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INTERACTIONS OF PATHOGENIC *NEISSERIAE* WITH EPITHELIAL CELL MEMBRANES

Alexey J. Merz[1] and Magdalene So
Department of Molecular Microbiology & Immunology, L220, Oregon Health Sciences University, Portland, Oregon 97201-3098; e-mail: somaggie@ohsu.edu

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■ **Abstract** The closely related bacterial pathogens *Neisseria gonorrhoeae* (gonococci, GC) and *N. meningitidis* (meningococci, MC) initiate infection at human mucosal epithelia. Colonization begins at apical epithelial surfaces with a multistep adhesion cascade, followed by invasion of the host cell, intracellular persistence, transcytosis, and exit. These activities are modulated by the interaction of a panoply of virulence factors with their cognate host cell receptors, and signals are sent from pathogen to host and host to pathogen at multiple stages of the adhesion cascade. Recent advances place us on the verge of understanding the colonization process at a molecular level of detail. In this review we describe the Neisseria virulence factors in the context of epithelial cell biology, placing special emphasis on the signaling functions of type IV pili, pilus-based twitching motility, and the Opa and Opc outermembrane adhesin/invasin proteins. We also summarize what is known about bacterial intracellular trafficking and growth. With the accelerated integration of tools from cell biology, biochemistry, biophysics, and genomics, experimentation in the next few years should bring unprecedented insights into the interactions of Neisseriae with their host.

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[1]Present address: Department of Biochemistry Dartmouth Medical School, Hanover, New Hampshire 03755-3844.

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INTRODUCTION

You can talk about leukemia all day long, because there's no specific cure, but the clap—you could whack it out in two days with all the antibiotics, so how come it's up there and stays up there?

Lenny Bruce, *How to Talk Dirty and Influence People*

Neisseria gonorrhoeae and *N. meningitidis* are the two pathogenic members of the *Neisseriae* family of gram-negative bacteria. *Neisseriae* cells assume a coccoid shape and occur in pairs. *N. gonorrhoeae* and *N. meningitidis* are closely related pathogens with only one habitat, humans, and no other known reservoirs. *N. gonorrhoeae* causes gonorrhea, and *N. meningitis* causes septicemia and meningitis. The last several years have brought enormous progress in our understanding of the interactions of these pathogens with host cells, and we are on the threshold of understanding, in molecular detail, "how come [Neisseria disease is] up there and stays up there." Genetic tools are well developed, and genome projects have provided, or will soon provide, complete sequence data for one *N. gonorrhoeae* strain and three *N. meningitidis* strains. The application of cell biological techniques has led to the identification of several classes of host cell receptors and is beginning to elucidate the signals that are passed between these bacteria and their host cells. In this review we concentrate on neisserial colonization of mucosal epithelia. We refer readers to several recent excellent reviews that highlight different aspects of neisserial pathogenesis and complement the present discussion (Booy & Kroll 1998, C Dehio et al 1998, Nassif et al 1999, Naumann et al 1999, Schryvers & Stojiljkovic 1999, Vogel & Frosch 1999).

Gonococcal Pathogenesis

A classic review of gonorrhea (Harkness 1948) describes the clinical manifestations of gonococcal urethritis. Sexually transmitted gonococcal infections typically occur on the mucosal epithelia of the male urethra or the female uterine cervix. GC

can also infect the rectum, throat, and conjunctiva of the eye. GC transmission generally occurs through direct sexual contact but indirect modes of transmission have been reported (Kleist & Moi 1993). Infection is frequently accompanied by an intense focal inflammatory response, and shedding of infected epithelial cells is common. In females, GC can ascend the urogenital tract, resulting in pelvic inflammatory disease (PID). Disseminated gonococcal infection (DGI) causes acute arthritis and, less often, myocarditis (Cucurull & Espinoza 1998, Masi & Eisenstein 1981).

Meningococcal Pathogenesis

Meningococci (MC) are facultative commensals because they colonize the nasopharynx of 3–30% of healthy individuals. However, MC can traverse the mucosal barrier and enter the bloodstream, causing septicemia, and can traverse the blood-brain barrier, resulting in fulminant meningitis (Booy & Kroll 1998, Nassif 1999, van Deuren et al 2000). In underdeveloped regions, epidemics of MC infection are major causes of morbidity and mortality, causing 10,000 or more deaths in a single outbreak (Hart & Cuevas 1997). The triggers of meningococcal disease are poorly understood, but environmental factors, bacterial genetics, and host genetics have all been implicated in MC pathogenesis.

Genetic Considerations

GC and MC encode diverse surface structures that can interact with host cells. However, the expression of these structures varies widely among the members of an infecting population—even if the population begins as a clonal isolate. Two general types of variation occur. In phase variation, the control of expression is binary: on or off. In antigenic variation, the variant protein changes in primary sequence. Both types of variation occur through heritable genetic mechanisms, which have been studied intensively and are reviewed elsewhere (Meyer et al 1994, Seifert 1996, Seifert & So 1988).

Phase and antigenic variation clearly facilitate microbial evasion of immune responses. However, it has become evident that the resulting polymorphisms influence all aspects of GC and MC biology: antigenicity, hydrophobicity and charge density, agglutination, resistance to microbicidal substances, motility, specificity of host cell adhesion and invasion, and nutritional requirements. Phase and antigenic variation thus can be thought of as mechanisms of cell differentiation because they cause phenotypic divergence within a cell lineage.

Phase variation was first described in *Salmonella* (Andrewes 1922), and comparable mechanisms exist in many (perhaps most) bacteria, as well as in pathogenic viruses and parasites. In GC and MC, phase and antigenic variation are so prevalent that they must be considered dominant features of neisserial infection (Parkhill et al 2000, Tettelin et al 2000). Indeed, these mechanisms are important enough that they may have drawn research interest away from more traditional mechanisms of physiological adaptation. Regulatory elements that sense and respond to environmental cues are well characterized in other bacterial pathogens and in

many cases are essential for virulence. However, the mechanisms of physiological adaptation are comparatively unexplored in GC and MC, despite strong evidence that at least some features of neisserial infection are elaborately choreographed.

Overview: Morphology of the Infection Process

Because GC and MC normally infect only human hosts, animal models for mucosal colonization by the *Neisseriae* have not been available, although a promising murine model for GC infection was recently introduced (Jerse 1999). Studies of neisserial interactions with epithelia have therefore relied heavily on ex vivo organ culture and primary or immortalized cell lines. Fallopian tube and nasopharyngeal organ cultures (FTOC, NPOC) are considered reference models for GC and MC infection of columnar epithelia (McGee et al 1983, Stephens et al 1983, Ward et al 1974). We begin by describing the infection process in these systems to provide a context for the more detailed discussion that follows. Infection in the organ culture models occurs as a fairly complex sequence of events. Correlated events occur in polarized T_{84} epithelial cells, as depicted in Figure 1 (Merz et al 1996; Merz & So 1997; Pujol et al 1997, 1999).

Adhesion and Invasion

Like enteropathogenic *Escherichia coli* (EPEC) (see Goosney et al 2000)), GC and MC initiate infection by attaching to host cells via surface-associated filaments called type IV pili (McGee et al 1981, Merz et al 1996, Mosleh et al 1997, Pujol et al 1997, Stephens & McGee 1981, Swanson 1973). In the first hours of infection, GC and MC typically attach as microcolonies of 10–100 diplococci. Attachment is accompanied by the elongation of host cell microvilli or filopodia toward the bacterial cells (Pujol et al 1999, Shaw & Falkow 1988, Stephens et al 1983, Ward & Watt 1972, Ward et al 1974); in thin sections, microvilli are often found anastomosing though the microcolonies (Figure 1*A*). Pilus-mediated attachment in the FTOC and NPOC models is restricted to nonciliated secretory epithelial cells (McGee et al 1983, Stephens & McGee 1981).

Type IV pili also mediate neisserial motility and dispersal over the epithelial surface (Pujol et al 1999; AJ Merz & M So, unpublished data). At later times (6–18 h) after infection, bacteria disperse from the microcolonies, pili disappear, and individual diplococci become intimately associated with the host plasma membrane (Figure 1*B,C*). At this time, host and bacterial membranes are often in such tight apposition that they frequently cannot be distinguished in transmission electron micrographs (McGee et al 1981, Shaw & Falkow 1988, Stephens et al 1983, Ward & Watt 1972, Ward et al 1974). One report suggests that intimately associated host and bacterial membranes actually fuse (Apicella et al 1996). At this stage, the surfaces of heavily infected cells are denuded of microvilli.

The region of contact between host and bacterial membranes enlarges (Figure 1*C*), and many bacteria are subsequently engulfed by epithelial cells (Apicella et al 1996, McGee et al 1981, Shaw & Falkow 1988, Stephens et al

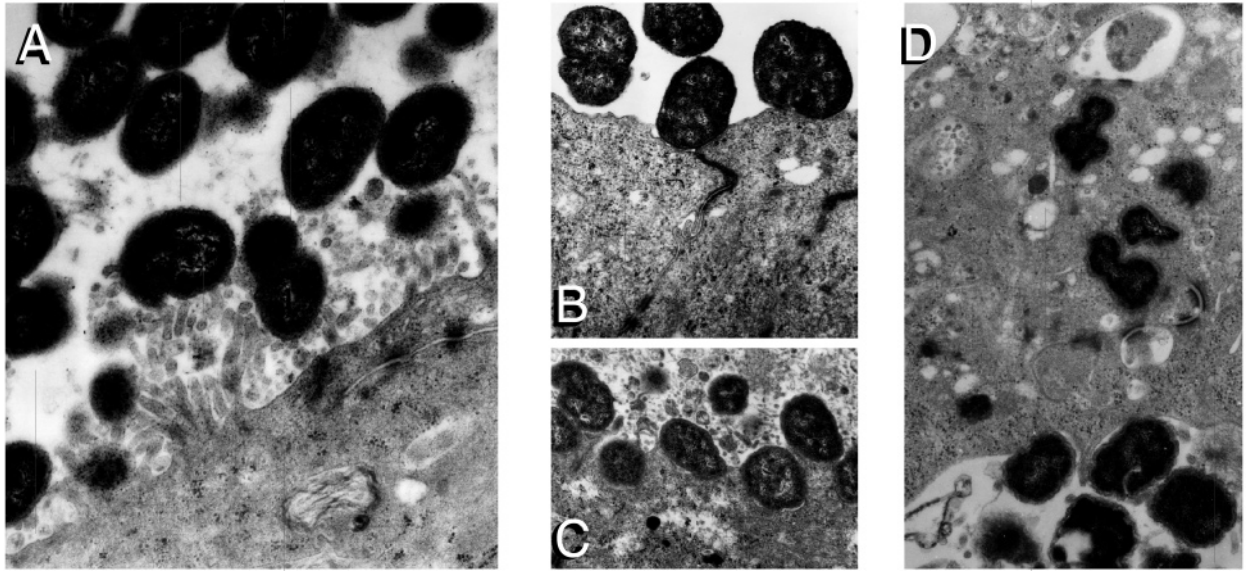

Figure 1 Electron micrographs of polarized T84 human epithelial cell monolayers infected with piliated, Opa^{-} *N. gonorrhoeae* strain MS11. At early stages of infection, *Neisseriae* adhere to the apical plasma membrane as microcolonies and bacteria are observed to be surrounded by a matrix of microvilli (*A*). Bacteria subsequently disperse from the microcolony and adhere as a monolayer. At this stage, bacterial and host cell membranes are tightly apposed (*B*, *C*). The region of contact between bacteria and host cell membranes enlarges (*C*) and bacteria are subsequently internalized (*D*). Bacteria traverse the cell and exit the monolayer via the basolateral membrane (*D*).

1983, Ward et al 1974). As detailed below, invasion is mediated through various outer membrane components that promote entry through multiple mechanisms.

Intracellular Life and Epithelial Traversal

Internalized bacteria survive and grow within the epithelial cells (Figure 1*D*) (Hopper et al 2000, Lin et al 1997). Both GC and MC are usually observed in vacuoles (Harvey et al 1997, Lin et al 1997, McGee et al 1983, Pujol et al 1997, Stephens et al 1983, Wang et al 1998), but there are persistent reports that GC reside in the cytosol of the host cell (Figure 1*D*) (Apicella et al 1996, Mosleh et al 1997, Shaw & Falkow 1988, Williams et al 1998). At 18 h (MC) or 40 h (GC) bacteria egress into subepithelial stromal tissues (Figure 1*D*) (Stephens et al 1983, McGee et al 1981). Epithelial traversal occurs by invasion and transcytosis through the cell. If GC and MC travel between epithelial cells, they do so without obvious disruption of lateral junctional complexes or epithelial barrier properties (Ilver et al 1998, Merz et al 1996, Pujol et al 1997, Stephens & Farley 1991, Wang et al 1998).

Cytotoxicity

Cell death, mediated at least partially through tumor necrosis factor-α (TNF-α) release, occurs at later times in the FTOC and NPOC models but is most severe in ciliated cells, which are not the major targets of colonization (McGee et al 1992). Cytotoxicity in polarized tissue culture models is minimal until bacteria reach the subepithelial compartment (Ilver et al 1998, Merz et al 1996, Pujol et al 1997, Wang et al 1998). Some shedding of epithelial cells is observed in GC-infected men and in a culture model of stratified epithelium (Apicella et al 1996, Mosleh et al 1997, Ward & Watt 1972). Neisserial components that contribute to cytotoxicity include LOS (endotoxin), peptidoglycan, the porin ion channel, and type IV pili (Dunn et al 1995; Gregg et al 1981; Melly et al 1981, 1984; Muller et al 1999; Stephens et al 1986; Virji et al 1982). A GC mutant with increased cytotoxicity to FTOC cells was recently characterized. The gene responsible for this phenotype is also present in MC and *N. lactamica*, but absent from other (commensal) *Neisseriae*, suggesting that GC may actively suppress its own toxicity (Arvidson et al 1999).

ATTACHMENT AND INVASION

Type IV Pili

Like many gram-negative bacteria, the pathogenic *Neisseriae* produce type IV pili, filamentous polymers 6 nm in diameter and up to several micrometers in length. Type IV pili are not merely passive sticky fibers but dynamic machines that participate in a surprising number of functions (Table 1). Nevertheless, it appears that a core set of mechanisms—fiber assembly and extension, fiber adhesion, fiber disassembly and retraction—can account for all these functions. Work with many

TABLE 1 Functions requiring type IV pili. This list is representative, not comprehensive

N. gonorrhoeae* and *N. meningitidis	**References**
Bacterial aggregation	Swanson et al 1971
Adhesion to host cells	Nassif et al 1993, Swanson 1973
Twitching motility (**T**)	Brossay et al 1994, Swanson 1978
Pilus retraction (**T**)	AJ Merz, M So & MP Sheetz, submitted
Dispersal from aggregates and loss of pili (**T**)	Pujol et al 1999
Host cell responses	
Cytosolic Ca^{2+} fluxes	Källström et al 1998
Exocytosis	P Ayala & M So, submitted
Cortical plaque formation (**T**)	Merz et al 1999
Cytotoxicity	Dunn et al 1995, McGee et al 1981
DNA transformation (**T**)	Sparling 1966, Wolfgang et al 1998a
Other bacterial species	
Type II secretion (*P. aeruginosa*)	Lu et al 1997
Bacterial aggregation (*V. cholerae*)	Kirn et al 2000
Complement resistance (*V. cholerae*)	Chiang et al 1995
Adhesion to host tissues (*Azoarcus* sp.)	Dorr et al 1998
Virulence gene regulation in vivo (*V. cholerae*)	Lee et al 1999
Twitching/social gliding motility (**T**; *M. xanthus*)	Wu et al 1997
Dispersal from aggregates (**T**; enteropathogenic *E. coli*)	Bieber et al 1998
Pilus bundle structural change (**T**; enteropathogenic *E. coli*)	Knutton et al 1999
Biofilm formation (**T**; *P. fluorescens*)	O'Toole & Kolter 1998
Cytotoxicity (**T**; *P. aeruginosa*)	Comolli et al 1999
Horizontal genetic transfer	
DNA transformation (**T**; *L. pneumophila*)	Stone & Kwaik 1999
Conjugation (**T**; IncI1 plasmids)	Yoshida et al 1999
Bacteriophage infection (**T**; *P. aeruginosa*)	Bradley 1974
Bacteriophage assembly (*V . cholerae* ΦVPI)	Karaolis et al 1999

T indicates that a requirement for *pilT* or twitching motility-related functions has been demonstrated.

bacterial species indicates that the assembly pathways and overall structures of type IV pili are broadly conserved (Dalrymple & Mattick 1987, Hoyne et al 1992, Patel et al 1991, Sauvonnet et al 2000). Type IV pili function in all three types of bacterial genetic transfer: transformation, conjugation, and phage transduction (Table 1), and may share evolutionary origins with filamentous bacteriophages (Karaolis et al 1999). Type IV pili also share striking similarities with bacterial type II protein export and DNA uptake systems (Fussenegger et al 1997). Here, we emphasize the role of neisserial type IV pili in colonization and disease processes.

Structure and Assembly of the Type IV Pilus Fiber

Much is now known about the structure of the *Neisseriae* type IV pilin subunit, which has been resolved to the 2.6 Å level, and the arrangement of pilins in the pilus fiber itself. The isolation of a number of mutants defective in pilus functions paves the way for biochemical and biophysical analyses of the pilus assembly process. Very recent studies demonstrating pilus retraction are beginning to shed new light on the dynamics of type IV pilus assembly and function.

The Pilin Subunit The type IV pilus fiber is composed primarily of pilin, an 18–22 kDa polypeptide. Pilin is synthesized as a precursor protein with an unconventional secretory signal sequence that is shared among many type II secretion factors and undergoes characteristic processing by the PilD prepilin peptidase/transmethylase. (Freitag et al 1995, Nunn & Lory 1991, Strom et al 1993). The result is a mature pilin subunit with an α-methylated phenylalanine residue at its N terminus (N-met-Phe). Other gene products that contain prepilin-type secretion signals are similarly processed by PilD or its homologues (Dupuy et al 1992; Nunn & Lory 1992; Strom et al 1991, 1994). At least some prepilin-like proteins have pilus-related functions, whereas others are involved in DNA transformation or type II secretion (Dubnau 1999, Pugsley 1993, Pugsley et al 1997, Russel 1998). Neisserial pilins are further post-translationally modified by *O*-glycosylation and phosphorylation at sites mapping to the exposed surface of the fiber (Forest et al 1999; Jennings et al 1998; Marceau et al 1998; Marceau & Nassif 1999; Parge et al 1995; Stimson et al 1995, 1996; Virji et al 1993b).

The structure of mature GC pilin has been solved to 2.6 Å resolution (Parge et al 1995). The pilin monomer contains a C-terminal globular head region and an N-terminal hydrophobic tail that folds into an unusually long α-helix. Analyses of *E. coli* cells producing GC prepilin-alkaline phosphatase fusions suggest that both prepilin and mature pilin exist as transmembrane proteins in the bacterial cytoplasmic membrane (Dupuy et al 1991). In this model the C-terminal globular domain of prepilin faces the periplasm, the hydrophobic α-helix spans the membrane, and the charged N terminus serves as a cytoplasmic anchor. PilD active sites face the cytoplasm, where they remove charged residues from the prepilin N terminus and methylate the α-N terminus. These modifications are hypothesized to facilitate the extraction of pilin from the cytoplasmic membrane and/or the incorporation of pilin subunits into the growing pilus fiber.

Structure of the Polymeric Fiber Fiber diffraction, cryoelectron microscopy, antigenic mapping, and molecular modeling indicate that pilin subunits polymerize into a right-handed helical cylinder with fivefold symmetry about the helix axis (Forest & Tainer 1997, Parge et al 1995). The hydrophobic pilin tails pack into the cylinder core where they form a helical coiled-coil bundle that probably accounts for the fiber's tensile strength. The globular pilin heads face outward, forming the surface of the cylinder. Within the globular domain, an invariant pair of

disulfide-linked cysteine residues anchors a ~30 residue loop. This loop is surface exposed and is structurally isolated from the core framework involved in polymerization. This loop is also the hypervariable region of pilin that exhibits the greatest primary sequence and antigenic diversity (Seifert 1996). Recent analyses of intragenic suppressor mutations in the pilin subunit support a similar model for fiber assembly in *Vibrio cholerae* (Kirn et al 2000).

Functional Consequences of Pilin Antigenic Variation The pilins of GC and MC undergo primary sequence variation in nature as well as in the laboratory (Hagblom et al 1985, Seifert 1994). Variation occurs through a variety of genetic mechanisms at extraordinary rates of $\sim 10^{-4}$ per cell division and is especially prevalent in regions of pilin that map to the fiber surface (Seifert 1996). Small alterations in the primary structures of neisserial pilins cause changes in immunoreactivity, post-translational modification, and adhesive function (Jonsson et al 1994; Marceau et al 1995, 1998; Nassif et al 1993; Rothbard et al 1985; Schoolnik et al 1984; Virji et al 1991, 1993b). Perhaps surprisingly, point mutations that abolish *O*-glycosylation of the MC pilus or phosphorylation of the GC pilus cause only minor effects on adhesion to host cells and other pilus-related functions. In contrast, other point mutations that do not appear to influence post-translational modification cause dramatic changes in host cell binding and tropism (Jonsson et al 1994; Marceau et al 1995, 1998; Nassif et al 1993; Rothbard et al 1985; Schoolnik et al 1984; Virji et al 1991). The relatively invariable *Pseudomonas aeruginosa* type IV pilin binds directly to host glycolipids via a conserved site (Lee et al 1994, Sheth et al 1994, Yu et al 1994). By analogy, the point mutations in GC or MC pilin might directly modify a receptor-binding site within the pilin subunit. Alternatively, these mutations might alter pilus adhesive function indirectly, for example by modifying the ability of pilin to interact with a minor subunit that acts as a high-affinity adhesin.

Interestingly, many neisserial pilin variants that promote strong adhesion to host cells also aggregate into laminar bundles, whereas variants that promote weaker adhesion tend to exist as single filaments (Marceau et al 1995). The type IV pili of organisms that do not exhibit high levels of antigenic variation (e.g. *V. cholerae*, *E. coli*, *P. aeruginosa*) also tend to occur as laminar bundles. Moreover, recent experiments with *V. cholerae* show that pilus bundling correlates with bacterial aggregation and colonization phenotypes (Kirn et al 2000). It is unclear how bundles promote neisserial adhesion. Bundles could promote bacterial aggregation, increase receptor avidity by oligomerizing binding sites, or increase pilus stiffness and tensile strength in shear or flow conditions. Bundles might also facilitate twitching motility by promoting coordinated fiber extension and retraction processes that would be unfeasible with less-ordered structures, as described below.

Proteins Involved in Fiber Assembly Type IV pilus assembly is hypothesized to occur within the cytoplasmic membrane or periplasm. (Fussenegger et al 1997,

Hultgren et al 1993, Pugsley 1993). Prepilin processing (described above) is necessary but insufficient for fiber assembly. Mutational analyses demonstrate requirements for additional components including lipoproteins, pilin-like proteins, and secretins, believed to form the large outer membrane channel through which the assembled fiber translocates. The evolutionary relationships and functions of these proteins are reviewed elsewhere (Koomey 1995, Lory 1998, Mattick et al 1996, Pugsley et al 1997, Russel 1998). Multiple gene products implicated in pilus assembly or function contain conserved ATP-binding motifs and are present in the bacterial cytoplasm or associated with the bacterial inner membrane. At least one member of this family, PilF, is essential for GC pilus assembly (Freitag et al 1995). The functions of these putative ATPases are not well understood, but genes encoding proteins with strong sequence homology have essential functions in bacterial type II and type IV secretion systems (He 1998, Lory 1998, Pugsley et al 1997, Russel 1998). Mutational analyses indicate that the ATP-binding motifs are generally essential for function; in vitro ATPase activity has been demonstrated for the *Agrobacterium tumefaciens* VirB11 protein, which is required for conjugal pilus function and DNA transfer (Christie et al 1989, Fullner et al 1996).

Role of PilC Proteins in Adhesion and Pilus Dynamics The *pilC1* and *pilC2* loci were originally identified as genes involved in structural and phase variation of GC pili (Jonsson et al 1991, 1992). PilC1 and PilC2 are large (110 kDa) proteins with conventional signal sequences (Jonsson et al 1991, 1992). The GC *pilC* loci undergo high-frequency on/off phase variation and appear to be functionally redundant. GC strains lacking either PilC1 or PilC2 have no obvious defects, but strains lacking both PilC1 and PilC2 bear few or no pili and are deficient in DNA transformation (Jonsson et al 1991, 1992, 1994; Rudel et al 1992, 1995a). A simple interpretation of these data is that PilC is a pilus assembly factor, but studies of the MC *pilC* loci suggest greater complexity.

Mutation of the MC *pilC1* gene alone results in a piliated, transformation-competent strain that is unable to adhere to epithelial cells (Backman et al 1998, Nassif et al 1994, Pron et al 1997, Rahman et al 1997, Rudel et al 1995b, Ryll et al 1997, Virji et al 1995a). MC *pilC1* thus has an essential role in pilus-mediated cell adhesion that is not fulfilled by *pilC2*. Cell binding and adhesion blockade experiments with purified GC PilC2 suggest that PilC may function directly as an adhesin (Rudel et al 1995b). Furthermore, immunogold electron microscopy experiments suggest that PilC is selectively localized at the pilus tip (Rudel et al 1995b). However, specific and saturable binding of PilC to host cells or receptors remains to be demonstrated, and different PilC localization results have been reported (Rahman et al 1997). The PilC proteins clearly have essential functions in adhesion, but their exact functions and sites of action remain controversial. Nevertheless, recent data may explain the assembly phenotypes observed with *pil*C mutants. As described below, PilC appears to antagonize pilus retraction rather than being required for filament assembly per se.

Twitching Motility, Pilus Retraction, and DNA Transformation

Type IV pili mediate twitching motility and social gliding motility, modes of migration along liquid-solid or liquid-air interfaces (Henrichsen 1975b, 1983; Lautrop 1961; Wall & Kaiser 1999). Twitching motility and related processes are implicated in a wide array of biological processes (Table 1).

Experimental Evidence of Pilus Retraction There is a strong correlation between twitching motility, piliation, and susceptibility to bacteriophages that adsorb to type IV pili (Bradley 1972a, b, 1974). In phage-binding experiments, phage-susceptible *P. aeruginosa* has many phage attached to the bacterial cell body and concentrated at the base of pili. In contrast, a nonsusceptible but piliated mutant has pili uniformly covered with phage and few phage bound to the cell body. These nonsusceptible mutants do not exhibit twitching motility. Bradley proposed that pilus retraction into the cell body mediates both phage infection and motility. The lesion in one nonmotile mutant was mapped to the *pilT* locus, which encodes a cytoplasmic or inner membrane-localized ATPase highly similar to ATPases required in pilus assembly, DNA transformation, and protein translocation (He 1998, Pugsley 1993, Whitchurch et al 1991). It is hypothesized that ATP hydrolysis by PilT mediates pilus disassembly from the fiber base, causing retraction (Whitchurch et al 1991).

Like *P. aeruginosa*, GC and MC engage in pilus- and *pilT*-dependant twitching motility (Brossay et al 1994, Henrichsen 1975a; Swanson 1978; Wolfgang et al 1998a). GC *pilT* mutants have more pili than the wild-type strain, adhere avidly (125–200% of wild-type levels) to epithelial cells, and do not twitch (Wolfgang et al 1998a). Interestingly, *pilT* mutants are not competent for the DNA uptake step of genetic transformation. *dud1* mutants, identified in a screen for piliated but nontransformable GC, have lesions in the *pilT* locus (Biswas et al 1989, Seifert et al 1988, Wolfgang et al 1998a). Moreover, homologues of pilin and *pilT* are required for DNA uptake even in species that lack type IV pili, suggesting that twitching motility and DNA uptake utilize a shared machinery.

Recent experiments confirm that GC PilT is involved in pilus retraction. A GC strain has been constructed that carries an IPTG-inducible *pilT* and null mutations in both *pilC* loci (Wolfgang et al 1998b). In the presence of IPTG, this strain acts like a *pilC1 pilC2* double mutant and lacks pili. Upon removal of IPTG, the strain is depleted of PilT and becomes hyperpiliated, producing more pili of greater length than any GC strain so far described. Despite the abundance of pili, the *pilC1 pilC2 pilT* phenocopy is unable to adhere to epithelial cells, is nonmotile, and is deficient in DNA transformation. These results have two major implications. First, GC pilus fibers that lack *pilC* do not support epithelial cell adherence, strongly corroborating earlier observations that MC PilC is essential for epithelial adherence. Second, these experiments establish that PilC is dispensable for fiber assembly, as suggested by earlier studies with undefined mutants

(Rudel et al 1995a). The PilC null mutants are nonpiliated in the presence of PilT, but hyperpiliated when PilT is depleted. The simplest model consistent with these results is that PilC acts as an inhibitor of PilT-mediated fiber disassembly rather than as a promoter of fiber assembly.

More recently, we used biophysical techniques to directly confirm that PilT-dependent pilus retraction occurs. Observations of GC crawling on glass coverslips demonstrate that twitching motility is active (not brownian diffusion), requires type IV pili and PilT function, and occurs at $\sim 1 \mu$m/s. With a laser tweezers apparatus we showed that piliated GC cells pull toward one another. Using a bead-based assay, we directly demonstrated that the type IV pili on individual diplococci retract with significant mechanical force and at the same speed as twitching motility. As expected, retraction depends on PilT function, and repeated retraction events are observed with individual diplococci, suggesting the successive retraction of multiple pili (Merz et al 2000).

Because *pilT* mutants are nonmotile but seem to assemble pili normally, it is unlikely that pilus extension alone can power motility. Interestingly, EPEC type IV pilus bundles change in thickness during colonization. These changes fail to occur with a nontwitching mutant (Knutton et al 1999). We suspect that single pilus fibers are too flexible to transmit extensile forces over substantial distances. However, the retraction of single fibers associated with rigid bundles might allow the bundles to be used as levers or might cause other changes in bundle structure (Knutton et al 1999).

Host Cell Receptors for Neisserial Type IV Pili

GC and MC pili mediate strong adhesion to several human cell types including epithelial cells, endothelial cells, and sperm cells (Buchanan & Pearce 1976, Gubish et al 1982, James-Holmquest et al 1974, Jonsson et al 1994, Nassif et al 1994, Pearce & Buchanan 1978, Stephens & McGee 1981, Swanson 1973, Swanson et al 1975, Virji et al 1991). The molecular basis for this adhesion has been studied intensively, but the first receptor for neisserial pili, CD46, was identified only recently (Källström et al 1997). CD46, or membrane cofactor protein (MCP), is a member of the superfamily of complement resistance proteins. At least six CD46 splice variants have been identified, and CD46 variants are expressed on nearly all human cells except erythrocytes. Interestingly, CD46 also serves as a receptor for measles virus (Dorig et al 1994) and for human herpesvirus 6 (Santoro et al 1999). Furthermore, measles virus-mediated crosslinking of CD46 on human monocytes results in decreased IL-12 expression and in the inhibition of cell-mediated immunity, suggesting that the use of this receptor allows microbial manipulation of host immune responses (Karp et al 1996).

Three lines of evidence support the hypothesis that CD46 is a biologically relevant pilus receptor. First, crudely purified GC or MC pili bind to native CD46 in an overlay assay. Pili prepared from a MC *pilC1* mutant fail to bind CD46 in the same assay, suggesting a requirement for PilC1 function. Second, purified CD46

ectodomain made in *E. coli* blocks adhesion of GC or MC to epithelial cells. Third, ectopic expression of some but not other CD46 splice variants allows otherwise nonpermissive cells to support adhesion by piliated GC or MC. The adhesion-supporting variants contain both the B and C exons in their exoplasmic domains. CD46 that contains the C but not the B domain does not support measurable adhesion by GC (Källström et al 1997). Consistent with this, an mAb (GB24) directed against an epitope proximal to the B domain inhibits neisserial adhesion to epithelial cells, whereas an mAb (TRA-2–10) directed against a more distal N-terminal epitope has little effect.

Adhesion to CD46 expressed in otherwise nonpermissive cells is weak compared with the adhesion supported by human epithelial cells. Furthermore, binding has not been demonstrated between CD46 and any specific neisserial molecule (e.g. pilin or PilC), and piliated GC can agglutinate human erythrocytes, which lack detectable CD46. It is possible that tight adhesion to CD46 requires a yet to be identified coreceptor. Alternatively, CD46 might require cell-type-specific post-translational modification to support strong adhesion. As described below, it is also possible that pili stimulate signal transduction though CD46, leading to host cell responses (e.g. the unmasking of a cryptic binding site on CD46 or a coreceptor) that reinforce an initial, weak adhesion.

Pilus-Mediated Signaling to Host Cells Several recent studies reveal that type IV pili and twitching motility function at multiple stages of colonization and involve complex host cell responses. We describe these functions in the order in which they are believed to occur during infection (Figure 2, see color insert).

Pilus-Triggered Ca^{2+} Signals Piliated GC and MC or semipurified pili trigger a cytosolic Ca^{2+} flux in human epithelial cells (Källström et al 1998). This host cell response has several notable features. First, the Ca^{2+} signal has a long latency, occurring $\sim$10 min after the addition of bacteria or pili. Second, the ability of MC or purified MC pili to trigger the Ca^{2+} flux depends on the presence of MC PilC1, which, as mentioned above, is required for strong pilus-mediated adherence. Third, the signal is blocked by antibodies directed against CD46. These controls suggest that pili are necessary for the Ca^{2+} signal but do not exclude the possibility that a copurifying component is also required. Moreover, depletion of intracellular Ca^{2+} stores or treatment with certain protein kinase inhibitors results in diminished bacterial adherence (Källström et al 1998). Thus GC and MC pili may elicit a host cell response that reinforces an initial, weak attachment via CD46. Recently the pilus-triggered cytosolic Ca^{2+} flux was observed to cause an exocytic event in the host cell, which results in the deposition of lysosomal membrane Lamp1 at the plasma membrane and the release of lysosomal contents into the extracellular medium (P Ayala, B Vasquez, S Clary, R Rodland & M So, submitted). It is tempting to speculate that one or more components released through pilus-triggered exocytosis mediate the Ca^{2+}-dependent adhesion observed previously (Källström et al 1998).

Pilus-Induced Cortical Plaques A second host cell response to pilus-mediated adhesion is a series of rearrangements in the cortical cytoskeleton and plasma membrane, beginning within minutes of attachment and culminating at 4–6 h post infection. These rearrangements include dramatic accumulations of phosphotyrosine, actin, ezrin, and a subset of transmembrane glycoproteins at GC or MC attachment sites in epithelial cells (Merz et al 1999, Merz & So 1997). Observations of living cells expressing green fluorescent protein (GFP) fusions to the membrane-cytoskeleton linker ezrin (see Bretscher et al 2000) reveal that cytoskeletal rearrangements initiate within minutes of GC attachment, beginning with the elongation of microvilli toward the microcolony (AJ Merz, S Lee & M So, unpublished results), as initially described in electron microscopic studies of colonization (Griffiss et al 1999, Pujol et al 1999, Shaw & Falkow 1988, Stephens et al 1983, Ward & Watt 1972, Ward et al 1974). The full set of rearrangements requires the expression of both type IV pili and PilT, suggesting a role for twitching motility. Plaque formation also occurs in the presence of MC capsule and is not limited to a single GC LOS biotype (Merz et al 1999, Merz & So 1997). Very recent experiments show that pilus-mediated actin rearrangements are enhanced by the presence of lacto-*N*-neotetraose moieties on GC LOS (Song et al 2000). In contrast, Opa, Opc, and MafB (see below) are dispensable for plaque formation (Merz et al 1999, Merz & So 1997). Because of their similarity to adhesive plaques formed during eukaryotic cell-cell and cell-substrate attachment, these structures are termed pilus-induced cortical plaques.

Do Type IV Pili Transmit Mechanical Signals to Host Cells? It is well established that mechanical forces applied to the plasma membrane dramatically enhance the formation of plaque-like structures and cytoskeletal anchoring during both cell-cell and cell-substrate adhesion (Sheetz et al 1998). We hypothesize that pilus retraction-induced mechanical tension accounts both for the elongation of microvilli during early infection and for the weak plaque formation exhibited by *pilT* mutants. In this model, a minimum of two signals would be transmitted through the pilus to the host cell. One signal would involve the binding of the pilus to the cell surface, presumably via CD46. The second signal would involve pilus retraction and the exertion of tensile forces upon the host plasma membrane. These retractile forces could be transmitted through both pilus-receptor interactions and (during microcolony formation) through outer membrane components such as Opa or LOS (Griffiss et al 1999, Song et al 2000). Direct measurements (Raucher et al 2000, Shao et al 1998) indicate that the forces required to elongate microvilli or to form membrane tethers (cylindrical membrane projections devoid of cytoskeletal components) are in exactly the same range (20–80 pN) as the forces generated though pilus retraction by individual diplococci (AJ Merz, M So & MP Sheetz, submitted). Even larger contractile forces would be exerted by an adherent microcolony of several dozen organisms.

Structures resembling cortical plaques are also induced by other bacteria. *Bartonella henselae*, the etiologic agent of cat scratch fever, produces adhesive pili

that may be type IV (Batterman et al 1995), and elicits a host cell structure (the invasome) strikingly similar to the neisserial pilus-induced cortical plaque (Dehio et al 1997). These findings imply that cortical plaque formation may be a feature common to colonization via type IV pili. The mechanical forces generated by pilus retraction may constitute a new class of bacterial signals to host cells.

Pilus Loss and Intimate Attachment As has long been observed in electron microscopic studies, at 8 to 16 h after infection GC and MC disperse from microcolonies, appear to lose their pili, and adhere tightly to the host cell plasma membrane. Moreover, MC pilus loss, bacterial dispersal, and intimate attachment are all blocked in a *pilT* mutant (Pujol et al 1997, 1999).

MC adhesion to host cells causes the transient up-regulation of PilC1 production and down-regulation of MC capsule (Deghmane et al 2000, Pron et al 1997, Taha et al 1998). PilC1 up-regulation occurs at the level of transcription, is required for strong pilus-mediated adhesion, and—interestingly—occurs upon cell contact even in the absence of pili (Taha et al 1998). Thus signals passing from bacterium to host and from host to bacterium may be important for strong type IV pilus-mediated adhesion.

One such signal may be mediated through the recently discovered *crgA* transcriptional regulator (Deghmane et al 2000). *crgA* is needed for MC attachment to host cells (Deghmane et al 2000). The severity of the attachment defect in the *crgA* null mutant is about as severe as the defect in an MC *pilC1* mutant, even at early times after infection. This raises two quandaries. First, *crgA* is apparently required specifically for intimate attachment. However, other experiments suggest that intimate attachment is dissociable from, and occurs after, efficient MC binding to host cells (Pujol et al 1999). Second, several studies have established correlations between MC *pilC1* expression and attachment efficiency, yet *crgA* is both required for attachment and negatively regulates *pilC1*. It is unclear how these apparent discrepancies can be reconciled. A partial explanation is suggested by the observation that capsule loss occurs upon MC attachment to host cells and that this loss of capsule is blocked in a *crgA* mutant (Deghmane et al 2000). At any rate, these exciting findings suggest that pilus-mediated attachment involves an unexpectedly complex sequence of signaling events, not only from bacterium to host, but from host to bacterium.

The Opa Proteins

The second major class of neisserial adhesins is encoded by the multigene *opa* family. GC strains typically harbor ~11 opa loci, whereas MC strains typically have 4–5 *opa* loci (C Dehio et al 1998). Specific *opa* repertoires vary from strain to strain, and until recently *opa*/Opa nomenclature was not standardized and was sometimes applied inconsistently. For example, Opa60 in GC strain MS11 is also known as OpaI or OpaH (Belland et al 1992, Bhat et al 1991, Bos et al 1997, Kupsch et al 1993, Swanson 1978). This situation has changed with the adoption

of a standardized nomenclature (C Dehio et al 1998, Malorny et al 1998). In the older GC literature, Opa proteins are referred to as Opacity proteins, protein II, or p.II. An excellent recent review summarizes the biological functions of the Opa family (C Dehio et al 1998).

Opa Phase and Antigenic Variation

All *opa* genes sequenced to date contain 5′ tandem repeats $[CTCTT]_n$ that cause high-frequency phase variable expression (Stern et al 1984, 1986). As a result, a given organism can reversibly express zero, one, or multiple different Opa proteins (Blake & Gotschlich 1984, Blake et al 1981, Poolman et al 1980, Walstad et al 1977). In addition, horizontal gene transfer promotes the formation of hybrid recombinant *opa* loci, both in the laboratory and in nature (Achtman 1994, Waldbeser et al 1994). The importance of Opa proteins in infection is underscored by the frequent Opa phase and antigenic variation observed during human infections (James & Swanson 1978, Jerse et al 1994); by detailed studies of *opa* microevolution during MC epidemics (Achtman 1994); and by the noninfectious phenotype recently reported for a GC strain with engineered deletions of all eleven *opa* loci (Cannon et al 1998).

Opa Function in Colonization

Different Opa proteins confer different phenotypes in host cell interaction assays (C Dehio et al 1998). Monoclonal antibody binding and limited proteolysis experiments indicate that Opas have eight transmembrane β strands and four surface-exposed loops. The relatively conserved regions of Opa primary structure map to transmembrane and periplasmic domains, whereas the relatively variable regions map to the first three surface-exposed loops (Blake et al 1981, Malorny et al 1998). In several studies with tissue culture cells, specific Opa proteins were shown to mediate either adhesion or invasion of epithelial cells. Subsequent experiments demonstrated that different cell lines gave different results for particular Opas, indicating that Opa expression is a determinant of cell tropism (Belland et al 1992; Bessen & Gotschlich 1986; Kupsch et al 1993; Makino et al 1991; Simon & Rest 1992; Swanson 1992, 1994; Swanson et al 1992; Virji et al 1993a; Waldbeser et al 1994; Weel et al 1991). The recent identification of two broad classes of Opa receptors has opened the way to a molecular understanding of Opa-mediated adhesion and invasion.

Opa Receptors on Host Cells

A number of signaling molecules serve as receptors for various Opa proteins. The binding of Opa variants to their cognate receptors initiates different signaling cascades, leading to entry of bacteria via distinct pathways—possibly also to different intracellular fates and/or infection outcomes.

Heparan Sulfate Proteoglycan Receptors The first class of Opa receptors are heparan sulfate proteoglycans (HSPGs) (Chen & Gotschlich 1996, van Putten & Paul 1995, Virji et al 1999). HSPG receptors bind a subset of Opa variants that have surface-exposed loops rich in positively charged amino acyl residues. The prototype Opa in this class is the GC Opa30 protein. Opa30 and related Opas mediate efficient binding to and invasion of conjunctival cells and a few other epithelial cell lines. This invasion is markedly reduced by competition with soluble polyanions including heparin, heparan sulfate, HSPGs, DNA, or by treatment of the cells with the enzyme heparatinase I. Binding to HSPGs and invasion of Chang cells is abrogated by replacement of the second loop (HV1) of Opa30 with the homologous region from a different Opa (Grant et al 1999).

Opa-HSPG Interactions and Lipid Signaling GC invasion of epithelial cells through Opa-HSPG interactions is mediated through a novel pathway that begins with the highly localized recruitment of HSPG receptors, F-actin, and tyrosine-phosphorylated proteins at the attachment sites (Grassme et al 1996, Merz et al 1999, Merz & So 1997). The binding of nonpiliated, Opa^+ GC stimulates at least two lipid hydrolysis enzymes, the phosphatidylcholine-specific phospholipase C (PC-PLC) and an acidic sphingomyelinase (SMase) (Grassme et al 1997). Both enzymes appear to be required for bacterial uptake (Grassme et al 1997). This system may represent a novel type of endocytosis that does not utilize clathrin-coated pits or caveolae (Grassme et al 1996). Recent experiments demonstrate that SMase treatment of cells is sufficient to trigger endocytosis of 15–30% of the plasma membrane and that this pathway does not require ATP (Zha et al 1998).

It is unclear whether Opa-HSPG interactions are sufficient or even necessary to activate PC-PLC and acidic SMase after GC binding to host cells (Grassme et al 1997). Opa-HSPG interactions are not sufficient to promote internalization or to trigger actin or phosphotyrosine accumulations in epithelial cells (Grassme et al 1996, Merz & So 1997), and mutations in GC porin proteins prevent Opa-HSPG-mediated invasion (Bauer et al 1999). In this context it is especially interesting that purified GC porins added to neutrophils dramatically influence the kinetics of PC-PLC activation through formylpeptide receptors and modify membrane trafficking (Haines et al 1991). Thus the possibility remains that Opa-HSPG interactions mediate only nonspecific adhesion and that porins or other molecules then trigger lipid hydrolysis and bacterial uptake (van Putten et al 1998a). The role of porins in invasion is discussed in greater detail below.

Adhesion and Uptake Though Vitronectin and Integrins A second pathway of uptake potentiated through HSPG-binding Opa (and Opc) proteins occurs through bacterial binding to vitronectin, which interacts with $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrin receptors on host cells (M Dehio et al 1998; Duensing & van Putten 1997, 1998; Gomez-Duarte et al 1997; Grinnell & Geiger 1986; van Putten et al 1998b; Virji et al 1994). Interestingly, the same integrin receptors are used directly by adenovirus (Wickham et al 1993). Opa-vitronectin interactions are strong enough to

promote adhesion and moderately efficient bacterial uptake into HeLa, CHO, or Hep-2 cells, which are nonpermissive for invasion in the absence of vitronectin. In this pathway, the aggregation of vitronectin is sufficient for uptake, which may involve protein kinase C and Src-family kinases (M Dehio et al 1998, Felsenfeld et al 1999). It is notable that vitronectin can also mediate the phagocytosis of apoptotic cells by professional and nonprofessional phagocytes (Aderem & Underhill 1999).

CD66 Family Receptors Recent work from several groups has shown that many different Opas bind to members of the large CD66/CGM1/CEA/BGP family of proteins (Bos et al 1997; Chen & Gotschlich 1996; Chen et al 1997; Gray-Owen et al 1997a, b; Hauck et al 1998; Virji et al 1996a, b, 1999; Wang et al 1998). CD66-related proteins can mediate cell-cell adhesion, are encoded by different genes, and are often produced as multiple splice variants (Hammarstrom 1999). Different CD66 family members are variably expressed on different cell types. Most family members encode transmembrane proteins, whereas a few encode peripheral membrane proteins with GPI (glycosylphosphatidylinositol) membrane anchors. All members of the CD66 family of proteins are glycosylated; however, Opa-CD66 binding occurs through protein-protein interactions. Other Opas bind to different but overlapping sets of CD66 variants. Single residue changes within Opa, in the so-called adhesiotopes, can change specificity for Opa binding (Virji et al 1999). Depending on the particular CD66 variant(s) and Opa protein(s) expressed, different host cell responses occur, including binding, uptake, and the activation of different signal transduction pathways.

Opa-CD66 Interactions and Signaling It is likely that the highly variable specificity and high affinity of Opa-CD66 binding will make Opa proteins choice reagents for dissecting the cell biology and signaling capabilities of the CD66 family. Experiments that led to the discovery of Opa-CD66 interactions were performed with neutrophils (Belland et al 1992, Naids et al 1991, Rest et al 1982). Thus far Opa/CD66-mediated signal transduction has been studied in depth only in professional phagocytes. Nonopsonic phagocytosis mediated by CD66 involves the activation of Src-like tyrosine kinases, Rac1, PAK, and Jun N-terminal kinase (Hauck et al 1998). This pathway is apparently distinct from the pathways that mediate antibody- and complement-mediated uptake. It is not yet known whether comparable signals mediate CD66-dependant uptake in epithelial or endothelial cells (Virji et al 1996a, Wang et al 1998).

The Opc Protein

The class V outer membrane protein was cloned in studies of MC antigens and renamed Opc because the cloned gene product has weak homology to Opa proteins. The *opc* gene is present in many but not all MC strains and is associated with virulence (Olyhoek et al 1991, Seiler et al 1996). *opc* homologs of undefined function

are also present in the GC genome (Zhu et al 1999). *opc* expression undergoes clonal variation via mutations in a poly-C tract within its promoter region. Spontaneous mutational variation in the number of cytidine residues changes expression levels or eliminates expression altogether (Sarkari et al 1994). Expression of *opc* in *E. coli* confers a weak adhesive phenotype. Expression of *opc* in non-encapsulated MC confers on the bacteria the ability to adhere to and invade endothelial cells independently of Opa and pili (Virji et al 1993a, 1992, 1994, 1995b). Opc can bind vitronectin (perhaps indirectly) and it has been proposed that, as with Opa30, vitronectin forms a molecular bridge between the bacterium and integrin receptors on the cell surface (Virji et al 1994).

Other Neisserial Adhesins and Host Receptors

In addition to type IV pili, Opa, and Opc, other potential adhesion systems have been identified in GC and MC. The biological functions of these systems are not well understood and are worthy of further attention. GC lipooligosaccharide (LOS) binds to asialoglycoprotein receptors on the cell surface (Porat et al 1995a, b). A contact-inducible GC activity may interact with lutropin receptors on FTOC or HEC-1-B cells (Chen et al 1991, Gorby et al 1991, Spence et al 1997). Finally, glycolipid-binding proteins exist in GC (Stromberg et al 1988). The gene encoding the GC glycolipid-binding adhesin (Paruchuri et al 1990) was recently recloned and named *mafB* (GenBank accession AF142582). Multiple sequences homologous to both *mafB* and the adjacent gene *mafA* are present in GC and MC (AJ Merz, unpublished data). These findings confirm that *mafB* is the prototype of a multigene family, as indicated by previous genetic analyses (Paruchuri et al 1990). The GC glycolipid-binding adhesins bind to lactose-containing asialoglycolipids such as globotetraosyl ceramide (GgO_4/asialo GM_1). Similar carbohydrate moieties are present in LOS in the GC outer membrane and on host cell glycolipids that serve as receptors for the type IV pili of *P. aeruginosa* (Lee et al 1994, Sheth et al 1994, Yu et al 1994).

Neisserial Anti-Adhesins and Adhesion Modulators

MC capsule and certain structural variants of GC LOS inhibit bacterial interactions with host cells. In both cases the anti-adhesive effects are due to the negatively charged carbohydrate sialic acid. MC capsule is composed of long polysialic acid chains, and both GC and MC LOS can be modified by the addition of terminal sialic acid moieties (Preston et al 1996, Reglero et al 1993, Smith 1991, Smith et al 1995). Remarkably, GC cannot synthesize sialic acid, and the sialyl donor for GC LOS is host-derived cytidinemonophosphate-*N*-acetylneuraminic acid (CMP-NANA) (Smith 1991, Smith et al 1995). Sialylation is catalyzed by a bacterium-encoded sialyltransferase. Because only some LOS variants can be sialylated, and the synthesis of these LOS variants is controlled by phase-variable enzymes, LOS sialylation is a variable phenotype. Similarly, enzymes that control MC capsule production are phase variable. Sialic acid moieties in either LOS or

capsule increase the bacterium's negative surface charge density and confer resistance to complement and to ingestion by professional phagocytes (Vogel & Frosch 1999). Capsule and sialylated LOS also inhibit Opa- and Opc-mediated adhesion to host cells (van Putten et al 1995; van Putten 1993; Virji et al 1993a, 1995b). In contrast, these carbohydrates do not strongly inhibit type IV pilus-mediated adhesion, presumably because pili extend far enough from the bacterial cell surface that electrostatic repulsion between bacterial sialic acids and the negatively charged host cell surface is negligible. In the presence of MC capsule or sialylated GC LOS, type IV pili appear to be indispensable colonization factors.

Neisserial Porins

The most abundant proteins in the outer membranes of GC and MC are porins. These ~30 kDa proteins form anion-selective ion channels that are essential for neisserial viability (Barlow et al 1989, Gotschlich et al 1987, Murakami et al 1989, Suker et al 1994, van der Ley et al 1991). The functions of porins in colonization remain unclear but over the last 20 years several findings have suggested that these proteins exert a pivotal influence on the outcome of host-bacterial interactions. Some evidence indicates that porins are capable of translocating into host cell membranes as active, gated ion channels (Lynch et al 1984, Mauro et al 1988, Mietzner et al 1987, Rudel et al 1996). Interestingly, the gating properties of certain porin alleles are modulated by nucleotide triphosphates, especially ATP and GTP. This sensitivity is asymmetric: NTPs change gating only if present on the side of porin that would face the host cell cytoplasm after translocation (Rudel et al 1996). Furthermore, the addition of purified porins to human monocytes triggers a Ca^{2+} flux into the cytoplasm from external sources. This signal is unlikely to be related to the pilus-induced Ca^{2+} flux. The porin-triggered Ca^{2+} response is rapid, occurring within 2 min after addition of porin, owing to influx of Ca^{2+} from the external environment (Muller et al 1999). In contrast, the pilus-induced Ca^{2+} transient occurs 10 min after addition of pili, owing to release of Ca^{2+} from intracellular stores, and can be triggered in the absence of extracellular Ca^{2+} (Källström et al 1998).

A role for porins in invasion was decisively demonstrated in experiments (van Putten et al 1998a) using isogenic GC strains carrying different porin alleles (Carbonetti et al 1988, 1990). Certain porin alleles, which tend to be found in strains recovered from patients with invasive, disseminated infections, conferred the ability to invade epithelial cells in the absence of pili or Opa. The porin-specific invasive phenotype was observed only at low concentrations of phosphate and was abolished by the presence of NTPs or polyphosphate at relatively low concentrations (van Putten et al 1998a). More recent mutational studies corroborate these findings and suggest that GC porins may also be required for invasion mediated by HSPG-binding Opa proteins (Bauer et al 1999). Further work should reveal whether porin translocation into epithelial cells is required for invasion and illuminate how porins work in combination with other colonization factors.

PASSAGE ACROSS THE EPITHELIUM

Our knowledge of how GC and MC behave inside cells is rudimentary. For example, as described above, some studies suggest that GC and MC reside within a vacuole, whereas others suggest that GC may escape into the cytosol. It is not yet clear whether these differences result from the different host cell types and/or different bacterial strains used in the studies or from differences in sample preparation for electron microscopy. Similarly, both GC and MC appear to replicate within host cells and are capable of traversing polarized epithelial cells without disrupting barrier function (Merz et al 1996, Pujol et al 1997). Certain Opa proteins promote transepithelial passage of GC and recombinant *E. coli* through polarized cells; however, the mechanistic details of this process remain to be defined. It is unknown to what extent the intracellular bacteria egress through basolateral versus apical membranes (Ring et al 1998). It is also not known how intracellular bacteria obtain nutrients. Studies are just beginning to address these questions.

What Is the Nature of the Neisserial Vacuole?

Lamp1 and Lamp2 are the most abundant transmembrane glycoproteins in mammalian lysosomes. During GC or MC infection, at least some GC are found in Lamp1-positive compartments, but Lamp1 levels dramatically decline during infection (Hauck & Meyer 1997, Lin et al 1997). Inspection of the Lamp1 sequence reveals the presence of a Pro/Ser/Thr-rich "hinge" region linking two predicted globular domains. Because highly similar sequences in human IgA1 serve as targets for IgA1 proteases secreted by both GC and MC (Mulks & Shoberg 1994, Plaut et al 1975), it was hypothesized that these proteases cleave Lamp1. Genetic analyses and in vitro cleavage experiments confirm this prediction (Ayala et al 1998, Hauck & Meyer 1997, Hopper et al 2000a, Lin et al 1997).

Further analyses show that neisserial infection decreases not only Lamp1 levels but also the levels of a variety of other lysosomal constituents (Ayala et al 1998), and that mutation of the gene encoding the IgA1 protease in GC reduces intracellular survival and slightly slows traversal of a polarized monolayer (Hopper et al 2000a, Lin et al 1997). These experiments demonstrate that GC and MC are capable of modifying the lysosome. In addition, recent data show that a significant proportion of Lamp1 cleavage by piliated GC and MC occurs not in the phagosome but at the cell surface as a result of a pilus-triggered exocytic event that deposits Lamp1 on the host cell plasma membrane at early stages of infection (P Ayala, B Vasquez, S Clary, K Rodland & M So, submitted). This raises the possibility that GC and MC modify their vacuole prior to entry. Modification of the neisserial vacuole may also facilitate access to the cytoplasm. In this context it should be noted that GC Opa proteins appear to bind to host pyruvate kinase, a cytoplasmic enzyme (Williams et al 1998).

In professional phagocytes, bacteria taken up through Opa-CD66 interactions (Hauck & Meyer 1997, Hauck et al 1998) or opsonic phagocytosis (Read et al 1996)

are capable of entering Lamp-positive compartments. IgA1 protease apparently cannot cleave the Lamp1 of phagocytes (Hauck & Meyer 1997), and encapsulated MC can avoid or delay entry into Lamp1-containing compartments (Read et al 1996). Curiously, encapsulated MC are more, not less, susceptible to killing once inside the macrophages (Read et al 1996).

Host cell membrane traffic can be modified by the addition of purified porins. GC porins inhibit formylpeptide-evoked exocytosis in neutrophils (Haines et al 1991), and recent experiments with latex beads suggest that phagosome maturation or fusion with lysosomes is also inhibited by GC porins. If porins can translocate into the vacuole membrane (Rudel et al 1996), they may equilibrate phagosomal pH and/or provide a portal for the exchange of small solutes between the cytosol and phagosome. It is not yet clear whether these results will apply with intact bacteria. The allelic exchange approach used to demonstrate porin function in invasion (van Putten et al 1998a) should be useful in this context.

CONCLUSIONS AND PROSPECTS

Recent progress has opened the way to a molecular understanding of neisserial attachment/invasion, persistence, transmission, and pathogenesis. The identification of receptors for neisserial adhesins is a major advance, as is the demonstration that clonal divergence through phase and antigenic variation allow select members of a population of infecting bacteria to bind to and signal through multiple host receptors (Figure 2). As described in this review, the host cell cortex undergoes dramatic and extensive morphological and biochemical changes in response to stimulation of these signaling systems. Clarifying how these signaling pathways promote specific biological responses is important.

Another key unresolved issue is whether the mode of GC or MC entry into a host cell influences its intracellular fate. For example, pathogenic *E. coli* producing the FimH fimbrial adhesin trigger accumulations of CD48, caveolin, and glycolipid-enriched domains at attachment sites on macrophages, resulting in bacterial uptake through an unconventional pathway that is not bactericidal. In this context, it is notable that FimH-mediated membrane rearrangements resemble the cortical plaques induced by neisserial type IV pili (Baorto et al 1997).

One question that is only beginning to be addressed is neisserial gene regulation during interactions with host cells. As recounted above, recent studies of contact-regulated gene expression in MC imply a regulatory complexity commensurate with the morphological complexity of the colonization process. Many specific issues remain unresolved. What targets of *crgA* regulation are required for MC adhesion? Does the GC *crgA* gene have a similar role? Is pilus retraction regulated, and if so by what signals? How do bacteria sense contact with host cells?

Recent evidence also suggests that GC can sense the intracellular environment. A screen for GC mutants with a *fast i*ntracellular *t*rafficking (*fit*) phenotype revealed that multiple loci regulate the speed of GC traversal of epithelial monolayers

(Hopper et al 2000b). Interestingly, one such mutant grows more rapidly than the wild-type parent strain within epithelial cells, suggesting that GC may take steps to lengthen their dwell time within cells (Hopper et al 2000b). Such a strategy may promote bacterial persistence, thereby facilitating transmission.

Additional regulatory loci probably mediate other interactions with host cells. In addition to the *fit* and *crg* loci, many two-component regulatory systems have been revealed in the neisserial genomic sequences (Parkhill et al 2000, Tettelin et al 2000), as well as a putative component of the autoinducer-2 quorum sensing system (Surette et al 1999). The elucidation of the signals, transducers, and effectors of host-*Neisseria* crosstalk remains a major challenge. This rich array of questions and the accelerating integration of tools from genetics, genomics, biochemistry, and cell biology suggest that the next few years will bring unprecedented insights into *Neisseria*-host interactions.

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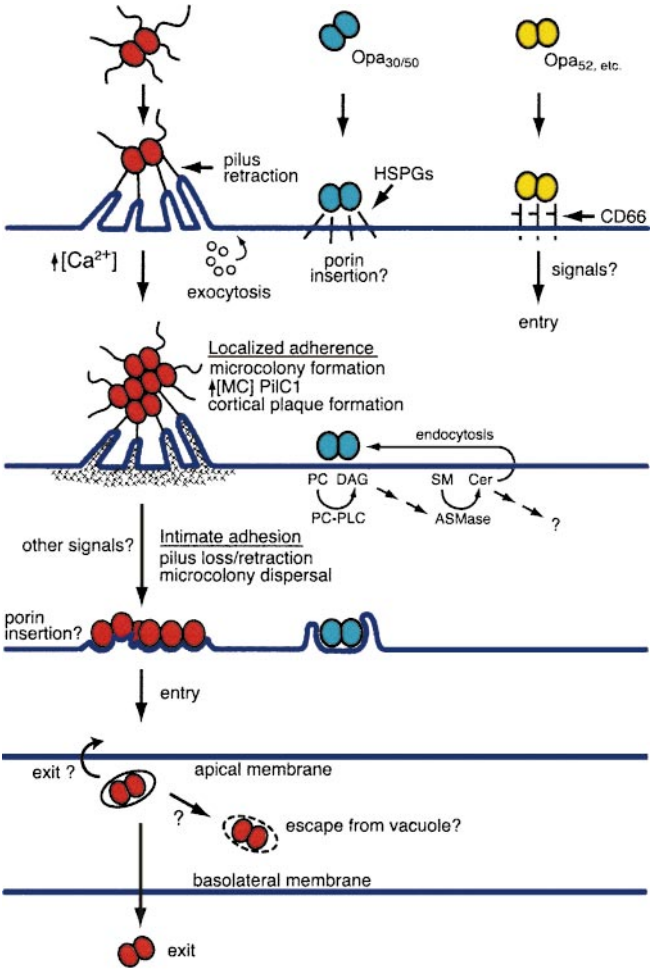

Figure 2 Neisseria induction of host cell signaling pathways via pili, Opa, and porin. HSPG: heparan sulfate proteoglycan; PC: phosphatidylcholine; DAG: diacylglycerol; PC-PLC: phosphatidylcholine-specific phospholipase C; SM: sphingomyelin; Cer: ceramide; ASMase: acidic sphingomyelinase.