriage state are still warranted to increase our knowledge of the population biology of meningococci and determine how protection against meningococcal disease is established.

**Acknowledgements**

S. P. Y. was supported by the EU-MenNet project of the European Commission Contract QLK2-CZ-2001-01436.

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