Colonization and invasion of human epithelia by Neisseria meningitidis. Bacterial surface variation and exploitation of host defense molecules

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Chapter 2

Invasion of Primary Nasopharyngeal Epithelial Cells by Neisseria meningitidis Is Controlled by Phase Variation of Multiple Surface Antigens

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Summary

We have investigated bacterial factors required for the entry of Neisseria meningitidis serogroup B into mucosal cells, using a novel in vitro infection model of primary cultures of human nasopharyngeal epithelium. An invasive meningococcal phenotype was obtained after several cycles of selection for intracellular bacteria with gentamicin. Invasive bacteria differed from those in the initial inoculum in that they lacked a capsule and pili, exhibited a non-sialylated low molecularweight type of lipopolysaccharide (LPS), and produced a new 28kDa opacity outer membrane protein. LPS revertants of the selected meningococci expressed a nonsialylated L3,(7,9) type of LPS and were also invasive, while after LPS sialylation bacterial entry was inhibited. Variants lacking the 28kDa opacity protein were poorly invasive. Coexpression of the outer membrane protein Opc and the 28kDa opacity protein strongly inhibited microbial invasion into the primary cultured nasopharyngeal cells. Conversely, meningococcal internalization by cells of various epithelial cell lines was correlated with the expression of Opc rather than the 28kDa opacity protein. Our data indicate that a concurrent phase switching of multiple phase variable bacterial surface components may be a prerequisite for meningococcal invasion into nasopharyngeal epithelium and that meningococcal class 5 proteins (Opa and Opc) may promote tissue tropism.

Introduction

The human nasopharynx is generally thought to be the natural habitat and reservoir for Neisseria meningitidis. Reported carriage rates for meningococci vary from 5 to 15% of the healthy population (5–7, 10, 25). Despite this high carriage rate, invasive disease due to this bacterium is relatively rare, suggesting that particular conditions are required to establish an infection. Recent studies indicate that clonal virulence as well as factors that increase host susceptibility, such as coincident respiratory infections (for a review, see reference 25), may affect the balance between carriage and disease. A still unanswered question is whether in the carrier state the bacteria (i) penetrate the mucosal barrier and are eliminated after gaining access to the bloodstream or (ii) are held at the level of the mucosal epithelium and only certain invasive meningococci are able to breach the mucosal barrier. The latter implies that only certain meningococcal phenotypes have the intrinsic ability to translocate across the nasopharyngeal epithelium. A major difficulty in defining putative invasion factors is that the
bacteria display a strong intra- and interstrain variation in their surface constituents. Ongoing phase switching and antigenic variation of factors such as capsule (7, 10), pili (2, 28), the class I porin (2, 38), lipopolysaccharide (LPS) (15), and class 5 outer membrane proteins (Opa and Opc) (4, 9, 20) during carriage and/or meningococcal disease make it nearly impossible to identify relevant pathogenicity factors through characterization of fresh human isolates.

One approach that may enable identification of factors required for bacterial penetration of the mucosa is the use of *in vitro* infection models. A widely accepted model for studying meningococcal colonization is the human nasopharyngeal organ culture model, which is particularly suitable for morphological analysis of the interaction of wild type or recombinant bacteria with isolated native tissue (26, 27). An alternative approach is to use monolayers of established epithelial cell lines without the complexity of the native tissue (33–35), but with the disadvantage that the cells often do not match those of the native tissue because they either are derived from anatomical sites other than the nasopharynx or have lost original characteristics during prolonged propagation. In the present study, we established a novel infection model to investigate the meningococcus host cell interaction. This human model system consists of monolayers of primary cultured nasopharyngeal cells and has the advantage of working with cells directly derived from the target tissue at the site of colonization. The obtained data indicate that in our infection model, a phase switching of multiple surface constituents may be required to establish an invasive bacterial phenotype and that, in contrast to what is found with various epithelial cell lines, opacity protein rather than Opc is associated with bacterial entry.

**Material and methods**

**Bacteria**

*N. meningitidis* H44/76 (B:15:P1.7,16) was originally isolated from the cerebrospinal fluid (CSF) and belongs to the ET5 complex, which has been associated with many cases of invasive meningococcal disease (7). Bacteria were grown on GC agar base (Difco Laboratories, Inc., Detroit, Mich.) containing 1%Vitox supplement (Unipath, Oxoid Laboratories, Basingstoke, United Kingdom) at 37 °C in a humidified atmosphere of 5% CO2 in air for 16 to 18 h. In some experiments, pellicle grown bacteria were used. Growth of meningococci as pellicles at the interface between Trypticase soy broth and air has previously
been shown to increase the diversity in expression of phase variable surface components such as capsule, pili, and opacity protein (22). Colony morphology was determined with a Zeiss stereomicroscope with oblique substage lighting.

LPS immunotyping was carried out by the whole cell enzyme linked immunosorbent assay technique (1, 24), using a panel of LPS type specific monoclonal antibodies (RIVM, Bilthoven, The Netherlands). All meningococcal variants used throughout the experiments were kept as single stocks, frozen in 10% glycerol broth on glass beads at -70°C. The bacterial phenotypes of the variants are given in Table 1.

Cell culture media.
Complete RPMI medium consisted of RPMI 1640 (LifeTechnologies, Paisley, Scotland) containing 2 g of NaHCO3 per liter, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.2), 1 mM L-glutamine, 100 mg of streptomycin per ml, 100 U of penicillin per ml, and 1.25% fetal calf serum (Boehringer, Mannheim, Germany). MCDB 151 medium (Sigma, St. Louis, Mo.) was prepared according to the manufacturer’s instructions. Prior to filtration (0.22-µm-pore-size filter; Millipore), phosphoethanolamine (5 x 10^-27 M), ethanolamine (5 x 10^-27 M), HEPES (20 mM), molybdic acid (10^-9 M), stannous chloride (5 x 10^-10 M), nickel II sulfate (5 x 10^-10 M), and 1% (vol/vol) of a 100x solution of mixed amino acids (stock solution II) were added. Stock solution II was made according to the method of Pechl and Ham (21). The medium was stored in portions of 100 ml at 4°C for up to 1 month. Prior to use, transferrin (10 µg/ml), insulin (5 µg/ml), epidermal growth factor (5ng/ml), hydrocortisone (0.2 µg/ml), isoproterenol hydrochloride (0.15 µg/ml), L-glutamine (1 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) were added and the medium was filtered. All hormones, antibiotics, and chemicals were from Sigma.

Culture of epithelial cells
Monolayers of primary epithelial cells were established from explants of human nasopharyngeal tissue which were collected from children (from 15 months to 9 years of age) who underwent adenoidectomy. The obtained tissue was washed several times in RPMI 1640 medium, cut into pieces (2 mm x 2), and rinsed again. The tissue pieces were transferred to 60-mm-diameter petri dishes (Nunc, Roskilde, Denmark) (10 pieces per dish) containing 4 ml of complete RPMI medium and incubated (37°C, 5% CO2) for 4 to 5 days with daily medium changes. Then the adenoid fragments were transferred to culture dishes (35 by 10 mm;
Meningococcal invasion of nasopharyngeal cells

Falcon, Becton Dickinson, Plymouth, England), and sufficiently small volumes of serum free MCDB 151 culture medium were added to prevent the tissue specimens from floating. This transfer resulted in release and migration of epithelial cells from the surface of the explants. Following attachment of viable cells to the plastic support, actively proliferating cells were observed within 2 to 3 days, and confluent monolayers of nonciliated cells with numerous microvilli and a typical epithelial cell morphology were obtained 3 to 4 days later. During culture, the MCDB 151 medium was changed every second day without disturbing the explants. The epithelial nature of cultured cells was confirmed by immunostaining using anticytokeratin antibodies and by the presence of tonofilaments as observed by electron microscopy. No fibroblast contamination was observed.

Cells were cultured in medium without antibiotics for at least 2 days prior to the start of the infection experiments. Experiments were performed within 2 days after confluence was reached and within 24 h after removal of the tissue fragments.

Chang human conjunctiva cells (ATCC CCL20.2), HEC1B human endometrium carcinoma cells (ATCC HTB133), and H292 human pulmonary mucoepidermoid carcinoma cells (ATCC CRL 1848) were cultured in RPMI 1640 medium with 5% fetal calf serum. Cells were grown on circular glass coverslips in 24 well tissue culture plates (Nunc) and used in the infection experiments just before reaching confluence.

Infection experiments

Bacterial adherence and invasion experiments with nasopharyngeal cells were carried out in 35-mm-diameter tissue culture dishes containing 1 ml of MCDB 151 medium. Experiments with the epithelial cell lines were done in 24 well plates containing 1 ml of RPMI 1640 plus 5% fetal calf serum per well. After addition of the meningococci (5 x 10^6 /ml) the cells were incubated for 3 h (37°C, 5% CO_2 ), washed with Dulbecco’s phosphate-buffered saline (DPBS) to remove non adherent bacteria, and then incubated for another 5 h in fresh medium. The cells were then washed five times with DPBS and fixed with 1% (wt/vol) paraformaldehyde–0.1% (vol/vol) glutaraldehyde in DPBS (30 min, 20°C).

Selection for invasive meningococci was done by performing infection experiments as described above with the following modifications, namely, pellicle grown bacteria (10^6 CFU/ml) of the parent strain H44/76 (designated H44/76#p) were used as an inoculum, and after 8 h of infection, the cells were washed and exposed to gentamicin (250 μg/ml) for 2 h to eliminate extracellular bacteria (17). Viable intracellular bacteria were obtained after lysis of
the cells using Saponin (1 ml of a 1% solution in DPBS for 15 min), by plating appropriate dilutions of the lysate on GC agar. Meningococci grown after this first cycle of selection were subjected to a second and third cycle of infection including exposure to gentamicin to further enrich for invasive bacteria. Bacterial adherence and invasion were examined by light microscopy after immunogold-silverstaining and/or crystal violet staining as previously described (32) and by electron microscopy. Variants expressing or lacking various surface components and revertants of the invasive variant (H44/76#1) in which individual components switched back to the parental phenotype were selected on the basis of a different colony opacity (Opa Opc) and by colony immunoblotting using L3,7,9 specific antibodies (15, 24). Exogenous LPS sialylation was achieved by growing bacteria in the presence of the substrate cytidine monophosphate N-acetylneuraminic acid (CMP-NANA; 5 μg/ml) (Sigma) and addition of this compound to the MCDB 151 medium (5 μg/ml) during the infection assay.

**SDS-PAGE and Western blotting (immunoblotting)**

Outer membrane fractions were obtained by Sarkosyl extraction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-denaturing and denaturing conditions (37). The separated proteins were either stained with Coomassie brilliant blue R250 or transferred onto nitrocellulose sheets as described elsewhere (19). To detect opacity proteins and Opc, blots were incubated with the Opa-specific monoclonal antibody 4B12/C11 and the monoclonal antibody A222/5b, respectively (both kindly provided by M. Achtman) and thereafter with alkaline phosphatase-conjugated anti-mouse immunoglobulins (Sigma). For the detection of pilin and the pilus-associated protein PilC, rabbit antisera raised against pilin (AK36) and against gonococcal PilC (AK203) (both kindly provided by T. F. Meyer) were used. Reactive proteins were visualized as described previously (37).

LPS was analyzed after proteinase K digestion of outer membrane fractions (14). Neuraminidase (Clostridium perfringens type V; Sigma) treatment of outer membrane fractions was performed as previously described (30). Samples were separated by the Tricine SDS-PAGE system (16). LPS bands were silver stained as described elsewhere (30).

**Electronmicroscopy**

Meningococcal polysaccharide was demonstrated by immunoelectron microscopy. Whole organisms were fixed in 1% (wt/vol) paraformaldehyde and incubated with serogroup B-
specific polyclonal antiserum (Reference Laboratory for Bacterial Meningitis, Amsterdam, The Netherlands) and protein A-gold as previously described (36). Meningococcal pili were visualized after negative staining with 2% aqueous uranyl acetate.

To perform electron microscopy on epithelial cells, monolayers of cells grown on circular plastic coverslips (13-mm diameter; Thermanox) or on collagen membranes (Cellagen; ICN Biomedicals) were fixed (24 h, room temperature) in 4% (wt/vol) paraformaldehyde-1% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Then the cells were washed with cacodylate buffer, post fixed (1 h, 4°C) in 1% OsO4 in the same buffer, dehydrated in increasing concentrations of ethanol, and embedded in epoxy resin (LX112). Ultrathin sections were cut with a diamond knife and examined in a Zeiss EM 10 C electron microscope.

Scanning electron microscopy of infected samples was performed after critical point drying and evaporation of platinum-iridium on paraformaldehyde-glutaraldehyde-fixed specimens as described previously (29).

### TABLE 1. Phenotypes of strain H44/76 variants used in this study

<table>
<thead>
<tr>
<th>Variant</th>
<th>Pili Type</th>
<th>Capsule</th>
<th>28kDa Opa</th>
<th>Opc</th>
<th>LPS</th>
<th>Colony opacity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H44/76</td>
<td>Type a</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>L3,7,9</td>
<td>--</td>
<td>CSF</td>
</tr>
<tr>
<td>H44/76#p</td>
<td>Type a</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>L3</td>
<td>--</td>
<td>H44/76#p select</td>
</tr>
<tr>
<td>H44/76#0</td>
<td>Type b</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>L3</td>
<td>--</td>
<td>H44/76#p select.</td>
</tr>
<tr>
<td>H44/76#1</td>
<td>Negative</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>L8</td>
<td>+</td>
<td>H44/76#1 col. blot</td>
</tr>
<tr>
<td>H44/76#1A</td>
<td>Negative</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>L3,8</td>
<td>++</td>
<td>H44/76#1 col. blot</td>
</tr>
<tr>
<td>H44/76#2</td>
<td>Negative</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>L8</td>
<td>+++</td>
<td>H44/76#2 col. blot</td>
</tr>
<tr>
<td>H44/76#2A</td>
<td>Negative</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>L3</td>
<td>+++</td>
<td>H44/76#2 col. blot</td>
</tr>
<tr>
<td>H44/76#3</td>
<td>Negative</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>L8</td>
<td>++</td>
<td>H44/76#3 col. blot</td>
</tr>
</tbody>
</table>

\[a\]--, not expressed; ++, strongly expressed.

\[b\] +, ++ and +++ degree of colony opacity; --, colony opacity masked by presence of capsule.

\[c\] Select., selected for invasion; col. blot, colony immunoblot.

\[d\] +, different (30kDa) opacity protein present.
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Results

Attachment and entry of *N. meningitidis* into cultured nasopharyngeal epithelial cells

To study the interaction of meningococci with the monolayers of cultured nasopharyngeal cells, we initially used a heavily piliated clinical CSF isolate of the meningococcal strain H44/76 (B:15;P1.7,16;L3,7,9). These bacteria however, poorly adhered to the nasopharyngeal cells (10 bacteria per cell after 8 h of incubation), and essentially no intracellular bacteria were seen in the light microscope. To increase the probability of obtaining bacteria that are able to adhere to or invade the nasopharyngeal cells, we used a heterogeneous population of pellicle grown meningococci (designated H44/76#p) as an inoculum. In order to enrich for invasive meningococcal variants, the monolayers of nasopharyngeal cells were exposed to gentamicin to eliminate extracellular bacteria (17). After three successive cycles of infection and exposure to gentamicin, a 100 fold increase of surviving bacteria was obtained (10⁴ CFU of the invasion enriched bacteria per ml and 10² CFU of the nonselected bacteria per ml). Examination of the colonies arising from the internalized bacteria by transillumination microscopy revealed opaque and blue-gray iridescent colonies. Both the blue-gray colony variant (H44/76#0) and the meningococci from the more opaque colonies (H44/76#1) survived in the presence of gentamicin following infection (10⁴ and 10⁵ surviving bacteria, respectively). Light microscopy revealed large numbers of intracellular bacteria (50 to 200 per cell, using nasopharyngeal cells from different donors) of variant H44/76#1, whereas bacteria of variant H44/76#0 were not seen inside cells. The latter bacteria had formed thick layers of tightly packed bacteria at the cell surface. These results were confirmed by electron microscopy (Fig. 1). The selection of the adherent, non-invasive variant H44/76#0 was not due to a decreased susceptibility to gentamicin, as all variants were equally susceptible to gentamicin (MIC, 8 mg/ml).

Different expression of capsule and pili by the invasive and noninvasive meningococcal variants

The molecular basis for the increased bacterium-host cell interaction was examined by systematic analysis of the composition of the bacterial cell surface, with emphasis on known variable components. Immunoelectron microscopy of the selected variants and the original CSF isolate using serogroup B-specific capsule polysaccharide antiserum and gold-
Meningococcal invasion of nasopharyngeal cells

FIG. 1. Transmission (a and b) and scanning (c to f) electron micrographs showing the localization of the invasive variant H44/76#1 (a, c and e) and the adherent, noninvasive variant H44/76#0 (b, d and f) obtained by the gentamicin selection procedure. Note that the invasive phenotype preferentially adheres at the central part of the host cell surface and interacts with microvillous projections from the host cells (e). The noninvasive adherent variant (H44/76#0) covers the entire cell surface (d) and forms a complex of tightly packed bacteria with intervening bundles of pili (f). Bars represent 1 μm (a and b) and 10μm (c to f)
conjugated protein A, revealed that both the parent H44/76 (not shown) and the adherent non-invasive (H44/76#0) bacteria positively labeled for the presence of capsule, in contrast to the invasive H44/76#1 (Fig. 2). This observation indicates that the presence of a capsule does not interfere with bacterial adherence but that it may hamper meningococcal entry into nasopharyngeal cells. This assumption was supported by results from the analysis of the few intracellular meningococci recovered from the cells after 36 h infection with the encapsulated parental isolate (H44/76). Eight of every ten bacteria examined were capsule negative, as demonstrated by colony immunoblotting (data not shown).
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Analysis of the state of piliation of the variants H44/76#0 and H44/76#1 by electron microscopy demonstrated that the invasive bacteria (H44/76#1) were pilus negative (Fig. 2c), excluding pili as an essential factor for bacterial entry. Microscopy of the selected adherent, non-invasive variant H44/76#0 (Fig. 2d) and the poorly adhering parent strain H44/76 (not shown) showed that both types of bacteria were heavily piliated, implying that expression of pili is not always associated with bacterial adherence. In an attempt to find an explanation for this phenomenon, we investigated the migration of the major subunit of the pili, pilin and looked for the expression of pilus associated protein PilC by SDS-PAGE and immunoblotting.

This showed that the adherent, non-invasive bacteria (H44/76#0) expressed a pilin of slightly higher apparent molecular weight and produced more immunoreactive PilC than did the poorly adherent parent H44/76 (Fig. 3), suggesting that variation in the pilus had occurred. This may have affected the binding properties of the assembled pili. It should be noted that also the non-piliated, invasive meningococci (H44/76#1) expressed both pilin and a low level of PilC (Fig. 3). The smaller size of this pilin in conjunction with the absence of assembled pili suggests that the invasive bacteria produced S-pilin (12).

FIG. 3. Western blot analysis of whole-cell lysates with antiserum against pilC and pilin, showing the presence of PilC protein and differences in electrophoretic mobility of pilin, in the different meningococcal variants. Lane 1, parent CSF isolate H44/76; lane 2, adherent noninvasive variant H44/76#0; lane 3, invasive variant H44/76#1; lane 4, variant expressing both Opa and Opc, H44/76#2.

LPS as a determinant of meningococcal entry into the nasopharyngeal epithelium

Examination of the LPS of individual variants by determining migration on SDS-PAGE and by LPS immunotyping revealed that both the parent strain (H44/76) and the strongly adherent, non-invasive H44/76#0 produced a high molecular weight LPS of the L3,(7,9) immunotype. Neuraminidase treatment of the LPS samples resulted in a slightly increased electrophoretic mobility of the upper faint band of this LPS, leaving a single band, indicating the presence of sialic acid. In contrast, the LPS of the invasive variant H44/76#1 had a much lower molecular
weight, was characterized as belonging to the L8 immunotype of LPS, and was resistant to neuraminidase treatment (Fig. 4). To evaluate whether the observed transition in LPS phenotype was essential for the bacterial entry process to occur, we selected LPS backswitchers (H44/76#1A) of the invasive variant by colony immunoblotting, using the L3-specific antibody Mn4A8B2. Immunotyping and SDS-PAGE showed that this selected variant (H44/76#1A) coexpressed both the L3,(7,9) and the L8 form of LPS. Invasion experiments demonstrated that H44/76#1A was only 10% less invasive than H44/76#1, suggesting that switching between these two LPS types is not decisive for bacterial invasiveness. However, when we tested the 45-kDa LPS component for its ability to be sialylated endogenously, we found that in contrast to the non-invasive variants H44/76 (parent) and H44/76#0, the LPS backswitchers (H44/76#1A) no longer endogenously added sialic acid to their LPS. When H44/76#1A was grown and tested for invasion in the presence of exogenous CMP-NANA (1.6 nmol/ml), LPS sialylation did occur (Fig. 4), and although adherence was unchanged, bacterial entry into the primary epithelial cells was reduced to only one-third (40 versus 122 intracellular bacteria with and without CMP-NANA, respectively). Thus, transition to a non-sialylated LPS phenotype rather than the length of the LPS molecules appears to be an important determinant of meningococcal invasiveness. It should be noted that the failure of the LPS backswitcher to endogenously sialylate its LPS was not necessarily the result of the nonencapsulated state of this variant, as spontaneous non-encapsulated variants obtained from the parental H44/76 strain by colony immunoblotting still endogenously incorporated sialic acid into their LPS (data not shown).

**FIG. 4.** LPS profiles of the various meningococcal variants before (-) and after (+) treatment with neuraminidase. LPS was visualized after silverstaining of Tricin-SDS-PAGE-separated protein K digests of outer membranes. Lanes: 1, H44/76; 2, H44/76#0; 3, H44/76#1; 4, H44/76#2; 5, H44/76#3; 6, H44/76#1A; 7, H44/76#1A grown in the presence of exogenous CMP-NANA.
Role of opacity protein and Opc in the attachment and entry of meningococci into primary epithelial cells

Comparison of the outer membrane composition of the parent strain and the selected variants by SDS-PAGE revealed identical outer membrane profiles, except for the presence of additional 30-kDa (H44/76#0) and 28-kDa (H44/76#1) protein bands (Fig. 5A).

Electrophoresis of the samples under non-denaturating and denaturing conditions revealed that the proteins were heat modifiable, suggesting that they belong to the family of opacity outer membrane proteins. This was confirmed by Western blotting using monoclonal antibodies directed against opacity outer membrane protein (4B12/C11) and against Opc (A222/5b). The 30 and 28-kDa proteins positively stained for opacity (Opa) protein (Fig. 5D). Opc was detected only in very low levels in both the invasive and noninvasive variants (Fig. 5C).

To further substantiate the apparent relationship between the expression of a new 28-kDa protein but not Opc and meningococcal entry into primary nasopharyngeal cells, we randomly picked 16 colonies from the population of bacteria that survived after the third cycle of infection and gentamicin treatment.

Fourteen of these showed the same phenotype as H44/76#1, including the expression of a 28kDa Opa protein, and all five of those that were tested in the infection assay showed equal
numbers of internalized bacteria compared with H44/76#1. The bacteria of the other two colony variants showed outer membrane profiles identical to that of the non-invasive variant H44/76#0 and lacked the 28-kDa protein. Bacteria of one of these colonies were used in the infection assay. They were unable to enter the epithelial cells, consistent with previous results. These data strongly suggest that invasion of meningococci into nasopharyngeal cells is associated with the expression of a 28-kDa opacity protein rather than with Opc, which has been implicated as playing a role in the entry of meningococci into epithelial cell lines and endothelial cells (34,35).

Colony immunoblotting of the invasive bacteria revealed that several colonies showed segments strongly positive for Opc. An additional infection experiment, including gentamicin treatment, did not enhance the number of Opc positive colonies, indicating that there was no selection for Opc expressing bacteria during passage over the primary nasopharyngeal cells.

To further investigate the role of Opc in the meningococcal invasion of nasopharyngeal cells, we selected two different, strongly Opc positive (Opc++) bacteria from our invasive variant (H44/76#1) by colony immunoblotting. Extensive analysis, including SDS-PAGE of denatured and non-denatured samples in the presence of 4 M urea to detect possible differences in Opa protein expression (4), demonstrated that the first variant, H44/76#2, differed from its invasive parent (H44/76#1) only by the additional expression of Opc (Fig. 5). When tested for its ability to enter nasopharyngeal cells, H44/76#2 was found to adhere strongly to the mucosal cells, forming lawns of bacteria, each partly covering single epithelial cells. Intracellular bacteria, however, were scarce (Fig. 6A), although the bacteria had all the other characteristics of the selected invasive phenotype, including the 28-kDa opacity outer membrane protein (Table 1). The second variant, H44/76#3, showed a phenotype similar to that of the invasive variant H44/76#1, except that Opc was present instead of the 28-kDa opacity protein. Within 2 h after addition to the monolayers, these meningococci strongly adhered as large clumps to the primary cells (Fig. 7). Although at the end of the infection assay intracellular bacteria were present, the numbers of internalized bacteria were only <25% of those obtained with variant H44/76#1 (<30 per cell in all experiments). Thus, Opc may confer moderate invasion of primary epithelial cells, but in a 28-kDa Opa positive background (H44/76#1), it inhibits meningococcal invasiveness and downregulation of Opc expression may be required to establish a highly invasive meningococcal phenotype.
FIG. 6. Interactions of a selected nasopharyngeal cell invasive variant (H44/76#1) and the variant coexpressing high levels of Opc (H44/76#2) with the primary cultured nasopharyngeal cells (nasoph.) and with several established human epithelial cell lines (Chang, H292, HEC-1B). The number of adherent (hatched bars) and intracellular (closed bars) bacteria were determined by microscopy and represent absolute numbers of bacteria per cell. Note that the variant that expresses only 28-kDa Opa (44#1) invades nasopharyngeal cells but is poorly ingested by the cell lines while the bacteria coexpressing high levels of Opc (44#2) invade the cell lines but are poorly ingested by the nasopharyngeal cells.

Tissue tropism of meningococcal class 5 proteins

To further ascertain the apparent cell-type-specific roles of opacity protein and Opc in meningococcal invasion, we tested both the variant that invaded nasopharyngeal cells (H44/76#1) and its non-invasive Opa++Opc++ counterpart (H44/76#2) for their ability to enter cells of several established human epithelial cell lines (Chang conjunctiva cells, HEC 1B endometrium cells, and H292 lung carcinoma cells). As shown in Fig. 6, all these cell lines favored adherence and internalization of meningococci coexpressing high levels of Opc protein (H44/76#2). Bacteria that expressed only the 28-kDa protein (H44/76#1) were much less invasive. This result is consistent with the proposed role of Opc in the invasion of epithelial cell lines (34, 35). To confirm that indeed the Opc protein, and not the combination of Opa and Opc, was necessary for invasion into these various cell lines, we tested the interaction of the Opc positive, 28-kDa Opa negative variant H44/76#3. The invasive properties of this variant were similar to those of variant H44/76#2, since 12 and 13 intracellular bacteria per cell, respectively, were seen within Chang epithelial cells. Thus, Opc, expressed either singly or in combination with the 28-kDa opacity protein, rather than
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the 28-kDa opacity protein alone is associated with meningococcal entry into various epithelial cell lines; this in contrast to the results obtained with the monolayers of nasopharyngeal cells.

Discussion

In the human host, its only known reservoir and source of transmission, \textit{N. meningitidis} resides in the nasopharynx. It seems reasonable to assume that at this site there is some unique property favoring meningococcal colonization. We developed a new cell system, monolayers of primary cultured epithelial cells of the human nasopharynx, to study the interaction between meningococci and nasopharyngeal cells. Our results demonstrate that capsulated meningococci can adhere to the cell surface in this system through a pilus-type-dependent process and that a concurrent transition in expression of multiple phase variable surface components is required to establish an invasive meningococcal phenotype. This phenotype differs in many of its characteristics in comparison to those of the parent strain. Our findings indicate that phase variation of various meningococcal surface components may be critical for the establishment of infection.

A primary finding in our experiments was that the expression of pili does not unequivocally lead to adherent bacteria. Comparison of surface components of the parental strain and the selected adherent, non-invasive variant (H44/76#0) showed that both were strongly capsulated but produced pilins of different electrophoretic mobilities (Fig. 3). This suggests that the dramatic difference in adherence between the parent strain and variant (H44/76#0) is caused by the expression of functionally different pili. This finding is consistent with observations made in studies using various epithelial cell lines (18, 33). In our cell system, expression of the appropriate pilus type (H44/76#0) resulted in a very rapid (in 15 min) adherence, resulting in multilayers of bacteria attached to the cell surface. The bacteria were so tightly packed that this probably hampered the efficacy of gentamicin. This finding implies that the bacteria surviving after exposure of infected tissue cells to gentamicin do not necessarily represent internalized bacteria. This means that at least with certain meningococcal variants this method is not appropriate to quantitate meningococcal invasion. Isolates of \textit{N. meningitidis} from systemic infections, like the parent strain used in this study, are invariably encapsulated (8) and mostly piliated (11) and express a long, sialylated form of
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LPS (15). The invasive meningococcal variant (H44/76#1) that we selected was capsule deficient, expressed S-pilin instead of assembled pili, and showed a non-sialylated LPS of the L8 immunotype. In addition, it possessed a 28-kDa opacity protein. This difference in phenotype probably reflects optimal adaptation of this variant to the nasopharyngeal cell environment.

Capsule appeared to be disadvantageous for penetration of the mucosal cell barrier (34), while it is crucial for bacterial survival during dissemination (13). A similar situation may exist for LPS sialylation. The selection for a low molecular weight L8 type of LPS at the surface of our invasive variants may be due to the fact that the longer L3,(7,9) type of LPS in many serogroup B and C meningococci is endogenously sialylated. Sialylated LPS has been reported to inhibit bacterial entry of both meningococci and gonococci, probably by hampering opacity protein function (13, 31, 34), while it favors escape from host defenses (30). Evidence that LPS sialylation and not the type of oligosaccharide moiety of LPS is a critical determinant of meningococcal entry was obtained by the finding that invasiveness of variants that reverted from an L8 to a non-sialylated L3,(7,9) type of LPS was unchanged. After sialylation of this L3,(7,9) form of LPS by the addition of exogenous CMP-NANA, bacterial entry was strongly reduced. Thus, a switch to a nonsialylated form of LPS rather than the expression of an L8 immunotype of LPS appears to be required to establish an invasive meningococcal phenotype.

Meningococcal invasion into the primary cultured nasopharyngeal cells was associated with the expression of a 28-kDa opacity protein, while coexpression of Opc negatively influenced the entry into this type of cells. This result is remarkable considering the invasion promoting role of Opc with various cell lines, as found in this study and reported earlier (34, 35). It should be noted, however, that not all meningococci can express the Opc protein, especially organisms belonging to the ET37 related serogroup C strains, and serogroup B bacteria of the A4 cluster do not possess the opc gene (3). Our data indicate that indeed Opc is associated with meningococcal entry into various epithelial cell lines. A low expression level of Opc may allow strong 28-kDa Opa-dependent meningococcal invasion of the primary nasopharyngeal cells, while a high level of Opc down regulates invasion of nasopharyngeal cells and favors internalization by the cells of several established epithelial cell lines. Therefore, it seems likely that phase variation of class 5 proteins (Opa and Opc), and in particular of Opc, confers tissue tropism with respect to the invasive properties of the bacteria. How Opc is able to inhibit Opa mediated invasion into nasopharyngeal cells is uncertain, but Opc mediated clumping of bacteria may be a critical factor (Fig.7). Using a number of
additional LPS and Opa Opc variants, we noticed that Opc mediated clumping of bacteria was much more pronounced in bacteria with an L8 type of LPS than with meningococci.

expressing a non-sialylated L3,(7,9) form of LPS With the present identification of the serogroup B meningococcal variant H44/76#1A, unable to endogenously sialylate its L3,(7,9) LPS component, there arises the possibility that these variants, when expressing high levels of Opc, adhere in a more dispersed manner than variant H44/76#3 and show good invasive abilities, as has been observed for comparable variants in group A meningococci (33, 35). Despite extensive efforts, such a variant [Opc++, L3,(7,9)] was not obtained. However, variant H44/76#2A, expressing both Opc and 28-kDa Opa and possessing a nonsialylated L3,(7,9) immunotype LPS, showed a more dispersed adhesion pattern and an increased invasion into nasopharyngeal cells compared with the L8 positive variant H44/76#2 (26 versus 10 intracellular bacteria per cell, respectively).

On the basis of our experimental results, in vitro observations made with the nasopharyngeal organ culture model (26), and characterization of carrier isolates from the human nasopharynx (15), we hypothesize that the following events occur during meningococcal colonization of the nasopharynx. Carriers are colonized with capsulated and non-encapsulated piliated bacteria. After the initial attachment, unknown factors promote a strong Opc expression and a loss of capsule and perhaps pili. By switching to a non-sialylated L8 immunotype [or in the absence of host-derived CMP-NANA to a nonsialylated L3,(7,9) form of LPS] and expression of an appropriate opacity protein, like the 28-kDa Opa in our
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Strain H44/76, meningococci gain the ability to enter the mucosal cells. This entry, however, seems to require a down regulation of the adhesive function of Opc. In contrast to phase variable Opa proteins, Opc can be expressed in different amounts (23). In this way, modulation of invasive abilities can be achieved without significantly influencing the adherence to the host cells, as both Opc and 28-kDa Opa sustain adherence. This role for Opc in adherence and invasion might explain why meningococci expressing large amounts of Opc protein are preferentially isolated from the nasopharynx of patients and healthy carriers compared with isolates from the bloodstream or CSF, which generally express small amounts of this protein (4).

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References


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