Type IV Pili of Pathogenic *Neisseriae* Trigger Cortical Rearrangements in Host Cells: Bacterial Adhesion as an Active, Multistep Process

By

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CERTIFICATE OF APPROVAL

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What I needed was some disease which hadn't been exploited yet. Cancer, muscular dystrophy and tuberculosis had been run through the wringer. Most people had benefited from their contributions – they had the same catharsis of guilt for their own health that Nobel, the man responsible for the killer, dynamite, must have had when he instituted the Nobel Peace Prize.

I needed a disease...

The clap! No one had ever exploited the clap! When the guy comes to your door for the Community Chest or the United Fund, do you ever say to him, "Hey, wait a minute, I'm gonna give you a donation, but how much of my buck is going to the clap?"

—Lenny Bruce

Not just money (from the NIH and the Tartar and Sears endowments), but time, patience, encouragement, and out-and-out labor have been given to me and my research over the last five years.

Maggie So gave me great freedom to pursue my ideas, tempered with stern warnings about dead ends and unfocused hypotheses; once in a while I even listened. She rarely asked that I do experiments directly related to her grants, but always demanded that I do the best work that I'm capable of. I hope I've approached her expectations. **Caroline Enns** kept my feet planted on solid experimental turf, and actually did benchwork with me. Together we obtained pivotal data that provided the basis for most of my subsequent work. I'm delighted that she shares authorship on Manuscript #III. **Jay Nelson** brought me back to asking biological questions whenever I wavered (no matter how enthusiastically), and at one point argued forcefully that I should stay at OHSU; he was right. **Eric Barklis** assumed a relentlessly contrarian and reductionist stance towards this project. More than anyone else, Eric forced me to rethink my ideas on a regular basis. I'm grateful that he's one of my teachers. **Jorge Crosa, Linda Kenney**, and **Fred Heffron** teach the best and most demanding course offered by this department. **Cindy Arvidson** taught me molecular biology. She is a great teacher. **Xavier Nassif** lit a fire under my ass. Repeatedly. **Igor Stoichovic** altered my sense of humor. Permanently. **Micah Worley, Jason Larson, Brian Ahmer, Erik Snapp, Kurt Heldwein**, and **Lan Lin** are friends and colleagues. I'm glad to have gone through grad school with them. **Jen Marino, Walt Anyan, Gene Powell, Scott Greacen, my parents**, and sibs **Chris** and **Greta** provided touchstones on the Outside. **Steff Zimsen** has gone through grad school and a lot more with me, shoulder to shoulder every step.

ABSTRACT

Neisseria gonorrhoeae and *Neisseria meningitidis* are major bacterial pathogens of humans. These closely related species initiate colonization of hosts by attaching to mucosal surfaces using several different adhesive structures, including various outer membrane adhesins and type IV pili. Type IV pili have long been thought to mediate the earliest attachment events, but their function has been considered to be as passive "sticky fibers". In this dissertation I present several lines of evidence showing that neisserial type IV pili have a far more dynamic role in the colonization process than had been previously appreciated. Using cell culture techniques, molecular genetics and high resolution fluorescence microscopy I demonstrate that type IV pili provoke rapid host responses and also alter the course of much later events in colonization. Together these data indicate that neisserial adhesion is a multistep process that requires active participation by both the bacterium and the host, and help to explain why the pathogenic *Neisseriae* deploy such a large array of different adhesins.

Chapter 1: INTRODUCTION

When I got home, though, there came sorrow. Too, too plain was Signor Gonorrhoea.

—James Boswell

...and actually, it's way up there on the charts. Or are you like a lot of subintellectuals who would say, "Well, no, I wouldn't ask about the clap because only bums get it. And Communists." 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?

Don't even say the word clap, man.

—Lenny Bruce

Overview: Neisserial Pathogenesis & Vaccines

Neisseria gonorrhoeae (gonococci, GC) causes gonorrhea and its complications. *Neisseria meningitidis* (meningococci; MC) causes gram negative septicemia and meningitis. These closely related pathogens share numerous virulence factors, colonize humans exclusively, and initiate infection at mucosal surfaces. Yet GC and MC cause very different diseases and have distinctive life cycles and population genetics.

Gonococcal pathogenesis

A classic review of gonorrhea (Harkness, 1948) describes the clinical manifestations of gonococcal urethritis, or gonorrhea. 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. A century ago, opthalmia neonatorum, infection of the infant eye during childbirth, was a leading cause of blindness. GC transmission generally occurs through direct sexual contact but less direct modes of transmission have been documented in the literature

(Kleist and Moi, 1993). GC generally adhere to mucosal surfaces but are also observed in the interior of epithelial cells, and at two to several days post infection bacteria are frequently found in the subepithelial stromal tissue. GC infection is frequently accompanied by an intense focal inflammatory response, and shedding of infected epithelial cells is common in infected individuals. In females, GC frequently ascend the urogenital tract to the oviducts. The resulting inflammation frequently results in loss of gamete transport function. Pelvic inflammatory disease (PID) is the leading cause of infertility among women of reproductive age in the United States, with financial costs in the range of one half billion dollars (U.S.) per year. GC and *Chlamydia trachomatis* infection are the leading causes of PID. Disseminated gonococcal infection (DGI) is a frequent cause of acute arthritis and, less often, of myocarditis (Cucurull and Espinoza, 1998; Masi and Eisenstein, 1981). Epidemiological data suggest that GC infection facilitates HIV transmission (Ghys et al., 1997; Laga et al., 1993).

Meningococcal pathogenesis

Meningococci (MC) are facultative commensals because they colonize the nasopharynx of 3-30% of healthy individuals. However, MC are also a leading cause of bacterial septicemia and fulminant meningitis (Booy and Kroll, 1998). In underdeveloped regions, epidemics of *N. meningitis* are major causes of morbidity and mortality, causing 10,000 or more deaths in a single outbreak (Hart and Cuevas, 1997).

The triggers of meningococcal disease are poorly understood, but environmental factors, bacterial genetics, and host genetics have all been implicated in pathogenesis. The incidence of meningococcal disease varies with season, and is highest

in individuals who have recently had upper respiratory tract infections. In northern temperate zones meningococcal disease tends to peak in late winter, and disease occurs significantly more often in individuals who have recently had influenza or flu-like viral infections (Booy and Kroll, 1998). In the sub-Saharan "meningitis belt" of Africa, meningococcal disease peaks in the dry season and tends to occur in individuals who have recently had infections of the upper respiratory tract, especially infections caused by *Mycoplasma* (Booy and Kroll, 1998; Hart and Cuevas, 1997). These observations imply that immune responses to respiratory infection and/or direct damage to upper respiratory epithelial barriers are important triggers of meningococcal pathogenesis. Bacterial factors are implicated in meningococcal disease, because particular MC strains, and particular constellations of genetic and serological markers, tend to predominate during disease outbreaks (Freimer et al., 1996; Gupta et al., 1996). However, it is not known how such factors influence disease processes. Host genetics are also implicated in susceptibility to meningococcal disease (Booy and Kroll, 1998). In particular, frequent outbreaks of both MC and GC septicemia occur in people who have genetic lesions that cause defects in complement function.

Neisserial vaccines

Vaccines broadly protective against the *Neisseriae* have been difficult or impossible to produce. A reasonably effective polysaccharide vaccine is available for MC serogroups A, C, and W135, but this vaccine is not effective in young children (a major target population) and does not protect against serogroup B or untypeable MC strains (Peltola, 1998). Vaccine development for GC has been even less successful; no vaccine

is available (Blake and Wetzler, 1995). A major reason for these difficulties is the unusual genetic and phenotypic plasticity of the *Neisseriae*, as described below.

Neisserial Genetics

The neisserial genetic system: optimized for generating variation

The present work is not primarily concerned with bacterial genetics, however the lifecycles, virulence characteristics, and evolution of the pathogenic *Neisseriae* are deeply linked to the neisserial genetic system (for reviews see: Cannon and Sparling, 1984; Seifert, 1992; Seifert and So, 1988; Sparling et al., 1986). GC and MC have exceptionally plastic genomes: new variants with heritable phenotypic differences arise at rates that might exceed 0.1 progeny per cell division. This plasticity, combined with an unusual facility for lateral gene transfer, results in rapid genetic and phenotypic divergence both within single infected hosts and at the level of neisserial population structure among many hosts (Achtman, 1994; Achtman, 1995). At least four interlocking mechanisms contribute to this extreme rate of evolutionary change.

First, GC and MC have highly efficient systems for species-specific DNA transformation and recombination. Around 2,000 copies of the 12 bp neisserial uptake sequence (*nus*) are scattered around the 2.2 Mbp GC and MC genomes (Goodman and Scocca, 1991). DNA carrying a *nus* element transforms GC and MC $>10^4$-fold more efficiently than DNA lacking this sequence. For DNA that carries the *nus* element, transformation frequencies of up 10^{-2} to per locus per cell division are observed (Goodman and Scocca, 1988; Seifert, 1996; Sparling, 1966). Lateral genetic transfer

among the pathogenic *Neisseriae* – especially among GC – occurs at extremely high frequencies, as shown by linkage analyses (Smith, 1993; Smith et al., 1993).

Second, a specialized DNA recombination system facilitates antigenic variation of the neisserial pilus. This system relies on the core transformation and recombination machinery that operates throughout the genome, but also utilizes specialized *cis*- and *trans*- acting loci (Koomey et al., 1987; Mehr and Seifert, 1997; Mehr and Seifert, 1998; Scocca, 1990; Seifert, 1996; Wainwright et al., 1997; Wainwright et al., 1994).

Third, the 5' regions of many neisserial genes comprise mononucleotide (e.g., G_n) or polynucleotide (e.g., $[CTCTT]_n$) repeats that are intrinsic mutational hotspots. The addition and deletion of these repeat units occurs at high frequency and is *recA*-independent, indicating that homologous recombination is not substantially involved. Instead, repeat units are added or deleted due to slipped-strand mispairing during DNA replication. (Belland, 1991; Belland et al., 1997; Belland et al., 1989; Bucci et al., 1999; Farabaugh et al., 1978; Stern et al., 1986; Stern and Meyer, 1987; Stern et al., 1984; Streisinger et al., 1966). The addition or deletion of repeat elements causes reading frame changes and introduces or deletes premature termination codons, resulting in the reversible on/off phase variation of gene expression. This type of phase variation occurs at frequencies of 10^{-6}–10^{-2} per locus per cell division. The genomic sequences of GC and MC, near completion at this writing, indicate that a large number of neisserial genes (> 100) contain 5' tandem repeats likely to cause phase-variable expression.

Fourth, most pathogenic MC strains (and many GC strains) appear to have lost the gene encoding the Dam DNA methyltransferase, resulting in inefficient DNA

mismatch repair (Bucci et al., 1999). Inefficient mismatch repair is known to increase the rate of tandem repeat-controlled phase variation (Bucci et al., 1999). In addition, inefficient mismatch repair dramatically increases homologous recombination among DNA sequences that have weak similarity, resulting in increased rates of lateral gene transfer (Matic et al., 1995; Rayssiguier et al., 1989; Vulic et al., 1997).

Together these (and probably other) mechanisms ensure that growing populations of GC or MC are not clonal. Instead, a population will contain an array of variants, each with distinct biochemical, physiological, and immunological properties. Such populations are analogous to the 'quasiclonal' RNA virus populations formally described by Eigen and others (Eigen, 1991).

Why are the Neisseriae *so variable?*

High frequency heritable phenotypic variation occurs even during a short gonococcal infection (Jerse et al., 1994; Schwalbe et al., 1985), and it has been suggested that a primary function for this variation is the evasion of adaptive immune responses. Empirical studies and mathematical models of parasite and virus populations within single hosts indicate that during an infection pathogen numbers rise, crash, and rise again. These fluctuations occur because clonal or quasiclonal microbe populations are recognized by adaptive immune responses, cleared and then replaced by new quasiclonal antigenic variants. Thus, strong empirical and theoretical bases support the view that the *ability* to rapidly produce phenotypic (especially antigenic) variants is a strongly selected trait in microbial pathogens.

More recent research has focused on additional consequences of neisserial

phenotypic variation. Variation of surface exposed proteins and carbohydrates is now known to dramatically alter the ability of GC and MC to attach to and enter host cells, to modify susceptibility to innate immune effectors such as complement, and to alter bacterial nutritional requirements. High-frequency phenotypic variation influences virtually all aspects of *Neisseria*-host interactions. It is probable that high frequency phenotypic variation confers the ability to colonize different microenvironments within host tissues: on the mucosal surface or inside epithelial cells; in male or female urogenital tract; at different sites within a single mucosal epithelium; or at the same site but at different times during the female menstrual cycle. The mechanisms that promote variability in the *Neisseriae* seem to have evolved in response to both negative selection imposed by immune predation, and positive selection for the ability to colonize and grow in divergent host microenvironments. Although the relative importance of these factors is not known, strong evidence supports the hypothesis that environmental fluctuations (immune dynamics and microenvironmental heterogeneity) have driven the evolution of neisserial genetic mechanisms that promote phenotypic variability.

Experimental manipulation of neisserial genetics

Neisserial genetic tools have been thoroughly reviewed (Seifert and So, 1991). The most significant advance since 1991 is the imminent completion of three neisserial genomic sequencing projects. The first genome was recently completed at the Sanger Center. In June, 1999, the genome of MC group A strain Z2491, was announced to be a single contig of 2,182,497 bp (http://www.Sanger.ac.uk/Projects/N_meningitidis). A

second MC genome, that of group B strain MC58, is in progress at The Institute for Genomic Research (http://www.tigr.org). Finally, the genome of GC strain FA1090 is being sequenced at the University of Oklahoma and is 98% complete at this writing, and more than 80% of the sequence has been assembled into ordered contigs (http://www.genome.ou.edu/gono.html).

The natural competence for DNA transformation exhibited by most GC and MC strains makes transformation the easiest route for introducing DNA, but certain conjugative shuttle plasmids can also be moved into the *Neisseriae* by mating with *E. coli*. Genomic data mining combined with PCR techniques and transformation make the targeted mutagenesis of almost any neisserial locus a trivial operation, and isogenic strain sets with targeted mutations in large numbers of neisserial genes should soon be available for analysis.

Neisserial Colonization Factors

Type IV pili

Like many other Gram-negative bacteria, the pathogenic *Neisseriae* produce type IV pili, fibrous polymers 6 nm in diameter and up to several μm in length. In GC and MC, type IV pili are implicated in diverse biological processes including bacterial agglutination, host cell binding and hemagglutination, twitching motility, cytotoxicity, and DNA transformation. In addition, other bacterial type IV pili are involved in 'social gliding' motility, bacteriophage susceptibility, conjugation, and "type II" virulence factor secretion via the main terminal branch of the general secretory pathway (Lu et al., 1997; Pugsley, 1993; Pugsley et al., 1997; Russel, 1998;

Wall and Kaiser, 1999).

Type IV pili were initially distinguished from other types of pili because the major type IV pilus protein, pilin, carries a distinct type of amino-terminal secretion signal and is subjected to characteristic posttranslational processing (Frost et al., 1978; Hermodson et al., 1978). For this reason, type IV pili have sometimes been referred to as "*N*-Met-Phe" pili (see below). Subsequent work with many bacterial species indicates that the assembly pathways and overall structures of type IV pili are broadly conserved (Dalrymple and Mattick, 1987; Hoyne et al., 1992; Patel et al., 1991).

A clue about the origin of type IV pili is provided by the recent discovery that the type IV pilus machinery of *Vibrio cholerae* is wholly embedded within a prophage integrated into the *V. cholerae* genome (Karaolis et al., 1999). This prophage, TCPΦ, is functional and produces transducing phage that confer piliation on nonpiliated *Vibrio* strains. TCPΦ particles are filamentous, form bundles similar to those formed by type IV pili, and–amazingly–contain surface-exposed TcpA, the *Vibrio* type IV pilin subunit (Karaolis et al., 1999). Filamentous phage extrusion and type IV pilus biogenesis have interesting mechanistic similarities (see below), and neisserial type IV pili are involved in DNA transformation. These parallels suggest that type IV pili and filamentous bacteriophages share common evolutionary origins.

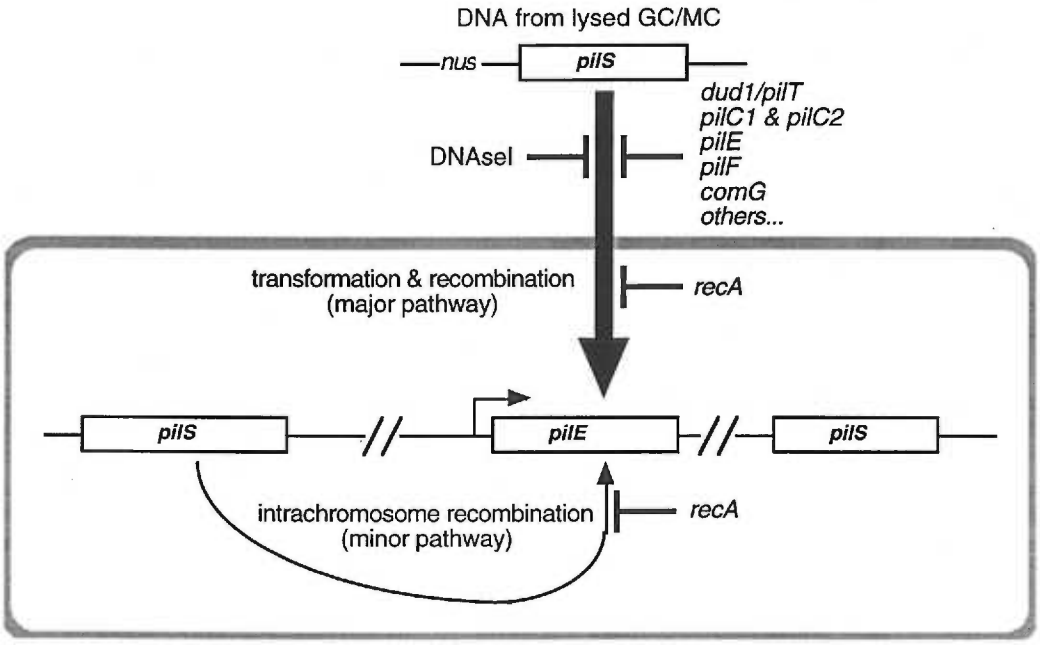
Discovery of neisserial pili and mechanism of pilin antigenic variation

Gonococcal pili were discovered because they promote bacterial cell-cell interactions, with the result that colonies of piliated and nonpiliated GC have different

morphologies. Kellogg and coworkers noticed that initial disease isolates are usually of the relatively compact type 1 and type 2 colony morphologies (Kellogg et al., 1963). Moreover, compact type 2 colonies were virulent upon human challenge, while less-compact type 4 colonies were not (Kellogg et al., 1968). The compact type 1 and 2 morphologies were correlated to GC piliation using electron microscopy (Jephcott et al., 1971; Swanson et al., 1971).

Subsequent work demonstrated that different GC strains produce pili with distinct molecular masses and immunoreactivities (Buchanan, 1975; Novotny and Turner, 1975; Zak et al., 1984), and that pilus-based GC vaccines were protective against the vaccine strain but not against other GC strains. Furthermore, upon passage in vitro, or upon infection of human volunteers, single GC clones throw off variants with structurally and immunologically distinct pili (Duckworth et al., 1983; Salit et al., 1980; Seifert et al., 1994; Swanson et al., 1987). Molecular cloning of a gene encoding GC pilin led to the discovery that many pilin genes and gene fragments are scattered around the GC chromosome, but that only one or two loci have functional promoters. Antigenic variation is now known to occur through the replacement of sequences at functional *pilE* (pilin Expression) loci with sequences from variant *pilS* (pilin Silent) loci that lack promoters (Haas and Meyer, 1986; Hagblom et al., 1985; Segal et al., 1985; Segal et al., 1986). Pilin antigenic variation is strictly dependant on the neisserial *recA* gene, a homologue of the *E. coli recA* gene which is essential for generalized homologous recombination (Koomey and Falkow, 1987; Koomey et al., 1987; Seifert, 1997). This process is largely dependant on DNA transformation, because *pilE* recombination frequencies are strongly reduced in the presence of DNAse

I or in piliated but nontransformable *pilT* (a.k.a. *dud1*) mutants (Seifert et al., 1988b). Because most recombination at *pilE* involves DNA taken up by transformation, the recombinant genotypes misleadingly appear to be products of nonreciprocal gene conversion events (Haas and Meyer, 1986; Seifert et al., 1988a).



More detailed genetic studies show that a specialized machinery exists to facilitate high frequency recombination of transformed *pilS* sequences into *pilE*, and that this machinery is partially distinct from the more generalized neisserial homologous recombination system (Howell-Adams et al., 1996; Mehr and Seifert, 1997; Mehr and Seifert, 1998; Wainwright et al., 1997; Wainwright et al., 1994). The neisserial system for rapidly generating many type IV pilin antigenic variants is unusual. Type IV pili are present in diverse gram negative bacteria, but most of these organisms express only one or a few pilin variants.

Structure of neisserial type IV pili

As might be expected from their range of biological functions, type IV pili are structurally complex. Pilus assembly requires > 10 specialized gene products as well as the functions of the bacterial general secretory pathway (GSP) (Duong et al., 1997; Pugsley, 1993; Pugsley et al., 1997). The type IV pilus fiber is composed mostly of pilin, an 18-22 kDa polypeptide. Many pilin antigenic variants are posttranslationally modified by O-glycosylation and phosphorylation (Forest et al., 1999; Marceau et al., 1998; Marceau and Nassif, 1999; Parge et al., 1995; Stimson et al., 1995; Virji et al., 1993b). The atomic structure of gonococcal pilin was recently solved to 2.6 Å resolution by Tainer's group (Parge et al., 1995). The pilin monomer comprises a C-terminal globular "head" region and an N-terminal hydrophobic "tail" that folds into an unusually long α-helix:

(Courtesy of K. Forest; see Forest and Tainer, 1997)

Fiber diffraction, cryoelectron microscopy, antigenic mapping, and molecular modeling by Tainer, Forrest and coworkers indicate that pilin subunits polymerize into a right-handed helical cylinder with fivefold symmetry about the helix axis (Forest

and 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 great stiffness and tensile strength. The globular pilin heads face outwards, 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 outside of 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). These structural studies provide a satisfying answer to the question of how enormous antigenic variability is accommodated at the surface of the pilus fiber without compromising the fiber's assembly properties or structural integrity.

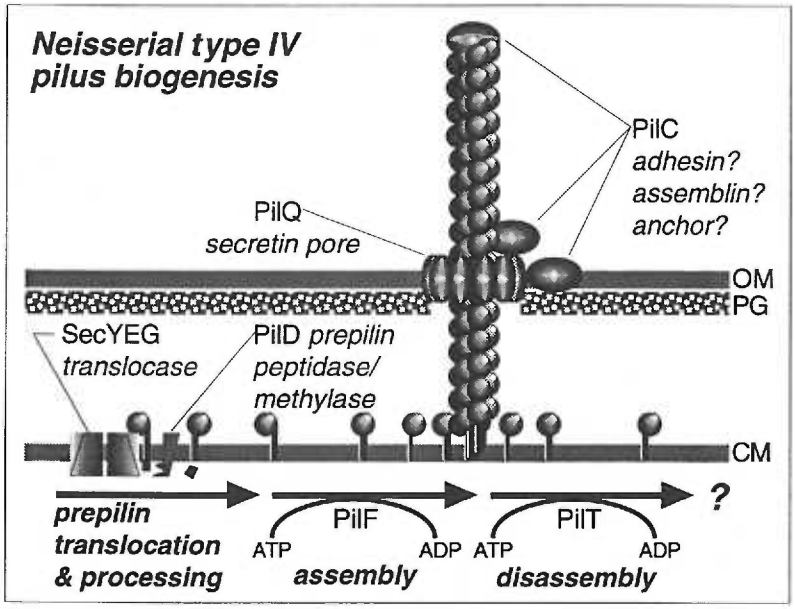
Functional consequences of pilin antigenic variation

The relatively invariable *Pseudomonas aeruginosa* type IV pilin is known to bind directly to host glycolipids via a conserved site (Lee et al., 1994; Sheth et al., 1994; Yu et al., 1994). In contrast, direct receptor binding has never been definitively shown for the far more variable pilin subunits of GC and MC. Nevertheless, small alterations in the primary structures of neisserial pilins can result in altered immunochemistry, altered glycosylation, and altered adhesive function (Jonsson et al., 1994; Marceau et al., 1995; Marceau et al., 1998; Nassif et al., 1993; Rothbard et al., 1985; Schoolnik et al., 1984; Virji et al., 1991; Virji et al., 1993b). Perhaps surprisingly, point mutations that abolish O-glycosylation of the MC pilus have only modest effects on adhesion to host cells. In contrast, other point mutations that do not appear to influence

posttranslational modification have dramatic effects on host cell binding and cell tropism (Jonsson et al., 1994; Marceau et al., 1995; Marceau et al., 1998; Nassif et al., 1993; Rothbard et al., 1985; Schoolnik et al., 1984; Virji et al., 1991). These point mutations might directly modify a receptor binding site within the pilin subunit, or they might alter pilus adhesive function via an indirect mechanism. Interestingly, many pilin variants that promote strong adhesion also aggregate into laminar bundles, while variants that promote weaker adhesion tend to exist as single filaments (Marceau et al., 1995). Bundles could increase pilus stiffness and tensile strength under shear or flow. Alternatively, bundles could facilitate coordinated extension/retraction processes (see below) that might not be possible with more disordered structural networks. Type IV pili from organisms that do not exhibit high levels of antigenic variation (e.g., *Vibrio*, *E. coli*, *Pseudomonas*) generally occur as filament bundles.

Assembly of type IV pili

Current models of type IV pilus biogenesis are based largely on the Lory and Mattick groups' studies of *Pseudomonas* pili and from the Pugsley group's studies of the type II secretion machinery (a.k.a. GSP main terminal branch), which has structural and mechanistic similarities to the type IV pilus. Recently, more progress has been made in experiments with *Neisseria*, particularly through work in Koomey's laboratory. A simplified cartoon model for type IV pilus assembly is shown below, and key findings that have led to this model are summarized below.



Transit of pilin across the bacterial inner membrane

It is not known how pilin is translocated across the bacterial inner membrane, but by analogy to type II secretion, pilin probably traverses the inner membrane via the inner membrane preprotein translocase (the SecYEG complex) (Duong et al., 1997; Pugsley, 1993). The bifunctional enzyme PilD is a polytopic inner membrane protein that is absolutely required for type IV pilus assembly and for type II secretion. PilD specifically recognizes an atypical secretion signal near the N terminus of prepilin, which PilD both cleaves and methylates (Freitag et al., 1995; Nunn and 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, some with homology to pilin, also contain prepilin-type secretion signals and are processed by PilD (Dupuy et al., 1992; Nunn and Lory, 1992; Strom et al., 1991; Strom et al., 1994). At least some of these proteins are required for pilus-related functions, while others are involved in type II secretion (Pugsley, 1993; Pugsley et al., 1997; Russel, 1998). Bacteria

that deploy type II secretion systems but lack type IV pili encode PilD homologues that can correctly process prepilin and can functionally complement *pilD* mutations (Dupuy et al., 1992).

Analyses of spheroplast preparations from *E. coli* cells producing GC prepilin-alkaline phosphatase fusions suggest that both prepilin and PilD-processed pilin monomers exist as type II 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 (prepilin peptidase and methyltransferase) active sites face the cytoplasm, where they remove charged residues at the prepilin amino terminus and then methylate the exposed α-amino terminus of pilin. These modifications eliminate the charged cytoplasmic anchor of prepilin, facilitating the extraction of pilin from the cytoplasmic membrane and/or pilin incorporation into the growing pilus fiber.

Assembly of type IV pili occurs prior to translocation across the outer membrane

Prepilin processing by PilD is necessary but insufficient for fiber assembly. Type IV pilin polymerization is thought to occur within the cytoplasmic membrane and/or in the periplasm, rather than at the outer membrane as for many other bacterial pili (Hultgren et al., 1993; Pugsley, 1993). Strong evidence supporting periplasmic or inner membrane assembly is provided by studies of the neisserial PilQ protein. PilQ is a member of the secretin protein family. Secretins are large, polytopic proteins found in homooligomers within the outer membrane. In two cases secretins have been shown

to form large-conductance pores through which assembled polymers are thought to be extruded (Marciano et al., 1999; Nouwen et al., 1999). Secretins are involved in flagellar assembly, contact-dependant type III secretion, and filamentous phage extrusion. Null mutations in *pilQ* are lethal if introduced into piliated GC. If an IPTG-inducible *pilQ* allele is introduced into GC, and *pilQ* expression is halted by removal of IPTG, the periplasm becomes distended and fills with pilin polymer. Long, thin membrane-bounded blebs are released into the medium, which contain high concentrations of mature pilin polymer (Dunham et al., 1998).

ATPases involved in fiber assembly

Several gene products that comprise Walker boxes (conserved ATPase motifs) are present in the bacterial cytoplasm and/or are associated with the bacterial inner membrane. The functions of these proteins are not well understood, but genes encoding proteins with strong sequence homology play essential roles in other bacterial type II (GSP main terminal branch) and type III (flagellar and contact-dependant) secretion systems (He, 1998; Pugsley et al., 1997; Russel, 1998). In all cases reported, mutations targeted to the predicted ATP-binding cassettes of these proteins produce phenotypes identical to null alleles. Mutations in *pilF*, a homologous gonococcal gene, result in a nonpiliated phenotype. PilF might catalyze translocation of pilin across the inner membrane, binding or unbinding of putative chaperones, filament polymerization, or other undefined assembly steps. Interestingly, mutations in the highly similar gene *pilT* yield very different phenotypes, as discussed below.

The enigmatic PilC proteins

The *pilC1* and *pilC2* loci were originally described by Jonsson and coworkers as genes involved in structural and phase variation of GC pili (Jonsson et al., 1991; Jonsson et al., 1992). Neisserial *pilC* loci contain 5' G_n repeats in the regions encoding the signal peptide which cause high-frequency on/off phase variation, and encode large (110 kDa) proteins with standard cleaveable signal sequences. The two GC *pilC* genes are functionally redundant. Phase-off variation or targeted mutation of either *pilC1* or *pilC2* yields no detectable phenotype. Early studies of GC *pilC* double mutants gave inconsistent results, but later studies have consistently shown that strains lacking both loci bear very few or no pili and are nontransformable (Jonsson et al., 1994; Jonsson et al., 1991; Jonsson et al., 1992; Rudel et al., 1995a; Rudel et al., 1992). A simple interpretation of these data is that PilC is a pilus assembly factor, but studies of the MC *pilC* loci suggested greater complexity. Mutation of the MC *pilC2* gene has no detectable phenotype, as in GC. Also as in GC, *pilC1 pilC2* double mutants are nonpiliated. However, 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). These results indicate that MC *pilC1* has a critical role in adhesion, and is not merely a pilus assembly factor.

In experiments by Meyer's group, hexahistidine-tagged GC PilC2, overproduced in a nonpiliated GC background, was used in cell binding and adhesion blockade experiments (Rudel et al., 1995b). These experiments suggested that PilC may function directly as an adhesin. In the same report, immunogold electron microscopy experiments suggested that PilC is selectively localized at the pilus tip. However,

specific and saturable quantitative binding of PilC to host cells has not been demonstrated, and divergent immunogold localization results have been reported by Jonsson's group (Rahman et al., 1997). Thus, both the specific adhesive activity and the subcellular location of PilC remain controversial. Nevertheless, recent data may explain the assembly phenotypes observed with *pilC* mutants: PilC appears to modulate the dynamics of pilus assembly, rather than being required for filament assembly *per se.*

Twitching motility and structural dynamics of type IV pili

Type IV pili have been implicated in "twitching" and "social gliding" motility (Henrichsen, 1983; Lautrop, 1961; Wall and Kaiser, 1999) These motility processes involve migration along phase interfaces, e.g., liquid-solid or solid-air (Henrichsen, 1975b). The mechanistic basis for twitching motility is unknown, but two lines of evidence suggest that twitching may involve pilus retraction and/or disassembly.

Twitching motility was first characterized in *P. aeruginosa* by Bradley, who noted a correlation between twitching motility, piliation, and susceptibility to bacteriophages that adsorb to type IV pili (Bradley, 1972a; Bradley, 1972b; Bradley, 1974). Bradley demonstrated that wild type *P. aeruginosa* had many phage attached to the bacterial cell body and concentrated at the bases of pili, while a nonsusceptible but piliated mutant had pili uniformly covered with phage but few phage on the cell body. Furthermore, the nonsusceptible mutants did not twitch. These results were consistent with the hypothesis that pili retract into the cell body. Later work by Mattick's group revealed that the lesion in one of these mutants mapped to the *pilT* locus, which

encodes a cytoplasmic or inner membrane ATPase similar to ATPases required in pilus assembly and in type II and type III secretion (He, 1998; Whitchurch et al., 1991).

Twitching was observed in gonococci and linked to the presence of pili in the 1970s (Henrichsen, 1975a; Swanson, 1978). In more recent experiments, Koomey's group identified a neisserial *pilT* homologue on the basis of its sequence homology to the *P. aeruginosa pilT* gene (Brossay et al., 1994; Wolfgang et al., 1998a). As in *P. aeruginosa*, GC *pilT* mutants have more pili than the wild type strain and adhere avidly (125-200% of wild type levels) to epithelial cells. However, *pilT* mutants do not twitch and are not competent for DNA transformation (Wolfgang et al., 1998a). Interestingly, *dud1* mutants obtained in a screen for piliated but nontransformable GC carry alleles of *pilT* (Biswas et al., 1989; Seifert et al., 1988b; Wolfgang et al., 1998a).

When an IPTG-inducible *pilT* allele was combined with null mutations in both GC *pilC* loci (see Manuscript II), a remarkable synthetic phenotype was obtained (Wolfgang et al., 1998b). In the presence of IPTG, the strain behaved like a *pilC1 pilC2* $pilT^+$ mutant, and was nonpiliated. Upon removal of IPTG, however, the strain was depleted of PilT. This *pilC1 pilC2 pilT* phenocopy was hyperpiliated, producing more pili of greater length than any GC strain so far described, but it was unable to adhere to epithelial cells, did not twitch, and was not competent for DNA transformation. These results have several implications.

First, the removal of PilT completely suppresses the piliation defect in the *pilC* double mutant, indicating that *pilC* is dispensable for fiber assembly. Second, GC pilus fibers that lack *pilC* cannot support epithelial cell adherence, strongly corroborating the data from MC which suggested that PilC plays a pivotal role in cell adherence.

Third, the phenotype of *pilC1 pilC2* strains can now be reinterpreted in terms of *pilT* function. These mutants are nonpiliated in the presence of PilT, but hyperpiliated in its absence. Because mutation of *pilT* confers a nontwitching phenotype, a simple model suggests that PilT could drive pilus *dis*assembly, possibly by using energy derived from ATP hydrolysis to remove pilin subunits from the base of the polymeric fiber. An interesting observation in this context is that pilin degradation occurs at higher rates in $PilT^+$ GC than in $PilT^-$ GC, whether or not PilC is present. In this model PilC would function as an *inhibitor* of *pilT*-mediated fiber *disassembly*, rather than as a promoter of fiber assembly. In the absence of PilC, PilT-mediated disassembly would act unopposed, causing an apparent defect in pilus assembly. Thus PilC appears to act both as a promoter of pilus-mediated adhesion and as an inhibitor of pilus disassembly – but precisely how it mediates these functions is not known.

Host cell receptors for type IV pili

GC and MC pili mediate strong adhesion to several human cell types including epithelial cells, endothelial cells, and sperm cells (Buchanan and Pearce, 1976; Gubish et al., 1982; James-Holmquest et al., 1974; Jonsson et al., 1994; Nassif et al., 1994; Pearce and Buchanan, 1978; Stephens, 1981; Swanson, 1973; Swanson et al., 1975; Virji et al., 1991). The molecular basis for this adhesion has been intensively studied, but the first receptor for neisserial pili, CD46, was only recently identified (Kallstrom 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 (Karp, 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 (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 a far-western type overlay assay. Pili prepared from a MC *pilC1* mutant failed 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. Also, certain monoclonal antibodies or antisera against CD46 inhibit adhesion to epithelial cells, while other monoclonal antibodies that bind different CD46 epitopes do not have an inhibitory effect. 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 (Kallstrom et al., 1997). (We have independently verified the latter result; A. Merz, T. Wong, and M. So, unpublished data.) Antibody-inhibition data are also consistent with a role for the B domain. A mAb (GB24) directed against an epitope proximal to the B domain inhibited neisserial adhesion to epithelial cells, while a mAb (TRA-2-10) directed against a more distal amino terminal epitope had little effect.

The reported adhesion to ectopically expressed "BC"variants is very weak

compared to adhesion supported by native epithelial cells. Further, 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 do not express CD46. For these reasons it is possible that CD46 is only a minor pilus receptor, requires a coreceptor, or requires cell-type-specific posttranslational modifications to support strong pilus-mediated adhesion. In this context it is interesting that CD46 holds a P/S/T-rich region that overlaps the apparently critical B domain:

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[...]VSTSSTTKSPASSAS-GPRPTYKPPVSNYP-GYPKPEEGILDSLDV-transmem.
     B domain       C domain      juxtamembrane
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This P/S/T region is similar to hinge regions present in human immunoglobulin A (IgA) and in human LAMP1, the major glycoprotein of degradative organelles within the endocytic pathway. IgA1 proteases secreted by GC and MC are known to cleave the IgA and LAMP1 hinges at P/T (or P/S) peptide bonds (Ayala et al., 1998; Kilian et al., 1995; Lin et al., 1997), raising the possibility that CD46 is also a target of IgA1 proteases. Consistent with this notion, piliated GC with null mutations in the gene encoding IgA1 protease adhere to epithelial cells somewhat more avidly than isogenic strains that produce the S/T selective IgA1 protease (S. Hopper, S. Clary and M. So, unpublished observations).

The Opa proteins

A second major class of neisserial adhesins is encoded by the multigene *opa* family. An excellent and up-to-date review summarizes the biology of this protein family (Dehio et al., 1998a). GC strains typically harbor ~11 *opa* loci, while MC strains typically

have 4-5 *opa* loci (Dehio et al., 1998a). Specific *opa* repertoires vary from strain to strain. In SDS-PAGE experiments Opa proteins migrate at 28-35 kDa, and share the unusual property of migrating more slowly after heating in the presence of SDS.

Opa nomenclature

Until recently *opa*/Opa nomenclature was not standardized. For example, The GC strain MS11 protein Opa60 is also known as OpaI or OpaH and is encoded by a single locus referred to either as *opa60*, *opaI*, or *opaH*. Moreover, at least one published Opa nomenclature table is inaccurate (Bos et al., 1997; Kupsch et al., 1993). This situation has changed with the adoption of a standardized nomenclature (Dehio et al., 1998a; Malorny et al., 1998). In the older GC literature, Opa proteins are referred to as Opacity proteins, protein II, or p. II. The Introduction, Manuscript III, and the Discussion of this thesis use the standardized nomenclature, while Manuscripts I and II were written prior to this standardization.

Opa phase and antigenic variation

All *opa* genes sequenced to date comprise 5' tandem repeats $[CTCTT]_n$ that cause high-frequency phase variable expression as described above (Stern et al., 1986; Stern et al., 1984). As a result a given organism can reversibly express zero, one, or multiple different Opa proteins (Blake and Gotschlich, 1984; Blake et al., 1981; Poolman et al., 1980; Walstad et al., 1977). In addition, DNA transformation and homologous recombination result in the formation of hybrid recombinant *opa* loci, both in the laboratory and in nature (Achtman, 1994; Waldbeser et al., 1994).

As with pili, Opa proteins mediate bacterial agglutination as well as binding to

host cells, and Opas were initially identified by their ability to cause the opaque GC colony morphology (Swanson, 1982). The importance of Opa proteins in infection is underscored by studies in which frequent Opa phase and antigenic variation have been observed to occur during human infections (Jerse et al., 1994); by very careful studies of *opa* microevolution during MC epidemics (Achtman, 1994); and by the noninfectious phenotype recently reported for a GC strain with targeted deletions of all eleven *opa* genes (Cannon et al., 1998).

Opa structure

Monoclonal antibody binding and limited proteolysis have been used to test models for Opa topology in the bacterial outer membrane. The results suggest that Opas have eight transmembrane beta strands and four surface-exposed loops. As might be expected, the relatively conserved regions of Opa primary structure map to transmembrane and periplasmic domains, while the relatively variable regions map to the first three surface-exposed loops (Blake et al., 1981; Malorny et al., 1998). Expression of functional Opas in *E. coli* was problematic for many years. More recently, translational fusion of Opa to the *E. coli* beta lactamase (Bla) leader sequence has allowed expression of Opa proteins with immunological, topological, and receptor-binding properties that are identical to the native proteins (Belland et al., 1992).

Opa function in colonization

Different Opa proteins confer different phenotypes in host cell interaction assays (Dehio et al., 1998a). To simplify the interpretation of experimental results, Opa

function has been studied mainly in nonpiliated neisserial strains or in recombinant *E. coli*. 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 and Gotschlich, 1986; Kupsch et al., 1993; Makino et al., 1991; Simon and Rest, 1992; Swanson, 1992; Swanson, 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 made possible a molecular understanding Opa-mediated adhesion and invasion of host cells.

Opa receptors on host cells

The first class of Opa receptors, heparan sulphate proteoglycans (HSPGs), were independently identified by van Putten's group and the Swanson/Belland group (Chen and Gotschlich, 1996; van Putten and Paul, 1995). HSPG receptors bind a subset of Opa variants that have surface-exposed loops rich in positively charged residues. The prototype Opa in this class is the GC MS11 Opa30 protein (a.k.a. OpaA in Manuscript II). Opa30 and related Opas mediate efficient binding to and invasion of Chang and a few other epithelial cell lines, and this invasion is markedly reduced by competition with soluble polyanions including heparin, heparan sulfate, heparan sulfate proteoglycans, DNA, or by treatment of the cells with the enzyme heparatinase I (Also see manuscript III). Interestingly, chondroitin sulfate and keratan sulfate moieties neither mediate binding to Opa30 nor block binding to HSPGs, suggesting that the

carbohydrate's negative charge density is critical to this interaction. 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). A more complex form of binding through the Opa30 molecules has also been discovered, in which fibronectin or vitronectin serves as a bridge between Opa-bound heparan or heparin and cell surface integrin molecules. These interactions are strong enough to promote moderately efficient bacterial uptake into HeLa, CHO, or Hep-2 cells, which are nonpermissive for invasion in the absence of vitronectin or fibronectin (Dehio et al., 1998b; Duensing and Putten, 1998; Duensing and van Putten, 1997; Gomez-Duarte et al., 1997; Grinnell and Geiger, 1986; van Putten et al., 1998b).

Recent work from several labs has shown that many different Opas bind to members of the large CD66/CGM1/CEA/BGP family of proteins (Bos et al., 1997; Chen and Gotschlich, 1996; Chen et al., 1997; Gray-Owen et al., 1997a; Gray-Owen et al., 1997b; Hauck et al., 1998; Virji et al., 1996a; Virji et al., 1996b; Wang et al., 1998). CD66-related proteins are thought to have roles in cell-cell adhesion, are encoded by different genes and are often produced as multiple splice variants. Different CD66 family members are expressed on different cell types. Most family members encode transmembrane proteins, while others encode peripheral membrane proteins with GPI (glycosylphosphatidylinositol) membrane anchors. Although all native CD66-related proteins are glycosylated, Opa-CD66 interactions are protein-protein interactions. Different Opas bind to different spectra of CD66 variants, and single amino acid changes within the Opa binding sites of CD66 can change specificity for Opa binding. Preliminary studies indicate that, 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 systems in host cells.

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 a large proportion (but not a majority) of MC strains and is weakly associated with virulence (Olyhoek et al., 1991; Seiler et al., 1996). A sequence similar to *opc* is present in the genome of GC FA1090 but the open reading frame appears to be truncated in this strain (unpublished observations). *opc* expression undergoes clonal variation *via* mutations in a polypurine tract. A poly-C tract is present within the 5' promoter region of *opc*, and 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 nonencapsulated MC confers the ability to adhere to and invade endothelial cells independently of Opa and pili (Virji et al., 1993a; Virji et al., 1992; Virji et al., 1994; Virji et al., 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 integrins on the cell surface (Virji et al., 1994). Like most MC isolates, the MC strains used in the present work lack the *opc* locus and do not produce detectable Opc protein (see Manuscript II).

Other neisserial adhesins

Besides type IV pili, Opa, and Opc, other potential adhesins have been identified in GC and MC. These include GC lipooligosaccharide, which may bind to asialoglycoprotein receptors on the cell surface (Porat et al., 1995a; Porat et al., 1995b), and a multigene family of glycolipid-binding outer membrane proteins identified in the So laboratory (Paruchuri et al., 1990; Stromberg et al., 1988). The GC glycolipid-binding adhesins bind to lactose-containing asialoglycolipids such as globotetraosyl ceramide (GgO4/asialo GM1). Similar carbohydrate structures are present on GC lipooligosaccharides, suggesting that the glycolipid-binding adhesins may mediate bacterial agglutination as well as binding to host glycolipids. Furthermore, the glycolipid-binding adhesins bear nearly perfect consensus prepilin leader sequnces (unpublished data). The same class of glycolipids can serve as receptors for the type IV pili of *Pseudomonas aeruginosa*. However, the GC glycolipid-binding adhesins seem to be functional in the absence of type IV pili or when expressed in *E. coli* (Paruchuri et al., 1990; Stromberg et al., 1988).

Neisserial antiadhesins and adhesion modulators

MC capsule and certain structural variants of GC lipooligosaccharide (LOS) inhibit bacterial interactions with host cells. In both cases the antiadhesive 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). This reaction is catalyzed by a bacterially-encoded silayltransferase. 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, the 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. 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; Virji et al., 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 lipooligosaccharide, pili are thought 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 are 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 observations have suggested that these proteins may exert a pivotal influence on the outcome of host-bacterial interactions. Most significantly, 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 that would face the host cell cytoplasm. These neisserial porins share these properties with the mitochondrial porin, VDAC (voltage-dependant anion channel) Rudel, 1996 #832. VDAC was recently shown to be a key mediator of cell survival and apoptosis through its ability to interact with the Bcl-2 family of survival factors (Green and Reed, 1998; Shimizu et al., 1999). Like VDAC, neisserial porins have been proposed to play a role in host cell apoptosis (Muller et al., 1999), but it is not clear that the pathogenic *Neisseriae* trigger apoptosis to any significant extent *in vivo*.

Another phenotype for the porins was recently demonstrated by van Putten's group. In elegant experiments using isogenic GC strains expressing different porin alleles, certain porin variants conferred the ability to invade epithelial cells in the absence of pili or Opa. The invasion-promoting porin alleles tend to be found in strains recovered from patients with invasive, disseminated infections. The porin-specific invasive phenotype was observed only at low concentrations of phosphate, and could be abolished by the inclusion of NTPs or polyphosphate at relatively low concentrations (van Putten et al., 1998a). Further work should reveal whether porin translocation into epithelial cells is involved in invasion, and how porin works in combination with other colonization factors.

The Present Work

When I began my thesis work a role for type IV pili in adhesion was well established, and functions for Opa proteins in adhesion and invasion were just beginning to emerge. At that time, two questions seemed to be of great importance. First, are there significant differences in neisserial interactions with different host cells? Up to that point only two epithelial cell lines had been commonly used, and neither of these had the simple columnar epithelial morphology possessed by many target cells for neisserial infection. Manuscript I describes the adaptation of the polarized T84 model to studies of GC and MC infection. An important result from this work was that type IV pili increased the ability of GC to not only adhere to, but to invade polarized T84 cells. This result stood in contrast to results obtained in earlier work with a conjunctival cell line (Makino et al., 1991), and indicated that the effects of pili on invasion depended on the particular host cell assayed. Because other proteins besides pili were then being defined then as invasion factors, a second major question became important: how do colonization factors interact during the infection process? To begin to answer this question it is useful to ask a slightly different question.

Why do the pathogenic Neisseriae *have so many adhesins?*

If we phrase the question this way, several different answers are possible. First, the different adhesins may be fully redundant. Combined with phase variation, redundancy would allow the bacteria to have adhesins available even through several rounds of immune predation. This answer may be partially correct but we already knew that some adhesins conferred different cell tropisms for adhesion and invasion.

Second, different adhesins might permit the colonization of different microenvironments. This answer also seems to be at least partially correct. Third, different adhesins might have specific, sequential roles during the colonization of specific sites. When I began my work very little evidence was available to support this last hypothesis, but it was appealing because it was already known that bacteria recovered from single infected sites *in vivo* often express multiple classes of adhesins (e.g. pili, one or more Opa proteins, and Opc). The tropism hypothesis did not provide a functional rationale for these observations, and the immune surveillance hypothesis seemed to be genuinely discordant with these observations.

Host responses to neisserial colonization

If multiple adhesins really are used sequentially, adhesion should occur as a multistep process. In fact there was evidence to support this view. Electron microscopy studies of human oviduct segments in organ culture (described in detail in the Introductions to Manuscripts I and III) identified distinct morphological stages in neisserial colonization: loose adhesion of small microcolonies; bacterial contact with microvilli that appeared to have elongated from the contact region; and tight association of single, dispersed bacteria with the host plasma membrane. It seemed likely that these stages would be mediated by different bacterial structures, and would involve active participation by both the bacterium and the host cell.

In Manuscripts II and III, I show that different adhesins do in fact mediate distinctive early host cell responses and that these responses are likely to have major influences on the later steps of colonization. These results are summarized in the

Discussion section of Manuscript III. In that Discussion, I propose a multistep model in which neisserial adhesion does in fact occur as an ordered series of events that are potentiated by different adhesins. In a final Conclusions section, I will integrate the results presented here with very recent data from other laboratories and suggest some directions for future work.

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Chapter 2: MANUSCRIPT I

Traversal of a polarized epithelium by pathogenic *Neisseriae*: facilitation by type IV pili and maintenance of epithelial barrier function

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Summary

Backgroound. Gonococci (GC) and meningococci (MC) are Gram-negative bacterial pathogens that infect human mucosal epithelia. We wish to understand the functions of specific bacterial components at each stage of mucosal colonization: adhesion, cell invasion, and traversal into subepithelial tissues. As no animal model of mucosal colonization by GC or MC is available, increasingly sophisticated *in vitro* approaches have been used to address these issues.

Materials and Methods. We adapted the polarized T84 human epithelial cell system to study GC and MC colonization. Epithelial barrier function was monitored by permeability to soluble tracers and with electrical resistance measurements. Polarized cells were used to assay bacterial traversal of the monolayers, and cells grown on plastic were used to assay adhesion and cell invasion.

Results. All pathogenic *Neisseriae* examined traversed the monolayers. The traversal times were species-specific and identical to times established previously in organ culture studies. In contrast to experiments with some enteric pathogens, transmigration by GC and MC was not accompanied by disruption of the epithelial barrier. GC mutants lacking type IV pili were compromised in adhesion, invasion and traversal of T84 cells.

Conclusions. Experiments with polarized T84 cells mimic key features of organ culture infections and reveal additional aspects of neisserial infection. Epithelial barrier function can be retained during bacterial traversal. Experiments with a nonpiliated GC mutant and its wild type parent indicated an unexpected role for pili in cell invasion. Our results are consistent with the hypothesis that bacterial adhesion, invasion, or both are rate-limiting for traversal across the epithelium.

Introduction

The genus *Neisseria* comprises several Gram-negative diplococci that colonize human mucosal tissues (1, 2). In most cases the relationship between host and bacterium is commensal but some species can pose serious health risks. *Neisseria gonorrhoeae* (GC) commonly colonize the mucosa and submucosal stroma of the conjunctiva, throat, urogenital tract, and rectum. Infection at the latter sites may be a mechanical cofactor facilitating the transmission of human immunodeficiency virus (3, 4). *Neisseria meningitidis* (MC) colonize the nasopharynx, usually without causing disease. In some cases, however, MC may traverse the mucosal barrier and enter the blood, leading to septicemia. Further, MC may cross the blood-brain barrier, resulting in meningitis.

Humans are the only natural host for GC and MC. As a result, no animal model of *Neisseria*-mucosa interactions is presently available. However, segments of human oviduct (FTOC; Fallopian tube organ culture) and nasopharynx (NPOC; nasopharyngeal organ culture) inoculated *ex situ* with GC and MC have yielded morphological descriptions of the initial steps of infection. Certain features are common to both GC and MC infection (5, 6). (i) Bacteria selectively adhere to secretory (nonciliated) epithelial cells, probably using pili. (ii) Bacteria intimately contact the host cell surface (close association). (iii) The region of contact enlarges until diplococci are surrounded by host plasma membrane. A fusion event is presumed to seal off the nascent vesicle. The engulfment process closely resembles classical 'zippering' phagocytosis (7). In tissue culture cells, bacterial entry is blocked by both microfilament-disrupting drugs and inhibitors of bacterial protein synthesis, implicating both host and parasite as active participants (8, 9). (iv) GC survive and multiply inside epithelial

cells. (v) GC are observed to traverse the length of the infected cell, and by 1-2 days after infection GC or MC are observed in the stromal matrix (5, 6).

The available infection models have advantages and limitations. Human volunteer studies have provided data implicating pili and opacity (Opa) proteins in colonization (10). But volunteer studies and organ culture are expensive and limited by the availability of volunteers or tissue specimens, so they cannot be used to compare many different bacterial strains. Cultured human cells grown on plastic have been used to dissect the function of a number of GC and MC surface components (11-16). However, cells grown on plastic do not replicate the polarized architecture of epithelial and endothelial cells *in situ*, and are therefore less useful for studying events after the early colonization steps. Moreover, none of the above models permits the isolation of viable bacteria from subepithelial spaces, and none of the *in vitro* systems facilitates studies of the mucosal inflammatory response, a hallmark of GC infection (1).

Many of these limitations can be overcome by culturing epithelial cells on permeable membrane supports which permit cells to feed from their basolateral surface and to polarize as in native tissue (17, 18). Both 'serosal' and 'mucosal' surfaces are experimentally accessible, permitting studies of transcytosis and protein trafficking (19, 20), barrier physiology (21, 22), bacterial infection (23, 24), and leukocyte diapedesis (25, 26). A recent report (27) describes a system that incorporates both an epithelial (HEC-1-B) and an endothelial layer (HMEC-1) to study early MC pathogenesis.

We adapted the polarized T84 colonic epithelial cell model to study *Neisseria*-mucosa interactions. GC and MC traversed T84 monolayers in distinct time courses identical to those observed in organ culture studies. In contrast to other studies with MC and with pathogenic *Escherichia coli* and *Salmonella* (23, 24, 27-29), traversal of epithelial monolayers by GC and

MC was not accompanied by disruption of the epithelial barrier. In addition, we show that pili facilitate not only GC adhesion but invasion and traversal of T84 monolayers. These latter features have not been reported previously.

Materials & Methods

Bacterial strains

GC MS11A has two pilin expression loci, *pilE1* and *pilE2* (30). To create a nonreverting *pilE* mutant MS11C3, a strain deleted in *pilE2* (31) was used as the parent in our constructions. To delete *pilE1* a 2.1 kb *Bsu*36I fragment from pNG1711 (30) was replaced with a 1.2 kb erythromycin resistance (Erm^r) cassette from pAT110 (32). This plasmid was linearized and used to transform MS11C3 (*pilE2*). A nonpiliated, Erm^r transformant was isolated and named MS11-306. PCR and Southern blotting verified that *pilE1* was deleted (not shown). MS11-306 is nonpiliated, and MS11A, F62, and MC8013.6 are piliated as assessed by immunoblot with mAb 10H5.1.1 (14) and by colony morphology or electron microscopy. MS11A and MS11-306 are Opa^- by immunoblot with mAb 4B12, generously provided by M. Blake (not shown). MC 249 and 250 are uncharacterized with respect to piliation and Opa/Opc production. For infection experiments bacterial inocula were grown for 18 hours on GCB agar with nutritional supplements at 37° C, 5% CO_2 as described (33).

Tissue culture

T84 cells from the American Type Culture Collection were grown between passages 55 and 70 in a 1:1 mix of Dulbecco's Modified Eagle's Medium (D-MEM) and Ham's F12 (Whittaker)

with 5% heat-inactivated fetal calf serum (Hyclone or Gibco BRL). For infection experiments this culture medium was supplemented with human transferrin except as noted (Boeringer-Mannheim; >90% iron-saturated) at 5 μg ml^{-1}. Cells were harvested and plated (10^6 cells cm^{-2}) on Transwell™ filters (Costar #3415) coated with rat tail collagen I (Collaborative Biotech) as described (21, 26). These monolayers localized human transferrin receptor predominantly basolaterally and exhibited classical junctional complexes and dense microvilli when stained for filamentous actin (not shown). Monolayer formation was routinely assayed by measuring electrical resistance with the ECL device (Millipore). In our hands monolayers took 7-14 days to exceed 600 Ω cm^2.

Adhesion and invasion assays

Adhesion and invasion were scored by washing, lysing, and plating of the infected cells before or after killing of extracellular bacteria with gentamicin. Assays were done as before (8, 33), except that DMEM-F12 supplemented as above was used instead of D-MEM, and GC broth containing 0.5% (w/v) saponin was used to lyse the epithelial cells. To decouple invasion efficiency from differences in bacterial adherence, invasion indices were derived by dividing the number of internalized (gentamicin-resistant, GR) CFU by the number of adherent (cell-associated, CA) CFU.

Monolayer traversal experiments

Polarized T$_{84}$ monolayers with resistances >600 Ω cm^2 were rinsed with medium, placed into fresh 24-well plates containing 0.5 ml of the invasion medium described above, and 100 μl

of invasion medium was added to the apical well. Bacteria were suspended in DMEM-F12 at 10^8 colony-forming units (CFU) ml^{-1}, 10 μl of which was added to the apical medium. Infected cells were incubated at 37°C, 5% CO_2. At intervals filters were moved to new 24-well plates containing fresh medium, and the old medium was plated on GCB agar with nutritional supplements.

To quantitate MC flux across monolayers the above protocol was followed but at 12 hours postinfection and at 1 or 2 hour intervals thereafter the basal medium was changed to DMEM-F12 with 5% FBS. The newly removed basal medium was diluted and plated to enumerate CFUs. At the end of the assay (24 hours postinfection) each filter was assayed for transepithelial resistance to verify that barrier function was intact ($\geq$500 Ω cm ^{2}). The time of traversal varied slightly with the batch of fetal bovine serum used, so each set of experiments shown was performed using a single lot of serum.

Permeability assay

Monolayer permeability to [^{3}H]mannitol was determined as described (34). Briefly, basal medium was removed and plated for CFU, filters were placed into new 24-well plates containing fresh basal medium, and the apical medium was replaced with 100 μl of DMEM-F12 containing $\sim 10^4$ c.p.m. of [^{3}H]mannitol (New England Nuclear). Filters were incubated for 60 minutes. At the end of this interval medium was removed from both chambers and counted with a liquid scintillation detector. Data are expressed as described (34).

Fluorescence microscopy

Actin staining was performed by fixing filters for 30 minutes in Zamboni's fixative (PAPF; ref. 35) at room temperature followed by permeablization in PBS with 0.1% goat serum, 0.01% azide, and 0.02% saponin. Actin was stained for 1 hour with BODIPY 581/591-phalloidin (Molecular Probes) diluted 1:200 from methanol stock made as recommended. Filters were rinsed extensively and mounted in glycerol. Images were acquired with a Leica inverted laser-scanning confocal microscope.

Statistics

Invasion indices, and raw CFU counts log-transformed to normalize variances, were compared using Student's 2-tailed, paired *t*-test where appropriate. Results from the traversal timecourse experiments were evaluated using the Mann-Whitney *U*-test when sample sizes were equal or the log likelihood ratio (*G*) test when sample sizes were unequal. Calculations were set up as recommended (36) and done using StatView 4.0 software for the Macintosh (Abacus products, Berkeley, California).

Results

T84 cells support adhesion and invasion of pathogenic Neisseria

The barrier (gate) function of epithelial cells is critical to tissue function *in vivo* (22). *In vitro*, it is a stringent indicator of monolayer integrity and epithelial polarity, and can be assayed by impermeability to small tracers or by transmonolayer electrical resistance. Only a few human epithelial cell lines have been successfully grown as polarized monolayers with high electrical resistance. For example HEC-1-B, a human endometrial carcinoma line commonly used to

study GC and MC infection, polarized morphologically when grown on Transwell™ filters coated with collagen I or Matrigel™ but in our hands did not attain high electrical resistance (data not shown). The T84 cell line, derived from a human colon carcinoma, is known to form polarized monolayers with robust barrier function (21). Additionally, colorectal epithelia are sites of clinical GC infection. We therefore asked whether T84 cells are appropriate for studying neisserial-epithelial interactions.

Adherence and invasion indices of GC for T84 cells grown on plastic were compared to those for HEC-1-B cells. Results indicated that GC MS11A (piliated, Opa^-) adhered to and invaded T84 cells at the same frequency as HEC-1-B cells (8, 9, 33). For example, at 7 hours postinfection strain MS11A had an invasion index of $\sim 10^{-3}$ in both HEC-1-B (ref. 33) and T84 cells (Table 1). Further, MS11A-14.1, an *opaH* mutant previously shown to be reduced 4.3-fold in its ability to invade HEC-1-B cells (33), was similarly reduced (3 to 6 -fold) in its ability to invade T84 cells (Table 1). These results indicated that T84 cells would be a useful model for GC-epithelial cell interactions. Similar molecular mechanisms govern many GC and MC interactions with host cells. For example, type IV pili, PilC, and Opa are involved in adhesion and invasion (1, 2). Therefore the T84 model may be appropriate for studies of MC-host cell interactions as well.

MC and GC traverse T84 cells in species-specific timecourses identical to those established in organ culture studies

In FTOC and NPOC studies, GC and MC entered epithelial cells at the apical region, traversed to the basal region, and egressed into the stromal matrix (5, 6). To determine

whether *Neisseria* spp. can traverse polarized T84 cells, we infected monolayers plated on collagen-coated Transwell™ filters. Only monolayers exceeding 600 Ω cm^2 were used in this study; most were >1000 Ω cm^2. Polarized T84 monolayers were infected with three clinical isolates of MC, one clinical isolate and two laboratory strains of GC, and a nonpathogenic *Neisseria* strain (see Methods). At 12-hour intervals, the medium from the basal well of each filter unit was plated to detect colony forming units (CFUs). Each strain was assayed in several independent experiments with 3-12 filters per strain per assay.

Results indicate that *Neisseria* strains traversed T84 monolayers with different kinetics (Fig. 1A). Most MC-infected filters yielded CFUs in the basal medium at 12-24 hours postinfection, although several filters yielded MC in the basal medium by 12 hours post-infection. In contrast, most GC-infected filters yielded CFU in the basal compartment significantly later, at 36-48 hours post-infection ($p < 0.001$; log likelihood ratios test). The traversal times observed for MC and GC in these assays are strikingly similar to times reported for FTOC and NPOC experiments: >18 hours for MC, and ~ 40 hours for GC (37, 38).

At the times assayed, *N. perflava* was only rarely detected in the basal chamber (Fig. 1A). *Escherichia coli* HB101 traversed T84 monolayers and reached the basal compartment within 12 hours, however this was accompanied by a nearly complete loss of epithelial barrier function (data not shown), consistent with experiments using MDCK cells (23).

Assays to determine the number of bacteria traversing the monolayers were also performed. Monolayers were infected with MC for a defined period (12 hours for MC; 30 hours for GC) then moved into fresh, basal medium lacking serum and transferrin. The basal medium from the previous interval was plated for CFUs at 1 or 2 hour intervals. In medium

without supplements GC and MC grow slowly (data not shown). Thus, our estimates of basal well CFUs should closely approximate the actual number of bacteria exiting the monolayer.

Results from an experiment with MC 8013.6 are shown (Fig. 1B). By 20 hours postinfection, in many wells 10^2-10^4 CFU/hour were entering the basal compartment. At this time 1-2 x 10^7 CFU were present in the apical compartment. Similar results were obtained with GC MS11A at ~40 hours postinfection (not shown).

Barrier function of infected monolayers

To determine whether the passage of bacteria across T_{84} monolayers affects epithelial barrier function, the electrical resistances of GC, MC, and *N. perflava* -infected filters were measured (Table 2). Results indicate that traversal could occur without large decreases in barrier function. For example, at 48 hours postinfection GC MS11A-infected monolayers were decreased an average of 15% from starting values. At this time bacterial traversal had already occurred in over 90% of the monolayers assayed. Interestingly, the nonpiliated mutant GC MS11-306 always caused larger decreases in resistance than piliated MS11A, even though it traversed the monolayers significantly later (Table 2; see below). This result indicates that a modest decrease in barrier function is not sufficient to permit bacterial traversal. At 24 hours postinfection MC 8013.6 decreased resistances by an average of 33% from starting values. At this time, the values obtained for MC-infected monolayers with detectable basal CFU ranged from 540 to 1800 Ω cm^2. By 48 hours postinfection, resistances of MC-infected monolayers declined by ~70% from starting values. This decrease is not surprising, as the basal medium of

these filters usually contained over 10^8 CFUs/ml at this time, contained by-products of bacterial growth, and was acidic (pH ≤ 6.5) compared to the controls.

To confirm the electrical resistance data, infected monolayers were tested for permeability to a small soluble tracer, [^{3}H]mannitol. Individual filters were assayed for both bacterial transit and permeability. As a positive control for permeability, barrier function in some uninfected monolayers was abolished by treatment with EDTA (10 mM, 15 minutes) prior to the assay. Results (Fig. 2) show that the MC 8013.6-infected monolayers had somewhat increased permeability compared to uninfected monolayers, although they were still several fold less permeable than the EDTA-treated controls. Similar results (Fig. 2) were obtained for MC strains 249 and 250, and GC MS11A (not shown). Bacterial transit and epithelial permeability are not correlated: in many samples with CFUs present in the lower chamber the barrier function was identical to uninfected controls. In other samples where permeability had increased, no bacterial transit was detected. We conclude that as GC and MC traverse the T84 monolayer, a functional epithelial barrier is maintained.

Cytoarchitecture of infected monolayers

To further characterize the effects of bacterial traversal on epithelial integrity, the arrangement of actin filaments in infected T84 monolayers was examined. Polarized monolayers were infected with MC 8013.6 for 40 hours, fixed and stained. Confocal optical sections at and just beneath the apical surface revealed that both uninfected and infected cells were circumscribed by the apicolateral actin band associated with *adherens* and *occludens* junctional complexes (39). In the filters shown (Fig. 3), bacteria had traversed the monolayers

at the time of fixation. Similar results were obtained for GC MS11A-infected monolayers (data not shown). These results provide further evidence for the maintenance of cell polarity and barrier function during and after bacterial traversal. In contrast, the apicolateral actin band in *Salmonella*-infected MDCK II cells was disrupted by 1 hour postinfection (29).

Role of pili in GC interactions with T84 cells

As GC type IV pili are thought to be essential for virulence in human volunteers (40, 41), we compared interactions of piliated GC MS11A with its isogenic nonpiliated derivative MS11-306 using T84 cells. In infections of T84 cells plated on plastic (fig. 4A), pili increased GC adherence by ~20-fold. At 5, 7.5, and 10 hours post-infection 50-70% of MS11A were cell-associated (CA) compared with only 2-4% of MS11-306. Surprisingly, pili also promoted GC invasion of T84 cells. At each time point 1-2 logs more gentamicin-resistant (GR) MS11A were recovered than MS11-306 (mean difference, 42.4-fold; $p < 0.0001$, *t-test*)(Fig. 4A). This result cannot be explained solely by differences in adherence. If the invasion data are expressed as an index adjusted to compensate for adhesion (GR/CA), MS11A invaded T84 cells much more efficiently than MS11-306 (mean difference, 10.1-fold; $p = 0.0011$, *t*-test)(Fig. 4B). Piliation has been observed to promote MC and GC adhesion to cultured cells (fig. 3A; refs. 14, 15, 42, 43). This is the first report in which pili are shown to enhance GC invasion of cultured cells.

GC MS11A and MS11-306 were next compared for their ability to traverse polarized T84 monolayers (fig. 4C). In these assays piliated MS11A appeared in the basal compartment about 12 hours earlier than MS11-306 ($p < 0.001$; Mann-Whitney 2-tailed *U*-test). We emphasize that nonpiliated GC were at least as numerous in the apical chamber as piliated GC, yet they

crossed the monolayers later (fig. 4C; data not shown). Thus, epithelial traversal is not simply a function of bacterial growth in the apical chamber. Together, these experiments provide evidence that pilus-mediated interactions with host cells, and possibly cell invasion, promote the traversal of GC across polarized T84 monolayers.

Discussion

The experiments reported here establish polarized T84 colonic carcinoma cells as a model for studies of epithelial infection by the pathogenic *Neisseriae*. Nonpolarized T84 cells grown on plastic are similar to the widely used HEC-1-B endometrial carcinoma cell line (8, 9, 33) in their capacity to support GC adherence and invasion. The time courses of GC and MC transit across polarized monolayers are species specific, and are identical to results obtained previously in organ culture studies (5, 6, 37, 38).

Transepithelial migration by pathogenic *Neisseriae* does not abolish the barrier function of polarized T84 monolayers. This conclusion is based on three types of experiments. First, monolayers which have been traversed by GC or MC are relatively impermeable to small soluble tracers (fig. 2). Second, they have high electrical resistances, in excess of 500 Ω cm^2. Resistances of $\sim$200 Ω cm^2 are typical for polarized, uninfected monolayers of CaCo2 or MDCK II cells, and are generally considered diagnostic of physiological barrier function (21). Third, apicolateral junctional complexes remain intact as judged by microfilament staining (fig. 3). The small reductions in barrier function that were observed could be due to modulation of paracellular pathways, intracellular pathways, or both

(22). The present experiments do not discriminate among these possibilities, and should therefore provide conservative estimates of junctional integrity.

How can the maintenance of a substantial barrier in infected T84 monolayers be reconciled with organ culture data showing that GC and MC cause cytotoxicity and sloughing of some cells? It should be emphasized that in organ cultures, GC and MC selectively adhere to and invade nonciliated epithelial cells, but that most of the evident toxicity occurs in ciliated cells (5, 6, 37, 38). This cell type-specific cytotoxicity is thought to be triggered by soluble bacterial components, particularly peptidoglycan and lipooligosaccharide, and to be mediated by the release of tumor necrosis factor-_ (1). Many tissues infected by GC (*e.g.*, conjunctival, uterine and colorectal epithelia) do not contain ciliated cells, and severe epithelial effacement is not generally noted in clinical cases of *Neisseria* infection. T84 cells may therefore be viewed as a model for the population of nonciliated cells that are colonized *in vivo*.

It is possible that the cellular targets for neisserial toxins are localized basolaterally and that the robust barrier present in the T84 system may prevent soluble bacterial toxins in the apical chamber from reaching basolateral cell surfaces. This hypothesis is consistent with our observation that barrier function is largely retained even when very many bacteria are present in the apical chamber, but is diminished after large numbers of bacteria accumulate in the basal chamber.

Pili are important for colonization of the urogenital tract *in vivo* and facilitate GC interactions with *ex vivo* FTOC cells (1, 2, 5, 40, 41). Consistent with these results, our data show that pili strongly enhance GC adhesion, invasion, and traversal of polarized T84 monolayers. Likewise, piliated MC 8013 traverses T84 monolayers more efficiently than an

isogenic nonpiliated counterpart (X. Nassif, personal communication). These results are consistent with the idea that bacterial adhesion, invasion, or both are rate-limiting for epithelial traversal.

Similar to our results with T84 cells, Makino et al. (42) reported that with Chang conjunctival cells, piliated GC are more adherent than nonpiliated strains. In contrast, while piliation causes a 1 log *increase* in invasion efficiency with T84 cells, Makino et al. (42) report a 1-2 log *decrease* with Chang cells. When our GC strains are assayed with Chang cells, the invasion efficiency of the piliated strain is similarly decreased (A. Merz, unpublished data). Thus this difference is not due to the particular GC strains used. Moreover, with A431 cervical carcinoma cells an intermediate result is obtained: piliation strongly affects adhesion but not invasion efficiency (C.G. Arvidson unpublished). The effect of piliation on GC invasion efficiency therefore depends strongly on the cells used, and ranges from a 1-2 log inhibition (Chang) to little or no effect (A431) to a 1 log enhancement (T84). We suggest that host cell-specific differences in the effects of type IV pili on GC invasion reflect a previously unrecognized aspect of the *in vivo* GC-host interaction. This possibility is being investigated.

Several approaches have been used to study *Neisseria*-host interactions *in vitro*. Cell culture has been used to identify numerous bacterial components as adherence and invasion factors. Such systems, although allowing screening of large numbers of samples, are unsuited for studies that require cells with polar morphology. FTOC and NPOC studies established key events in GC and MC infections of mucosal epithelia. These *ex vivo* systems, composed of native tissue segments, made possible certain studies of neisserial infection such as ciliotoxicity, times of trafficking and cytokine release. However, such systems require technical

specialization and are limited by the increasing scarcity of appropriate tissue. The T84 system overcomes many of these difficulties. It will be interesting to use this model to dissect the cell biology of neisserial passage across epithelial barriers and to further elucidate the reciprocal roles of host and parasite in this important pathogenic process.

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Tables

Table 1. Adhesion and invasion of T84 cells grown on plastic by *Neisseria* strains, after a 7 hour infection. Invasion was measured with a gentamicin protection assay (see Methods). Mean and S.D. of three to six replicates from a representative experiment are shown.

	Cell-associated CFU (CA)	Gentamicin^r CFU (GR)	Invasion index (GR/CA)
GC MS11A	1.19(.48) x10^6	1.25(.87) x10^3	1.1 x10^{-3}
GC MS11A-14.1	1.33(.28) x10^6	5.53(1.2) x10^2	3.5 x10^{-4}
N. sicca	1.23(.18) x10^7	4.80(1.3) x10^2	3.9 x10^{-5}

Table 2. Changes in transepithelial electrical resistance of infected T84 monolayers. Values shown are means from 2-4 assays with 6-18 replicates per strain per assay. hpi, hours postinfection; ND, not done.

	24 hpi	48 hpi
GC MS11A	ND	-15%
GC MS11-306	ND	-54%
MC 8013.6	-33%	-70%
N. perflava	+11%	ND

Table 3. Bacterial strains used in these studies.

Strain		Reference
N. gonorrhoeae:		
MS11A	P^+, Op^-	Segal,*et al.*, 1985
MS11-306	Δ*pilE1*::Erm, Δ*pilE2*, Op^-	This manuscript
MS11A-14.1	P^+, Op^-,*opaH*::Cat	Waldbeser, et al., 1994
F62		Clark et al., 1987
N meningitidis:		
249, 250	Blood and cerebrospinal fliud isolates from a single patient; encapsulated; serogroup C	This manuscript
8013.6	P+; highly adhesive to cultured epithelial and endothelial cells; encapsulated; serogroup C	Nassif et al., 1993
Others		
N. perflava NRL-30015		
N. sicca NRL-30016		Dr. Joan Knapp, CDC

Figure Legends

Fig. 1. (A) Timecourse of traversal across T84 monolayers by different *Neisseria* strains. 10^6 CFU of each of the indicated strains were inoculated into the apical wells of polarized T84 monolayers. At 6, 12, or 24 hour intervals, the filters were moved to wells with fresh medium and the medium from the old wells was plated on supplemented GCB agar. Points show the percentage of wells for a given strain that had detectable CFU in the lower chamber during each interval. The numbers in parentheses after each strain designation show the number of monolayers assayed. (*) designates the time taken by MC to traverse NPOC epithelia (ref. 3). (**) designates the time taken by GC to traverse FTOC epithelia (ref. 4). (B) Flux of MC 8013.6 into the basal compartment. At 12 hours postinfection medium in the basal wells was replaced with unsupplemented DMEM-F12 to limit the rate of bacterial growth. At intervals filter units were moved to fresh basal medium and the old medium was plated. 12 additional monolayers were infected with *N. perflava* and did not yield CFU during any of the intervals. A representative experiment is shown.

Fig. 2. Permeability to [^{3}H]mannitol of individual infected monolayers. The far left column shows the mean ± S.D. (n=6) of control wells, either uninfected (UI) or uninfected but treated for 15 minutes with 10 mM EDTA. Both positive and negative controls were done at different times after infection with similar results. The other columns show results for single wells infected with the indicated strains for different amounts of time. Filled circles (•) represent samples in which bacteria had crossed the monolayer. Open circles (o) represent samples in which no bacteria were detected in the basal medium. One of two independent experiments.

Fig. 3. Actin staining at 40 hours postinfection in T84 cells inoculated with MC8013.6 (A) or uninfected control (B). Confocal optical sections of apicolateral regions of the monolayers were made with a 40x objective. The photos show an area of 73 x 73 μm. One of three experiments.

Fig. 4. Effects of pili on GC interactions with T84 monolayers. (A) Adherence and invasion of (•) MS11A and (o) MS11-306 assayed with T84 cells grown on plastic. Tot, total; CA, cell-associated; GR, gentamicin resistant. Each point shows mean + S.E. of determinations from 3-4 independent experiments, each done at the least in triplicate. (B) Invasion indices. Data from (A) are shown normalized against adherence (intracellular CFU/cell-associated CFU). (C) Timecourse of traversal across T84 monolayers by the same strains. Data are presented as in Fig. 1.

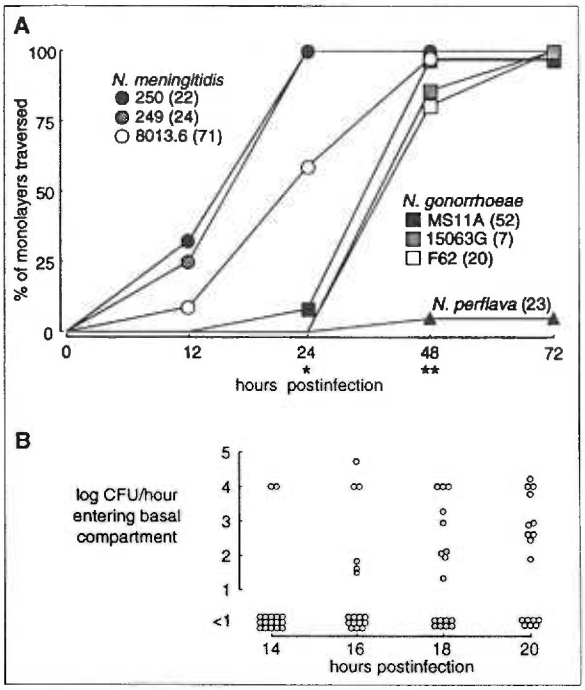

FIG. 1. Traversal across T_{84} monolayers by different *Neisseria* strains.

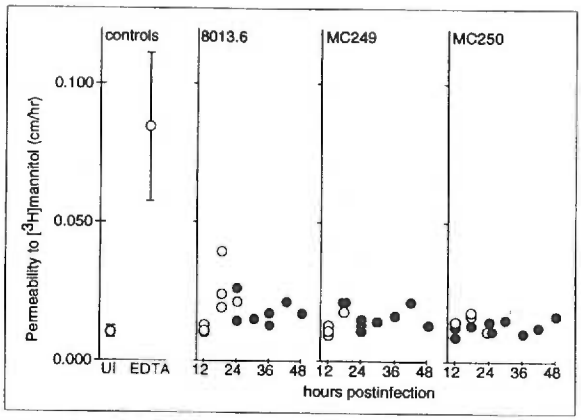

FIG. 2. Permeability to [^{3}H]mannitol of individual infected monolayers

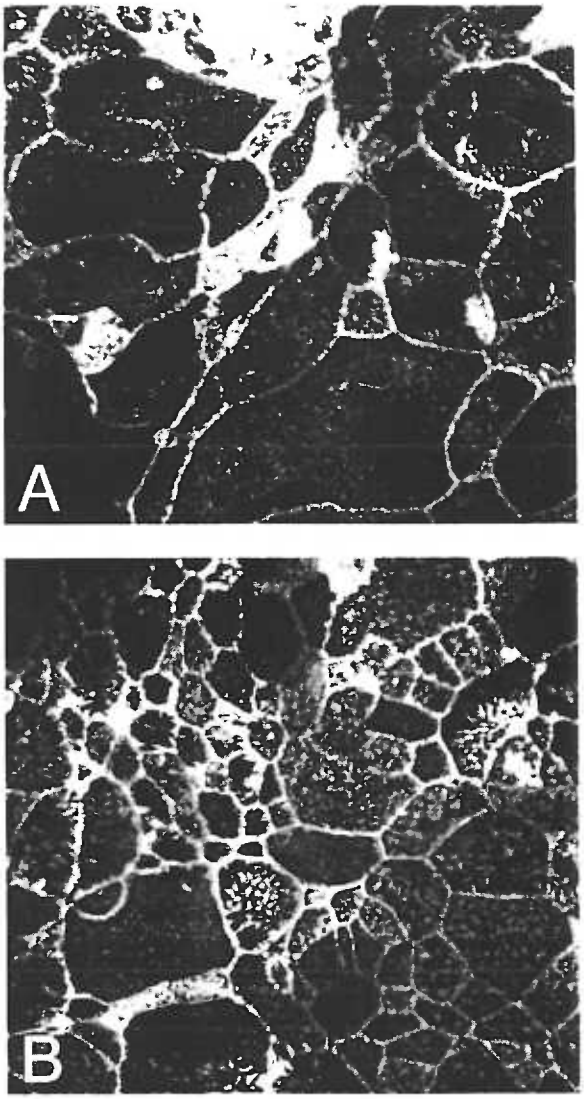

FIG. 3. Actin staining at 40 hr in control T_{84} cells (A) or in cells infected with MC8013.6 (B).

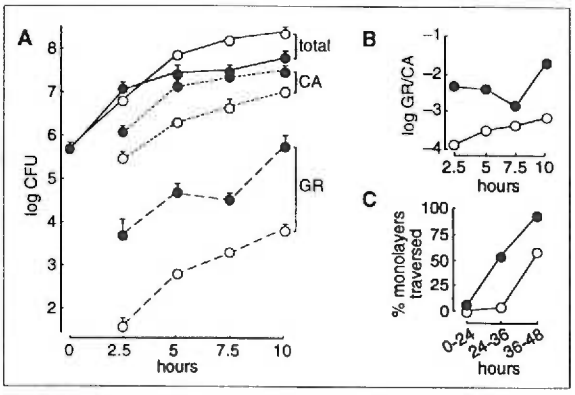

FIG. 4. Effects of pili on GC interactions with T_{84} monolayers.

Chapter3: MANUSCRIPT II

Attachment of piliated, Opa⁻ and Opc⁻ gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine phosphorylated proteins

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Summary

Attachment of piliated *Neisseria gonorrhoeae* or *Neisseria meningitidis* to A431, Chang, HEC-1-B, or polarized T84 cells triggers rearrangements of cortical microfilaments and the accumulation of phosphotyrosine-containing proteins at sites of bacterial contact. Actin stress fibers and the microtubule network remain unaltered in infected cells. The rearrangements reported here are triggered by piliated, Opa– and *opc*– strains and also by nonpiliated GC that produce the invasion-associated OpaA protein. Thus, neisserial adhesion *via* either of at least two different adhesins can trigger cortical rearrangements. In contrast, these rearrangements are not triggered by nonadherent GC or MC strains, by heat-killed or chloramphenicol-treated GC, or by *Escherichia coli* recombinants that adhere to cells *via* GC OpaA or OpaI fusion proteins, suggesting that additional neisserial components are involved. Immunoblot experiments did not detect consistent increases in the phosphorylation of specific proteins. Possible biological implications of these *Neisseria*-induced cortical rearrangements are discussed.

Introduction

It is now widely accepted that microbial attachment to host cells is not simply a passive process by which microorganisms initiate colonization. Rather, it can be a necessary prelude to further interactions between the newly associated partners. Attachment can potentiate or directly stimulate host cell responses including cytokine and chemokine release, toxicity, and microbial uptake (7-9, 15-17). Results from several systems show that adhesion to host cells can occur as an ordered process in which multiple microbial adhesins sequentially engage different receptors on the host cell surface (e.g., ref. 27). Such processes might be comparable to cell-cell

and cell-substrate adhesion cascades which occur during normal metazoan growth and immune function (6). If this is the case, similar events, including cytoskeletal rearrangements and the activation of host signal transducers, should occur soon after microbes adhere to host cells. In several cases this prediction appears to be valid. For example, attachment of enteropathogenic *Escherichia coli* to host cells results in accumulation of filamentous actin and tyrosine phosphorylation of host proteins (10, 27). Similar phenomena occur when bacteria producing the *Yersinia enterocolitica* Inv protein adhere to and invade epithelial cells *via* _1 integrin receptors and upon colonization of enterocytes by *Helicobacter pylori* (30, 37).

The pathogenic *Neisseria* species *N. gonorrhoeae* (gonococci, GC) and *N. meningitidis* (meningococci, MC) possess multiple components that promote bacterium-host cell interactions (19, 23). The type IV pilus may be an essential virulence factor in GC and appears to be absolutely required for adhesion to host cells by encapsulated MC (19). Opa and Opc constitute a large family of structurally related outer membrane proteins. Opa and Opc variants bind to different receptors on host cells, as well as to serum factors and neisserial lipooligsaccharides. In the absence of pili and capsule, Opa or Opc variants are sufficient to confer adhesive and, under some conditions, invasive phenotypes (19, 23). The presence of pili can either inhibit or promote invasion, depending on the cell line assayed (14, 18). Functional interactions between pili and Opa or Opc may occur but have not yet been thoroughly dissected.

Recently, Grassmé and coworkers observed microfilament rearrangements in epithelial cells after attachment of nonpiliated GC that express certain invasion-associated Opa proteins (4). In this report we show that the attachment of MC as well as GC to cultured epithelial cells results in localized rearrangements of the cortical actin cytoskeleton at the sites of bacterial

adhesion. We show that actin rearrangements occur not only with a very invasive GC strain (nonpiliated, OpaA+), as reported (4), but also with much less invasive GC and MC strains (Opa– and Opc–) that adhere to cells using type IV pili. We show that these strains trigger dramatic accumulations of phosphotyrosine-containing proteins at contact sites. Finally, we show that these cortical rearrangements occur not only at the dorsal cortex of cells cultured on coverslips, but at the apical surface of polarized epithelial cells grown on permeable supports. *Neisseria*-induced cortical rearrangements are therefore likely to occur *in vivo*, and are probably not an artifact of culture conditions. Our results indicate that extensive cortical rearrangements occur in several different cell types after GC or MC attachment *via* either pili or an invasion-associated Opa protein.

Results and Discussion

Infection experiments and bacterial strains. A431 and Chang cells were maintained in RPMI 1640 (Life Technologies) with 10% heat-inactivated fetal bovine serum at 37° C (Hyclone or Life Technologies), 5% CO2. HEC-1-B cells were grown as described (34). For microscopy experiments, cells were plated at about 15% confluence onto ethanol-washed, autoclaved glass coverslips (#1.5, Fisher Scientific) 48 hours prior to infection experiments. T84 cells were grown, polarized, and prepared for microscopy as described (18). GC and MC strains were grown overnight on GC agar with Kellog's supplements, and *E. coli* were grown on Luria-Bertani (LB) agar with carbenicillin added to 100μg/ml. For infection experiments, bacteria were resuspended and diluted into unsupplemented D-MEM (Life Technologies), then added to epithelial cells at a ratio of ~10 colony forming units per cell.

MC *opc* genotypes (Table 1) were evaluated by Southern hybridization of chromosomal DNA using a probe made from pEB501, a gift from M. Achtman. GC and MC Opa and Opc phenotypes (Table 1) were assayed by immunoblot with mAb 4B12, a gift from M. Blake, and mAb B306, a gift from M. Achtman. Opa+ or Opc+ strains were included in the blots as positive controls. GC MS11 AM13 and its derivatives (Table 1) contain deletions in the *pilC2* locus generated by transformation and allelic exchange with mutated *pilC2* DNA, in which a 544 bp *EcoRV* fragment is replaced by a chloramphenicol-resistance cassette. MS11 AM13 produces pilin as assayed by immunoblot with a mAb against the conserved SM1 epitope, and piliated colonies arise spontaeously at a frequency of ~10-3 to 10-4. Transformation of the piliated revertants with DNA encoding a disrupted *pilC1* locus gives rise exclusively to nonpiliated colonies. These experiments indicate that MS11 AM13 and its derivatives have intact *pilC1* loci that are "off" phase variants. For a description of analagous MC strains, see ref. 22.

Adhesion via pili triggers Opa and Opc-independant cortical actin rearrangements. A previous report indicated that nonpiliated GC which produce invasion-associated Opa proteins could elicit actin rearrangements. We wished to determine whether similar rearrangements are elicited when GC or MC attach to cells*via* type IV pili. Cells cultured on glass coverslips were infected with piliated GC or MC for various times, then fixed and stained for actin microfilaments using BODIPY 581/591 or rhodamine -phalloidin as described (18). Samples were rinsed in PBS and mounted in 50 mM Tris, pH 8.0 diluted 1:9 in glycerol, with *n*-propyl gallate (Sigma) added to a final concentration of 20 mg/ml (NPG-TBG) (12). Photographs were

made using a Nikon microscope and Kodak TMAX 400 film used at exposure index 800 or 1600).

In A431 (Fig. 1), HEC-1-B, and Chang cells (not shown), distinct microfilament accumulations were found at the periphery of adherent, piliated diplococci. Tightly packed organisms (microcolonies) triggered the assembly of a microfilament meshwork resembling structures previously observed beneath adherent enteropathogenic *E. coli*, *Y. enterocolitica*, *H. pylori*, or fibronectin-coated latex beads (5, 26, 27, 30, 37). Isolated diplococci were associated with actin accumulations less frequently than small microcolonies. These actin rearrangements appeared highly localized and were confined to the cell cortex. The overall distribution of actin stress fibers and perijunctional actin were unchanged in infected cells. Actin rearrangements were visible within 1 hour after infection in A431 cells. Similar experiments suggested that the microtubule network was not altered in infected A431 cells (11).

Bacterial components required for actin rearrangements. Several different GC, MC, and *E. coli* strains were assayed for the ability to cause actin rearrangements in A431 cells (Fig. 1; Table 1). Only strongly adherent strains triggered actin accumulation. Piliated, Opa- GC adhered strongly and caused microfilament accumulation, as did encapsulated, piliated, *opc*- MC 8013.6. Together, these results indicate that GC or MC attachment *via* type IV pili triggers actin rearrangements, that these rearrangements can occur in the absence of Opa or Opc, and that they are not inhibited by the presence of the MC polysialic acid capsule.

In contrast, nonpiliated (GC MS11 AM13, AM13.1R, or Cm23) or piliated but poorly adhesive (MC 8013 *pilC1*::Km) derivatives of the same strain did not trigger actin accumulation (Table 1). Additional experiments were done with piliated, Opa- GC that were heat-killed (50°

C, 30 minutes) or chloramphenicol-treated (10μg/ml, added 20 minutes prior to inoculation onto cells). Under each of these conditions large numbers of filamentous structures, presumed to be type IV pili, were visible by immunofluorescence using a rabbit antiserum raised against whole, piliated gonococci. At longer times after chloramphenicol treatment, fewer and fewer piliated GC were observed, but shed pili remained visible in the cultures, and appeared to form large, rigid bundles that ranged from 2–25 μm in length. These treatments therefore did not appear to cause pilus disassembly *per se*, but they did potently inhibit (> 100-fold) pilus-based GC attachment to A431 cells. Even when many treated bacteria were added to the cultures, only a few diplococci were observed associated with host cells, and microcolonies did not form. The few adherent diplococci were almost never found associated with actin accumulations Table 1). These experiments suggest that pilus-mediated adhesion requires ongoing bacterial protein synthesis, and indicate that actin rearrangements may only occur under conditions that promote efficient bacterial attachment.

Experiments on Opa-mediated adhesion buttress this interpretation. Consistent with previous results (4) we found that nonpiliated (*pilC1,* Δ *pilC2*), OpaA+ GC MS11 AM13.1 caused actin accumulation (Table 1). Both the Opa– parent and an Opa– revertant from this strain were poorly adhesive and failed to cause actin accumulation. When extremely large numbers of the Opa– revertant were used to infect A431 cells, a small fraction of the organisms that did adhere triggered microfilament accumulation (Table 1). These adherent GC may have been spontaneous Opa+ phase variants in the culture, which arise at a frequency of ~ 10-2 (19).

Further experiments indicated that adhesion *per se* was not sufficient to trigger actin accumulation (Figs. 1 b and c; Table 1). A431 cells were infected with *E. coli* recombinants that

expressed either OpaA or OpaI fusion proteins. Consistent with results reported for OpaA fusions (4), actin accumulations were found associated with fewer than 1% of adherent *E. coli* producing either the OpaA or OpaI fusion proteins (n=200 colonies in two independent experiments). Similarly, sheared GC pili either alone, or pili coated onto polystyrene beads under either aggregating or nonaggregating conditions failed to elicit phosphotyrosine or actin accumulation (unpublished data). Together, these results suggest that additional GC and MC components besides the primary Opa and pilus adhesins may be required to elicit cortical actin rearrangements in host cells.

Pilus-mediated actin accumulation occurs in the presence of cytochalasin D. To further characterize the actin-containing structures beneath piliated, Opa- GC, cells were treated with the microfilament-disrupting agent cytochalasin D (CCD) either before or during infection with GC MS11A (Fig. 2). The concentration of CCD used, 5 μg/ml, potently inhibits internalization of GC by epithelial cells (1, 2, 31). Dimethyl sulfoxide (DMSO), used to dissolve CCD, was added to control cells. Infected cells were then fixed and stained to detect microfilaments as before. A Leica laser scanning confocal microscope was used to obtain sets of optical sections, which were then assembled into "extended focus" projections using the brightest point algorithm in NIH Image software (v. 1.60 running under MacOS v. 7.5).

Infected cells treated with DMSO alone beginning at 20 minutes prior to infection were indistinguishable from untreated infected cells (Fig. 2a). Treatment with CCD during the final 20 minutes of a 3 hour infection (Fig. 2b) completely disrupted stress fibers and partially disrupted the actin band associated with the *adherens* junctional complex. However, the accumulation of microfilaments beneath adherent GC microcolonies was not diminished by

this treatment. Longer treatment with CCD, beginning 20 minutes prior to the 3 hour GC infection (Fig. 2c), resulted in the nearly complete disassembly of *adherens*-associated microfilaments and in the loss of cell-cell adhesion. This treatment also caused the movement of the bulk of polymeric actin to the perinuclear region. Surprisingly, microfilaments still accumulated beneath adherent GC. It should be noted that CCD treatment disrupts many actin-containing structures, but does not change the ratio of polymeric to monomeric actin in cultured cells (20). Together, these experiments suggested that the mechanism responsible for microfilament accretion at sites of pilus-mediated GC adhesion differs from the mechanisms responsible for the formation of stress fibers and *adherens*-associated microfilament bands.

GC and MC cause actin rearrangements at the apical surface of polarized epithelial cells. Cells cultured on glass or plastic acquire a relatively nonpolarized architecture, because they are unable to feed from their basolateral surfaces as they would *in vivo*. These conditions also promote the formation of cytoskeletal structures (stress fibers) not found in epithelial cells *in vivo*. Consequently, it was possible that the microfilament rearrangements observed above were artifacts arising from the culture conditions used. To address this possibility, infection experiments were performed using T84 cells grown on permeable supports. This system yields highly polarized epithelial sheets that have many characteristics of native epithelia, including a robust barrier function, and which support GC and MC adhesion, invasion, and epithelial traversal (18). In infections of polarized T84 cells, piliated GC or MC interacted initially with microvilli, which in many cases appeared elongated after bacterial contact (Figs. 3a-3a**). At later times after infection the infected cells were relatively denuded of microvilli, but many diplococci were still observed to associate with actin-containing structures. *Neisseria*-associated

microfilament structures at the cell surface became less punctate and more continuous at longer times after infection (compare Fig. 3 a** to Fig. 3b). These observations are consistent with the hypothesis that cortical actin rearrangements occur after GC or MC adhere to native epithelial cells, and are not morphological artifacts arising from standard culture conditions.

GC or MC attachment triggers accumulation of phosphotyrosine-containing proteins.

Phosphotyrosine-containing proteins concentrate at sites of cell-cell and cell-substrate attachment, in microvilli, and at sites of cytoskeletal remodeling (13). To determine whether tyrosine phosphorylation might be involved in the assembly of actin-containing structures beneath adherent GC, A431 cells were infected as before and indirect immunofluorescence microscopy was used to examine the distribution of phosphotyrosine. After infection, samples were fixed for 20 minutes at room temperature in picric acid paraformaldehyde (PAPF; ref. 17) then blocked and detergent extracted for 30 minutes in isotonic phosphate buffered saline (PBS) containing 3% normal goat serum (Gibco BRL), 10 μg/ml saponin (Aldrich), and 100 μg/ml sodium azide. Primary antibodies were diluted in blocking buffer, added to samples, and incubated overnight at 4° C in a moist chamber. Polyclonal rabbit sera 11507 and 8547 (22) react against whole GC and MC respectively and were diluted 1:1500. Phosphotyrosine was labeled using mAb 4G10 at 2.5 μg/ml, generously provided by B. Druker. After rinsing in PBS and reblocking, secondary antibodies (Texas Red-conjugated goat anti-rabbit and BODIPY FL-conjugated anti-mouse; Molecular Probes) were diluted 1:250 and added to samples for 1-2 hours at 25° C, then extensively rinsed prior to mounting as above. Staining and imaging controls included the substitution of primary mAb with isotype-matched antibodies of different specificity, incubation of uninfected cells with anti-GC and -MC sera,

and omission of primary antibodies to exclude artifacts caused by immunological cross-reactivity or optical bleed-through.

As expected, in uninfected cells phophotyrosine-containing proteins were concentrated at focal adhesions, at cell-cell junctions, and in microvilli (Fig 4a; ref. 13). The attachment of either GC MS11A or MC 8013.6 triggered the formation of dense foci of phosphotyrosine labeling in A431 and HEC-1-B cells (Fig. 4 b and c). Similar results were obtained using Chang cells and polarized T84 cells (data not shown). By 4 hours postinfection >90% of adherent GC MS11A were associated with phosphotyrosine accumulations in A431 cells (n = 200 colonies, 2 independent experiments). No antiphosphotyrosine labeling of GC or MC was observed in the absence of epithelial cells. Digital analysis of images obtained by conventional (nonconfocal) microscopy indicated that areas of A431 plasma membrane with attached GC were often >20-fold more intensely fluorescent than adjacent areas devoid of bacteria. As in the case of actin accumulation, phosphotyrosine accumulation was elicited by GC adhering *via* either type IV pili (Fig. 4 b) or via OpaA (Table 1). However, phosphotyrosine accumulations were seldom found beneath *E. coli* producing the OpaA fusion protein and virtually never found beneath *E. coli* producing the OpaI fusion protein (Table 1; Figs. 4 d and e). Thus, as in the case of actin rearrangements, bacterial adhesion appears to be necessary but not sufficient to trigger cortical phosphotyrosine accumulation.

Analysis of tyrosine phosphorylation after attachment of piliated GC or MC. The foci of phosphotyrosine accumulation at sites of bacterial adhesion could have formed through *de novo* phosphorylation of one or more proteins, through aggregation of existing phosphotyrosine-containing proteins, or both. To address whether *de novo* phosphorylation is

triggered by neisserial attachment, immunoblot experiments were performed using the mAb against phosphotyrosine (Fig. 5).

A431 cells were grown as above to ~85% confluence on 100 mm dishes, and left uninfected or inoculated with piliated, Opa- GC MS11A (m.o.i. 100). At 4 hours postinfection the cells were rinsed twice with ice-cold PBS and once with ice-cold TSA (10 mM Tris, pH 7.3, 140 mM NaCl, 0.025% w/v NaN3). Cells were lysed in 0.4 ml ice cold RIPA-V buffer (TSA with 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecylsulfate, 1 mM sodium vanadate, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. Lysates were scraped loose, the dishes were rinsed with an additional 0.4 ml of RIPA-V, and the combined lysates were cleared by centrifugation (16,000 x g, 20 minutes, 4° C). Protein contents of the lysates were determined with the BCA assay (Pierce). Samples were diluted into 1.2x SDS-PAGE sample buffer, boiled for 5 minutes, and loaded onto linear 5-20% gradient SDS-PAGE gels. Approximately 10% of the total protein in infected cells was bacterial. For fig. 4, 25 μg of protein was loaded onto each lane except for the GC only lane, which contained 3 μg. Proteins were electroblotted onto PVDF membranes (Boehringer-Mannheim), blocked with 3% w/v gelatin in TSAT (TSA with 0.02 % v/v Tween-20), and incubated with mAb 4G10 in TSAT with 1% BSA. mAb 4G10 was detected with alkaline phosphatase-conjugated goat anti-mouse followed by NBT-BCIP chromogen (Boehringer).

In these experiments no consistent changes were observed in the phosphorylation of particular proteins after infection with GC MS11A or MC 8013.6. Similar results were obtained using several different protocols for making cell lysates, with cells infected at different m.o.i. (multiplicity of infection; from 20-200), at times ranging from 30 minutes to 4.5 hours

postinfection, and using a different mAb against phosphotyrosine (data not shown). In addition, Triton X-100 -soluble and -insoluble fractions were immunoblotted to determine if any tyrosine-phosphorylated species migrated to the detergent-insoluble (i.e., cytoskeletal) fraction as a result of MC attachment. No consistent differences were observed between infected and uninfected cells in these experiments (data not shown). In two experiments, increases were noted in the intensity of Triton-X100-insoluble proteins migrating at 105-120 kDa, However, these differences were not consistent from one experiment to the next. In the blot shown (Fig. 4), 2 or 3 faint bands are visible in the lane with GC lysates alone; these bands may be due to weak cross reactivity of the secondary reagent with GC components. These bands appeared only after relatively long periods of chromogen development.

These experiments suggested that the phosphotyrosine-containing structures observed by microscopy might have arisen largely or completely through the aggregation of proteins that were already phosphorylated. It should be noted, however, that *de novo* phosphorylation may have contributed only a small fraction to the total cellular pool of phosphotyrosine residues, and therefore might have been difficult to detect in immunoblots of crude lysates.

Significance of GC and MC-induced cortical rearrangements. Our observations indicate that attachment of piliated GC or MC elicits substantial rearrangements in the host cell cortex directly beneath sites of bacterial contact. These rearrangements include the accumulation of filamentous actin and proteins containing phosphotyrosine, and occur in A431, HEC-1-B, Chang, and polarized T84 cells. In the case of polarized T84 cells the rearrangements seem to occur in at least two steps. First, GC or MC interact with microvilli, which appear to elongate upon contact with the bacteria. Subsequently, the T84 cell surface becomes relatively denuded

of microvilli, while adherent organisms remain associated with actin accumulations. Similar structural changes are observed by electron microscopy of GC and MC -infected T84 cells (A.M., R. Jones, P. Stenberg, and M.S., unpublished observations), in infected organ cultures (32, 33), and in urethral epithelial cells from GC-infected patients (25, 35). Together, these data strongly suggest that cortical rearrangements are a general feature of GC and MC adhesion to epithelial cells.

We also observe that dramatic accumulations of phosphotyrosine form beneath adherent GC and MC. In preliminary experiments, however, changes in the tyrosine phosphorylation of specific host cell proteins are not detected by immunoblot. These data are consistent with the interpretation that the phosphotyrosine accumulations beneath adherent GC and MC arise largely or entirely through the aggregation of proteins that are already phosphorylated, rather than through changes in tyrosine kinase or phosphatase activity. Alternatively, it is distinctly possible that such changes do occur but are difficult to detect against the background of total cellular phosphotyrosine in immunoblots of crude lysates.

Regardless of whether *de novo* tyrosine phosphorylation occurs after neisserial attachment, rearrangements at the cell cortex have the potential to alter or modulate the activity of several signal transduction systems in the host cell. Tyrosine phosphorylation appears to promote the assembly of modular signaling complexes that include components such as protein kinases and phosphatases, lipid kinases, and lipid hydrolases (24). It will be interesting to determine whether other signaling pathways are modulated following neisserial attachment.

In agreement with another report (4), we observe that nonpiliated GC which produce an invasion-associated Opa protein also trigger actin rearrangements in epithelial cells. In

addition, our results indicate that both actin rearrangements and phosphotyrosine accumulation are triggered by GC or MC that adhere using type IV pili but lack Opa or Opc. Therefore, neisserial attachment *via* either of at least two different adhesins elicits apparently similar changes in the host cell cortex. In control experiments, however, *E. coli* that adhered *via* either of two GC Opa variants elicited either very minor cortical rearrangements or none at all. Thus additional neisserial components may be required to trigger the formation of actin and phophsphotyrosine clusters.

Cortical rearrangements also occur after attachment of *Y. enterocolitica*, enteropathogenic *E. coli*, *H. pylori*, or fibronectin-coated beads. In these cases both microfilaments and tyrosine phosphorylated proteins accumulate at the contact region, while the microtubule distribution remains more or less unaltered (5, 26, 27, 30, 37). By contrast, *Salmonella typhimurium* causes the accumulation of microtubules as well as microfilaments at contact sites (3).

Invasion of epithelial cells by GC is strongly inhibited by cytochalasin D (1, 2, 31), implicating actin microfilaments in the uptake process. Indeed, it has been suggested that actin accumulation is an early signal for gonococcal invasion (4). However, our data show that both actin and phosphotyrosine accumulation can occur in the absence of invasion. Invasion of A431, Chang, or HEC-1-B cells by the Opa– strain MS11A is minimal until several hours postinfection, yet this strain triggers rapid (<1 hour postinfection; data not shown) changes in microfilament and phosphotyrosine distribution. Although actin rearrangement is probably necessary for GC invasion, it is evidently not sufficient. This conclusion is further supported by the observation that both piliated, Opa– GC (Fig. 1) and nonpiliated, OpaA+ GC (4)

trigger actin accumulation even in the presence of cytochalasin D at concentrations that potently inhibit invasion.

The host cell responses reported here might also be involved in triggering the inflammatory response. Attachment of enteropathogenic *E. coli* or *S. typhimurium* to the apical surface of polarized T84 epithelial monolayers elicits the basolateral release of neutrophil chemoattractants, including interleukin-8 (15, 16, 28). Similarly, very recent data indicate that the attachment of GC MS11 to HeLa cells elicits cytokine expression and activation of the host transcription factor NF_B (36). It is conceivable that such events could occur generally upon attachment of pathogenic microbes to apical epithelial surfaces, and that the phosphotyrosine accumulations observed upon attachment of GC and other microbes represent part of the signal which elicits this putative response.

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Figure Legends

FIG. 1. Staining of actin microfilaments in infected A431 cells at 4 hours postinfection. Cells were infected with (a, a*) MC8013.6 (piliated, *opc*–) or with *E. coli* producing OpaA (b, b*) or OpaI (c, c*) fusion proteins. Panels show phase contrast images (a-c), or the same cells stained for filamentous actin (a*-c*).

FIG. 2. Effects of the microfilament-disrupting agent CCD (5 μg/ml final) on GC-induced actin accumulation. A431 cells were infected with GC MS11A (piliated, Opa–) for 3 hours, then fixed and stained for actin polymer as for Fig. 1. (a) Infection carried out in the presence of dimethyl sulfoxide (DMSO) vehicle. (b) CCD present during the final 20 min. of infection. (c) CCD added 20 min. prior to infection. These images are "extended focus" projections. Each image is derived from 9 confocal optical sections taken at 1.0 μm intervals. Arrows indicate the locations of GC microcolonies as determined by phase contrast microscopy. Scale bar: 15 μm.

FIG. 3. Staining of microfilaments at the apical surface of polarized T84 cells infected with MC 8013.6 for 8 hours (a) or 40 hours (b). For the 8 hour time point three views of the same area are shown. In (a) an extended focus projection is shown. This image is derived from 9 optical sections taken at 0.5 μm intervals. In (a*) an enlarged area of the same projection is shown. In (a**) a single optical section from the stack used to compute (a) is shown. In this section, taken at the level of the cell surface, the outlines of adherent diplococci are visible. (b) shows the microfilament distribution at 40 hours postinfection. This is an extended focus projection of 3 optical sections taken at 0.5 μm intervals. Arrows point to regions of *Neisseria-*

induced microfilament rearrangements. Asterisks (*) in the figure indicate regions where rearrangements are not visible.

FIG. 4. Localization of phosphotyrosine at 4 hours postinfection. (**a, a***) Uninfected cells. Note that phosphotyrosine is concentrated at cell-cell junctions and at focal adhesions at the termini of actin stress fibers. (**b, b***) A431 cells infected with GC MS11A (piliated, Opa–) for 4 hours. In this low-magnification view, phosphotyrosine accumulations are visible beneath almost all adherent GC microcolonies. (**c, c***) HEC-1-B cells infected with MC 8013.6 (piliated, *opc*–). (**d, d*** and **e, e***) A431 cells infected with *E. coli* producing either OpaA (d) or OpaI (e) fusion proteins. Arrowheads in (d) indicate possible sites of phosphotyrosine accumulation beneath adherent bacteria. Such accumulations were present beneath $<5\%$ of *E. coli* that produced the OpaA fusion. The above images were aquired by conventional fluorescence microscopy except for (c) and (c*), which are "extended focus" projections of five confocal optical sections taken at 0.5 μm intervals.

FIG. 5. Immunoblot analysis of tyrosine phosphorylation. From left to right: uninfected A431 lysates (UI); GC MS11A lysates; uninfected A431 lysates mixed with lysates of GC MS11A; and A431 cells infected with GC MS11A for 4 hours.

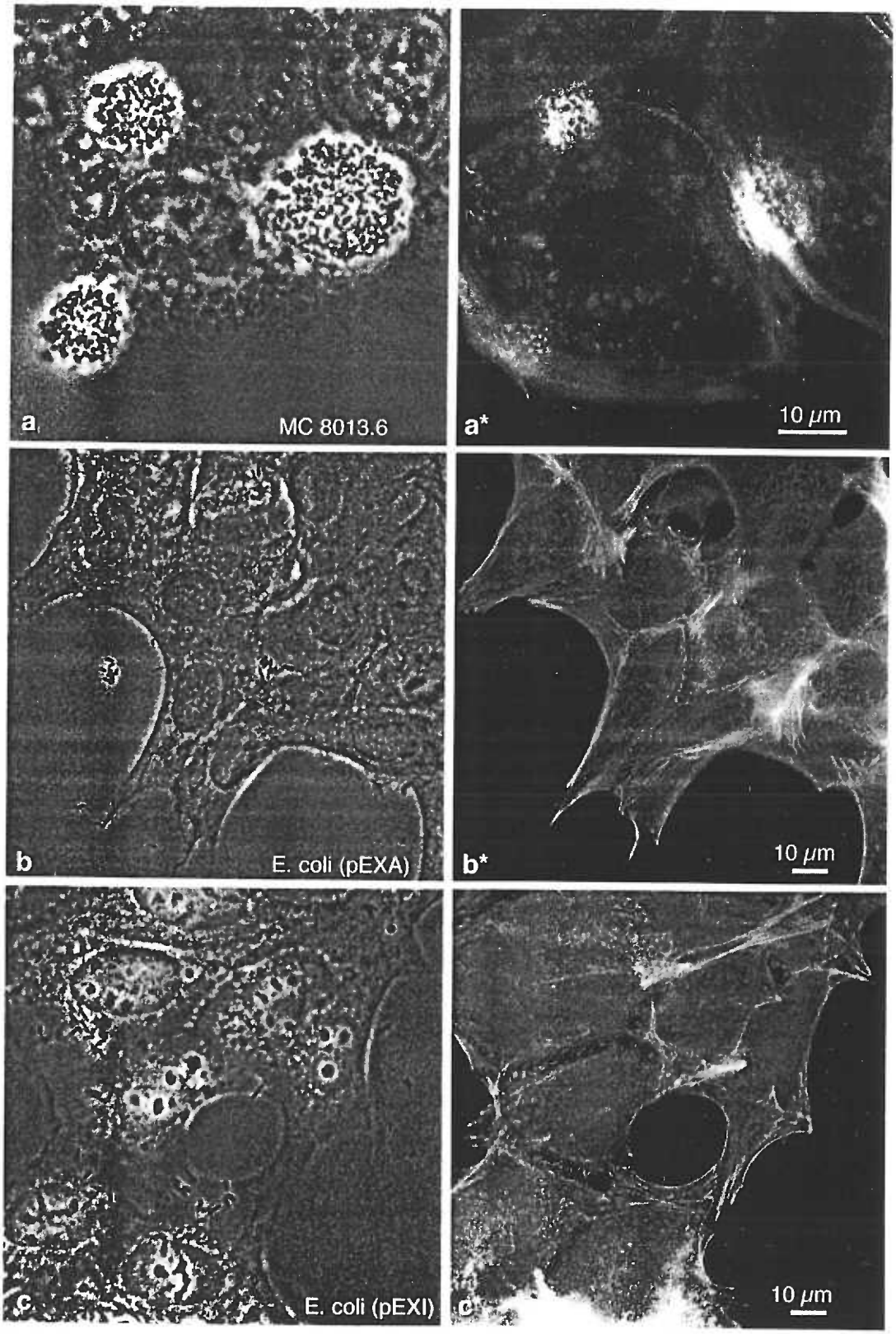

FIG. 1. Staining of actin microfilaments in infected A431 cells at 4 h postinfection.

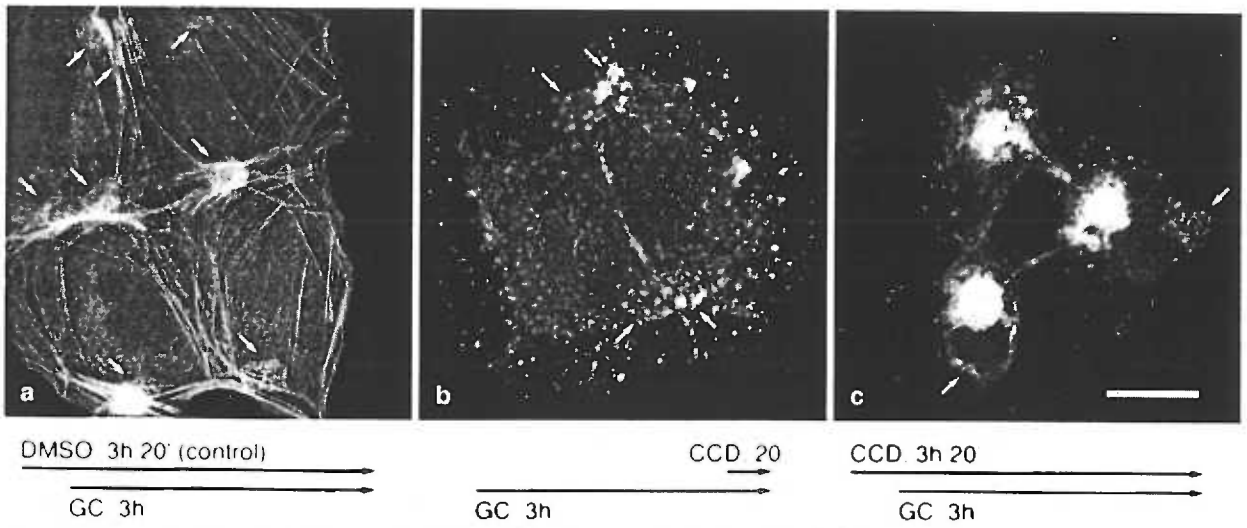

FIG. 2. Effects of the microfilament-disrupting agent CCD (5-μg/ml final concentration) on GC-induced actin accumulation.

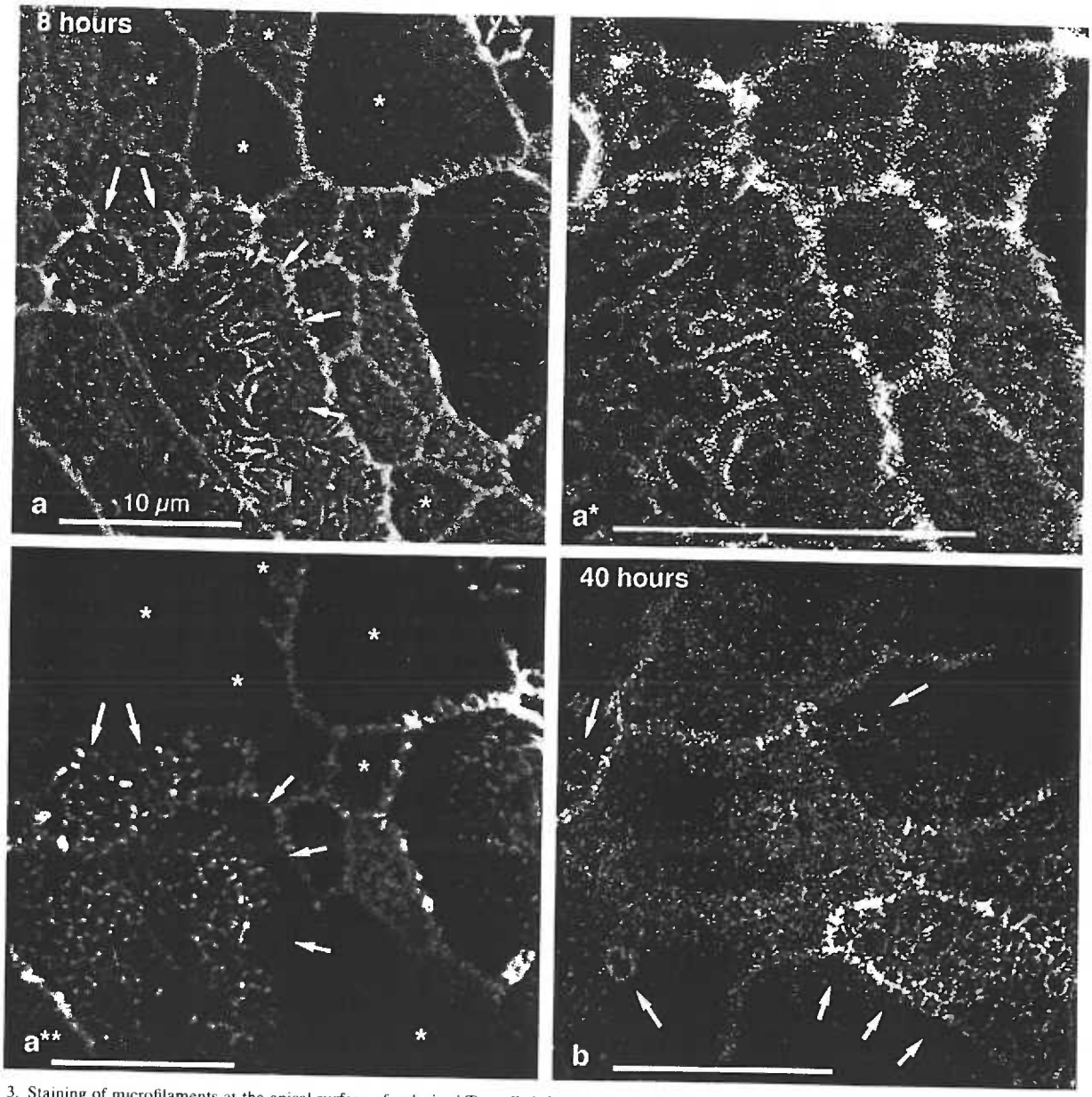

FIG. 3. Staining of microfilaments at the apical surface of polarized T_{84} cells infected with 8013.6 MC for 8 h (a) or 40 h (b).

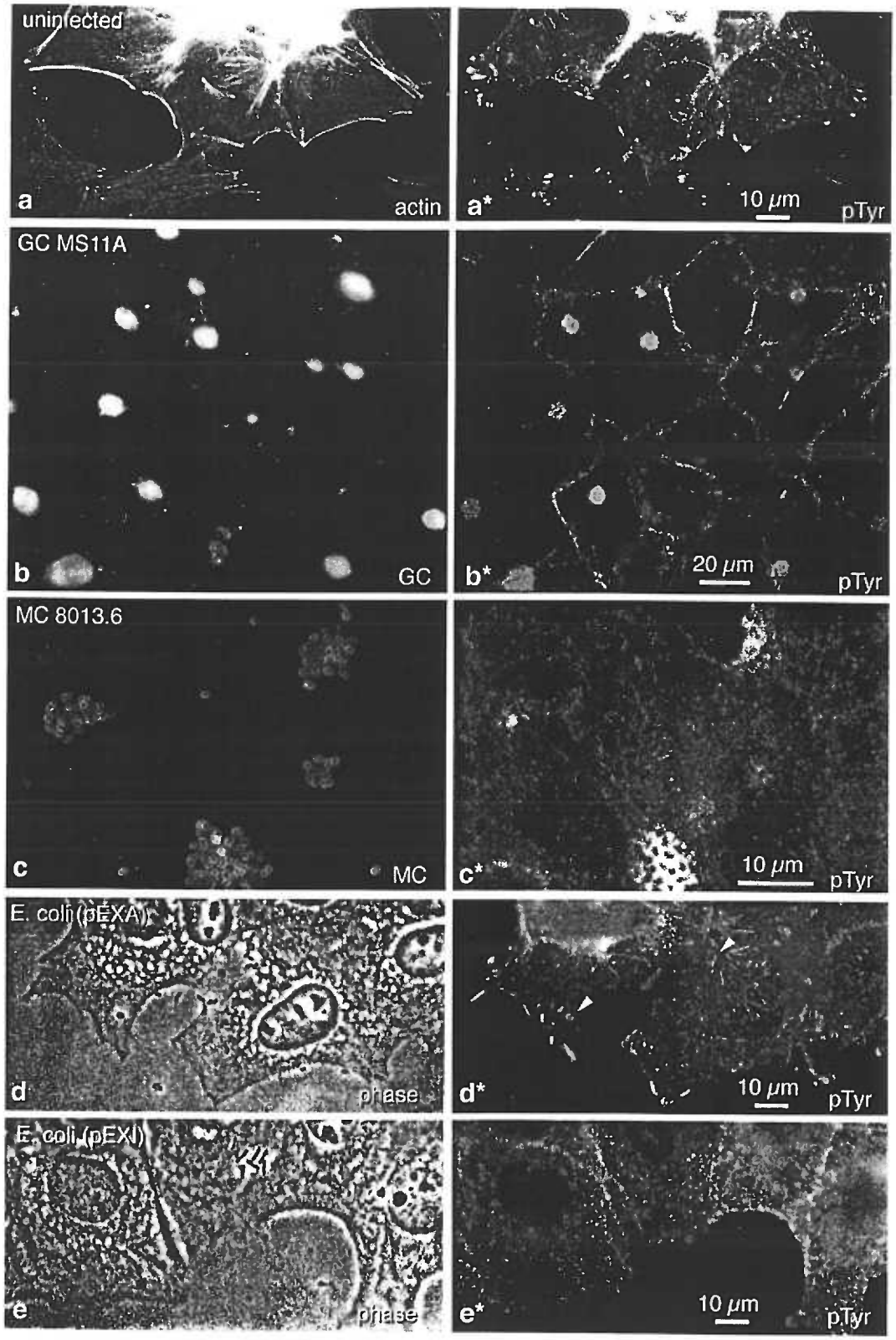

FIG. 4. Localization of phosphotyrosine (pTyr) at 4 h postinfection.

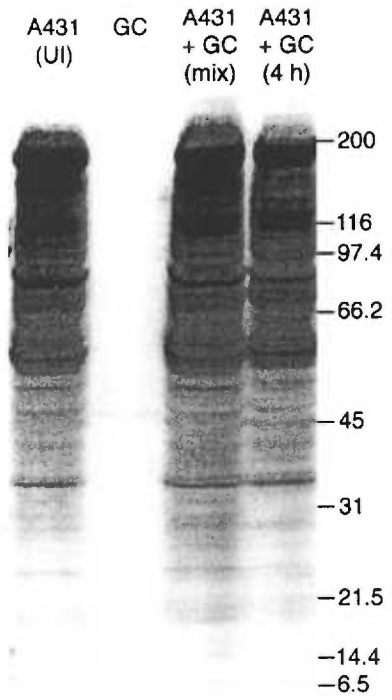

FIG. 5. Immunoblot analysis of tyrosine phosphorylation.

Chapter 4: MANUSCRIPT III

Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells

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Summary

The pathogenic *Neisseriae*, *N. meningitidis* and *N. gonorrhoeae*, initiate colonization by attaching to host cells using type IV pili. Subsequent adhesive interactions are mediated through the binding of other bacterial adhesins, in particular the Opa family of outer membrane proteins. Here it is shown that pilus-mediated adhesion to host cells by either meningococci or gonococci triggers the rapid, localized formation of dramatic cortical plaques in host epithelial cells. Cortical plaques are enriched in both components of the cortical cytoskeleton and a subset of integral membrane proteins. These include CD44v3, a heparan sulfate proteoglycan that may serve as an Opa receptor; EGFR, a receptor tyrosine kinase; CD44 and ICAM-1, adhesion molecules known to mediate inflammatory responses; f-actin; and ezrin, a cytoskeletal component that tethers membrane components to the actin cytoskeleton. Genetic analyses reveal that cortical plaque formation is highly adhesin-specific. Both *pilE* and *pilC* null mutants fail to induce cortical plaques, indicating that neisserial type IV pili are required for cortical plaque induction. Mutations in *pilT*, a gene required for pilus-mediated twitching motility, confer a partial defect in cortical plaque formation. In contrast to type IV pili, many other neisserial surface structures are not involved in cortical plaque induction, including Opa, Opc, glycolipid GgO_4 binding adhesins, polysialic acid capsule, or a particular lipooligosaccharide variant. Further, it is shown that type IV pili allow gonococci to overcome the inhibitory effect of heparin, a soluble receptor analog, on gonococcal invasion of Chang and A431 epithelial cells. These and other observations strongly suggest that type IV pili play an active role in initiating neisserial infection of the mucosal surface *in vivo*. The functions of type IV pili and other neisserial adhesins are discussed in the specific context of

the mucosal microenvironment, and a multistep model for neisserial colonization of mucosal epithelia is proposed.

Introduction

The closely related Gram-negative bacteria *Neisseria gonorrhoeae* (gonococci, GC) and *Neisseria meningitidis* (meningococci, MC) are causative agents of gonorrhea and meningitis (Meyer et al., 1994; Nassif and So, 1995). Both species initiate infection by colonizing mucosal epithelial cells, a process that has been studied intensively using organ and cell culture systems. Several adhesins have been identified in GC and MC, and receptors have been identified for a subset of these. Type IV pili are fibrous structures that mediate the initial adhesion of GC and MC to epithelial cells, and may be required for pathogenicity *in vivo* (Buchanan et al., 1977; Cannon et al., 1996; Kellogg et al., 1968; Kellogg et al., 1963; Nassif et al., 1993; Seifert et al., 1994; Swanson, 1973). GC pili are implicated in twitching motility, which requires both pilus assembly and the *pilT* locus (Wolfgang et al., 1998a; Wolfgang et al., 1998b). Neisserial pili exhibit high frequency phase (on/off) and antigenic (primary structure) variation (Seifert, 1996). Both antigenic variation at *pilE* and phase variation at *pilC* can result in the assembly of pili with altered binding properties (Meyer et al., 1994; Nassif and So, 1995; Seifert, 1996). Human CD46 (membrane cofactor protein, MCP) has been identified as a host receptor for neisserial type IV pili (Kallstrom et al., 1997).

The Opa outer membrane proteins are encoded by a family of unlinked genes that are independently phase-variable (Dehio et al., 1998a; Meyer et al., 1994; Nassif and So, 1995). This manuscript uses the unified Opa nomenclature recently suggested by Achtman et al. (Dehio et

al., 1998a; Malorny et al., 1998). Particular Opa variants confer the ability to invade certain epithelial cell lines. One set of invasion-associated Opas, exemplified by Opa30 from GC strain MS11, contains putative surface-exposed loops rich in basic amino acids. These Opa variants bind to a variety of polyanionic molecules, including heparan sulfate proteoglycans on the host cell surface, and to vitronectin. Moreover, soluble heparin, heparan sulfate, and DNA bind to these Opas and potently inhibit Opa-mediated adhesion and invasion by nonpiliated GC (Chen et al., 1995; Dehio et al., 1998b; Gomez-Duarte et al., 1997; Swanson, 1992a; Swanson, 1992b; Swanson, 1994; van Putten et al., 1997; van Putten and Paul, 1995). Many other Opa variants expressed by either GC or MC recently have been shown to bind receptors in the CD66 family, which includes biliary glycoprotein, carcinoembryonic antigen and nonspecific cross-reacting antigen (Bos et al., 1997; Chen and Gotschlich, 1996; Chen et al., 1997; Gray-Owen et al., 1997a; Gray-Owen et al., 1997b; Virji et al., 1996a; Virji et al., 1996b; Wang et al., 1998). GC, MC, or recombinant *Escherichia coli* expressing the appropriate Opa proteins can invade cells that express large amounts of CD66. There is also evidence for Opa binding to neisserial lipooligosaccharides (Blake et al., 1995) and to serum components (Dehio et al., 1998b; Duensing and Putten, 1998; Duensing and van Putten, 1997; Gomez-Duarte et al., 1997; Virji et al., 1994). Opc, a protein weakly related to Opa, is present in some MC strains. A similar protein appears to be encoded in the genome of GC strain FA1090. Opc permits invasion of endothelial cells by nonencapsulated MC and can bind human vitronectin (Virji et al., 1994). Other potential adhesins have been identified in GC and MC, including lipooligosaccharide (Porat et al., 1995a; Porat et al., 1995b) and multiple glycolipid-binding adhesins (Paruchuri et al., 1990). These and other components await further characterization.

In several cases bacteria appear to initiate adhesion cascades, ordered processes in which multiple adhesins sequentially engage different receptors on a single host cell (Hoepelman and Tuomanen, 1992; Hultgren et al., 1993; Isberg, 1991). These bacteria may exploit not only host cell receptors, but signalling pathways and dynamic functions normally used by host cells to establish adhesion to other cells and to substrates. For example, in both normal metazoan cell adhesion and in microbe–host cell interactions, attachment is often accompanied by formation in the host cell of structures containing cytoskeletal components, tyrosine-phosphorylated proteins, and signaling molecules. In addition, subsets of membrane associated proteins and glycolipids may become highly concentrated at these sites, processes known as clustering, capping, or plaque formation (Adams and Nelson, 1998; Singer, 1992; Yamada and Geiger, 1997). Cortical rearrangements can be a prerequisite for subsequent events, such as the initiation of T-cell receptor signaling (Dustin, 1998; Monks et al., 1998; Singer, 1992), the full activation of integrin-mediated signaling from focal adhesions (Yamada and Geiger, 1997), or fimH-mediated survival of *Escherichia coli* within macrophages (Baorto et al., 1997).

GC and MC interactions with host cells may also follow a multistep cell adhesion pathway in which initial attachment of the bacteria triggers host cell responses that facilitate subsequent adhesive interactions. Genetic studies indicate that GC and MC type IV pili inhibit bacterial invasion of some cell types but enhance invasion of others (Makino et al., 1991; Merz et al., 1996; Pujol et al., 1997; Virji et al., 1995). These results suggest that pili modulate the function of other neisserial adhesins in a manner that depends on the host cell.

Electron microscopy data are also consistent with a multistep adhesion process. GC or

MC initaially attach *via* pili, and form small aggregates or microcolonies. At this stage host microvilli often appear elongated and can be seen contacting the bacterial cell envelope. At later times the bacteria and host cell surfaces become tightly apposed, a stage referred to as 'close association'. At this stage the host and bacterial membranes are only a few nanometers apart, the host cell cortex immediately beneath the bacteria is amorphous and electron-dense, microvilli have largely disappeared from the host cell, and the bacteria adhere as individual organisms rather than as microcolonies (McGee et al., 1983; Pujol et al., 1997; Shaw and Falkow, 1988; Stephens and Farley, 1991; Ward and Watt, 1972).

More recent studies using molecular markers and fluorescence microscopy are consistent with the electron microscopy data. At early stages the bacteria attach as microcolonies, accompanied by microvillus elongation and rearrangements of cortical actin filaments (Giardina et al., 1998; Grassme et al., 1996; Merz and So, 1997; Pujol et al., 1997), as well as the accumulation of tyrosine-phosphorylated proteins at sites of bacterial attachment (Merz and So, 1997). Accumulations of actin and phosphotyrosine occur with piliated (P^+) Opa^- GC or MC, or with nonpiliated (P^-) $Opa30^+$ GC, and are thus potentiated by *Neisseriae* that adhere *via* at least two distinct adhesin-receptor combinations (Merz and So, 1997). Furthermore, recent reports indicate that GC adhering *via* different adhesins trigger the activation of different signal transduction systems in host cells (Grassme et al., 1997; Hauck et al., 1998; Kallstrom et al., 1998). At later times after infection, bacteria adhere as individual organisms that lack immunodetectable pili (Pujol et al., 1997), and microvilli disappear from the host cell surface (Merz and So, 1997).

In this report we present two sets of observations which support the hypothesis that

neisserial type IV pili are the initiators of a multistep adhesion cascade. First, we demonstrate that the attachment of P^+ GC or MC to epithelial cells triggers rapid rearrangements within the host cell cortex. These rearrangements result in the formation of plaques comprising both components of the cortical cytoskeleton and a specific subset of transmembrane glycoproteins, including receptors known to be involved in cell adhesion, signal transduction and the inflammatory response. Cortical plaque formation is shown to be adhesin-specific. Plaque formation occurs only in the presence of type IV pili and is partially dependant on the *pilT* locus, suggesting a role for pilus-mediated twitching motility. Plaque formation does not require several other neisserial surface structures including Opa. Furthermore, invasion assays indicate that P^+ GC are at least 100-fold more resistant than P^- GC to the effects of heparin, a potent inhibitor of Opa-mediated invasion of A431 and Chang epithelial cells. Together these results demonstrate that neisserial adhesion via type IV pili triggers specific rearrangements at the host cell surface which may have modulatory effects on subsequent *Neisseria*-host interactions.

Results

Piliated GC cause major rearrangements in the epithelial plasma membrane. In previous work, the attachment of GC and MC to cultured epithelial cells was shown to trigger the assembly of f-actin and phosphotyrosine-rich structures at the sites of bacterial attachment (Merz and So, 1997). These results raised the question of whether rearrangements elicited by GC and MC are confined to the region beneath the host cell plasma membrane, or whether changes also occur within the plasma membrane itself. To address this question, the subcellular localization of

several transmembrane glycoproteins was examined after GC and MC infection. A431, Chang, or HEC-1-B human epithelial cells were grown on coverslips, infected with P^+ GC or MC, fixed, and processed for indirect immunofluorescence microscopy as described in Experimental Procedures.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase. EGFR associates with the cortical cytoskeleton, is present at the medium-exposed surface of A431 cells grown on plastic or glass, and is a major phosphoprotein of A431 cells (den Hartigh et al., 1992; Landreth et al., 1985; van Bergen en Henegouwen et al., 1992). Indirect immunofluorescent staining of uninfected A431, HEC-1-B, and Chang epithelial cells demonstrated EGFR on microvilli, at the leading edge of cell protrusions, and at lateral cell-cell junctions, as previously reported (Lichtner and Schirrmacher, 1990; van Bergen en Henegouwen et al., 1992). The amount of EGFR observed at lateral cell-cell junctions correlated with the degree of culture confluence, but EGFR was observed on the medium-exposed surfaces of all three cell lines even in confluent cells. When epithelial cells were infected with MC8013.6 (P^+ Opa^- *opc*) or GC MS11A (P^+ Opa^-) dramatic clusters of EGFR were found associated with microcolonies of GC cells (Fig. 1a). Some EGFR clusters were visible within 1 hour postinfection, and by 4 hours postinfection, >90% of adherent GC microcolonies were associated with EGFR accumulations. The *Neisseria*-associated EGFR clusters were very similar in appearance to 'caps' formed in antibody-mediated crosslinking experiments (Khrebtukova et al., 1991; Singer, 1992). Identical results were obtained using either monoclonal or polyclonal antibodies against EGFR, and using A431, Chang, or HEC-1-B cells (see Experimental Procedures). These results are consistent with our previous work

demonstrating that phosphotyrosine-containing proteins are recruited to the sites of GC and MC attachment (Merz and So, 1997).

CD44 comprises a broadly expressed family of variant transmembrane proteins in mammalian cells that are synthesized from differentially spliced mRNA transcripts from a single genetic locus (Sherman et al., 1994). CD44 variants are extensively and differentially glycosylated, and have functions in cell-cell and cell-matrix adhesion, in the presentation of growth factors and chemokines, and in the induction of inflammation (Sherman et al., 1994). The localization of the entire population of CD44 molecules was examined using two different mAbs that recognize epitopes common to all CD44 variants (panCD44). In uninfected cells, panCD44 had a subcellular distribution similar to that of EGFR, with the highest concentrations at lateral cell-cell junctions and at cell protrusions, but with reactivity visible over the entire surface of most cells. Immunofluorescent staining of infected cells revealed dramatic concentrations of panCD44 associated with adherent microcolonies of GC MS11A or MC8013.6 (Figs. 1b and 2a). panCD44 accumulations were observed by 1 hour postinfection, and by 4 hours postinfection, $>90\%$ of adherent microcolonies were associated with CD44 accumulations. Identical results were obtained with A431, Chang, or HEC-1-B cells (see Experimental Procedures).

In both human keratinocytes and in the A431 cervical carcinoma cells used in the present experiments, a subset of CD44 molecules contains the variant 3 (v3) exon. In these variants the v3 domain of the mature protein is decorated with both heparan and chondroitin sulfate (Bennett et al., 1995; Jackson et al., 1995; Milstone et al., 1994). Multiple heparan sulfate proteoglycans have been shown to serve as receptors for a subset of Opa proteins including

MS11 Opa30, and to be recruited to the sites of GC attachment when Opa30 is produced (Chen et al., 1995; van Putten et al., 1997; van Putten and Paul, 1995). To determine whether CD44v3 is recruited by GC in A431 cells, indirect immunofluorescence was performed using a specific mAb directed against the v3 domain. The results (Fig. 1c) show that CD44v3 variants form clusters beneath adherent P^- $Opa30^+$ GC MS11 AM13.1, as would be predicted for a known heparan sulfate proteoglycan. However, clusters of CD44v3 were also observed beneath P^+ Opa^- GC MS11A. CD44v3 clusters were observed both beneath microcolonies and beneath linear structures that stain with anti-GC antiserum. These linear fluorescent structures are very likely to be type IV pili, because such structures are only rarely observed with *pilC* null mutants and are never observed with *pilE* null mutants. *pilC* and *pilE* null mutants have few or no pili when examined by transmission electron microscopy (Meyer et al., 1994; Nassif and So, 1995). The above results suggest that cortical rearrangements triggered by piliated GC result in the recruitment of at least one host molecule that subsequently interacts with a GC outer membrane adhesin.

ICAM-1, an adhesion molecule induced by inflammatory stimuli, is upregulated in cultured epithelial cells infected with *Salmonella typhimurium* (Huang et al., 1996). In cells infected for 4 hours with GC (P^+ Opa^-), ICAM-1 staining was visible in bright, punctate clusters beneath adherent GC microcolonies (Fig. 2b). The staining pattern of the ICAM-1 clusters was similar to that observed with panCD44 and EGFR, however the overall distribution of ICAM-1 appeared to be somewhat more punctate. Similar results were obtained using both A431 (not shown) and Chang cells (Fig. 2b).

Transferrin receptor (TfR) is the transmembrane glycoprotein receptor for the serum

protein transferrin. TfR cycles rapidly and continuously between the plasma membrane and early endosomes. In marked contrast to CD44, EGFR, and ICAM-1, little or no colocalization of TfR with adherent GC was observed (Fig. 3). Less than 20% of adherent P^+ Opa^- GC MS11A were associated with accumulations of TfR. Similar results were obtained with MC, and when cells were observed by conventional wide-field or confocal fluorescence microscopy. The absence of TfR clusters at the bacterial attachment sites indicates that the GC and MC-induced plaques comprise only a specific subset of the proteins in the epithelial membrane.

Together these experiments demonstrate that binding to host epithelial cells by P^+ Opa^- GC MS11A triggers the recruitment of specific proteins within the plasma membrane. GC-induced clusters of transmembrane panCD44 and EGFR were observed when infected cells were not treated with detergent or were fixed using a different protocol, indicating that the observed structures are not artifacts of the fixation or detergent treatments used (see Experimental Procedures). Furthermore, experiments performed with multiple cell lines yielded similar results, suggesting that the clustering of transmembrane proteins may be a general consequence of the attachment of piliated GC and MC. We refer to these clusters of proteins as cortical plaques because of their resemblance to cortical plaques that form during other eukaryotic cell adhesion events.

Neisseria-triggered membrane protein clustering correlates with cytoskeletal rearrangements and is not prevented by cytochalasin D. P^+ Opa^- GC and MC trigger actin accumulation at sites of bacterial attachment (Merz and So, 1997), and EGFR, CD44, and ICAM-1 have all been identified as proteins that interact with the cortical cytoskeleton. In contrast TfR, which does

not associate with adherent GC or MC, is not thought to associate with the cytoskeleton. Double-label immunofluorescence microscopy was therefore used to compare the distribution of f-actin with that of panCD44, ICAM-1, and EGFR in infected epithelial cells. At three hours post infection, dramatic clusters of panCD44, EGFR, and ICAM-1 were associated with adherent microcolonies of P^+ Opa^- GC MS11A (Figs. 2a-c). In each case, cortical f-actin accumulations were found to coincide with the membrane protein-enriched plaques at sites where GC had attached (Figs. 2a and c; data not shown).

The distribution of the cortical cytoskeleton component ezrin also was examined. Ezrin is expressed in epithelial cells and is concentrated at the cell cortex in microvilli and in other cell protrusions. It is thought to serve as a physical link between the f-actin cytoskeleton and many transmembrane and membrane-associated proteins, including panCD44 and ICAM-1, and binds to several signaling molecules (Bretscher et al., 1997). When infected cells were examined using indirect immunofluorescence, dramatic foci of ezrin staining were observed beneath adherent microcolonies of P^+ GC (Fig. 2b) or MC (not shown). In double-label immunofluorescence experiments ezrin closely colocalized with ICAM-1 (Fig. 3b), panCD44, and EGFR (not shown) at the sites of bacterial attachment. Thus at least two components of the cortical cytoskeleton, f-actin and ezrin, are concentrated in cortical clusters associated with adherent P^+ Opa^- GC, along with a subset of transmembrane glycoproteins.

EGFR has been shown to associate with the cortical cytoskeleton, possibly through direct binding to f-actin (den Hartigh et al., 1992; Landreth et al., 1985; van Bergen en Henegouwen et al., 1992). These data suggested that disruption of the actin cytoskeleton might alter the morphology of GC-triggered cortical plaques (Figs. 2c-e). We first tested whether

preformed clusters of EGFR were sensitive to the f-actin disrupting agent cytochalasin D (CCD). Treatment with 5 μg ml^{-1} CCD during the last 20 minutes of a 3 hour GC MS11A (P^+ Opa^-) infection resulted in the disassembly of all f-actin stress fibers (Fig. 2d; Merz and So, 1997). In addition, the distribution of EGFR was altered. In the control cells the distribution of EGFR was somewhat punctate due to the presence of microvilli (Fig. 2c), but after 20 minutes of CCD treatment, the distribution of EGFR was much more homogenous over the cell surface (Fig. 2d). However, although CCD treatment produced changes in the subcellular localization of both f-actin and EGFR, neither the preformed EGFR clusters nor the f-actin accumulations beneath adherent GC were disrupted by this treatment. Thus, CCD treatment did not result in the disassembly of preformed GC-triggered f-actin or EGFR clusters.

Experiments were also performed to determine whether CCD could inhibit the formation of new EGFR clusters, by beginning CCD treatment 20 minutes prior to a three hour infection (Fig. 2e). The results indicated that EGFR still accumulates beneath adherent GC, even though the actin cytoskeleton is massively perturbed by CCD treatment (fig. 2e; Merz and So, 1997). We conclude from these results that GC-triggered cortical rearrangements of EGFR are either f-actin-independent, or that the rearrangements involve a CCD-insensitive pool of f-actin (Morris and Tannenbaum, 1980). This contrasts with GC internalization by host cells, a process strongly inhibited by the same concentration of CCD (Shaw and Falkow, 1988).

Neisserial components involved in cortical plaque formation. To further define the bacterial requirements for cortical plaque formation, a panel of GC and MC strains was evaluated for

the ability to elicit cortical plaques (Table 1). To simplify scoring of the samples, EGFR and panCD44 were chosen as diagnostic markers for plaque formation. These molecules are abundant at the A431 cell surface and form especially bright, visible clusters under adherent GC MS11A or MC 8013.3. Cells were infected for 3-4 hours because at these times nearly all adherent microcolonies of GC MS11A or MC8013.6 were associated with concentrations of panCD44 and EGFR (see Figs. 1b and 4a for examples). The criteria used to score cortical plaque formation are described in Experimental Procedures.

P^+ neisserial strains that do not produce detectable Opa or Opc proteins (GC MS11A and FA1090, and MC8013.6) caused cortical plaque formation (Table 1). These results indicate that Opa and Opc are not necessary for cortical plaque formation.

The P^+ GC strain MS11A 24-1 carries defined mutations in at least two loci that encode glycolipid binding adhesins, and is defective in binding to GgO_4 (gangliotetraosyl ceramide) in an *in vitro* overlay assay (Paruchuri, 1988). This strain induced cortical plaques similar to those elicited by its isogenic wild type parent MS11A (Table 1). Therefore, the GgO_4-binding adhesins are not required for cortical plaque formation. Additional loci homologous to those mutated in MS11A 24-1 have been detected in GC and MC by DNA hybridization and in DNA sequence database analyses (Paruchuri, 1988; unpublished results). At present these other loci cannot be excluded as participants in the formation of cortical plaques.

P^+ strains with different lipooligosaccharide structures (e.g., GC MS11A and FA19), and both capsulated (MC 8013.6) and nonencapsulated *Neisseriae* (all GC strains used), all caused plaque formation (Table 1). These results suggest that particular carbohydrate structures

present on the neisserial surface are neither required for, nor inhibitory of, cortical plaque formation.

Several GC and MC strains carrying mutations in loci important for type IV pilus assembly and function were assayed. Null mutations in *pilE*, which encodes pilin, the major pilus subunit, result in a nonpiliated (P^-) phenotype. GC MS11-306 is P^- (_ *pilE1* _ *pilE2*) and Opa^-, and is 20 to 50-fold reduced in adherence to epithelial cells relative to its P^+ parent strain. At very high multiplicities of infection (MOI; 200) significant numbers of GC MS11-306 adhered to host cells, probably due to the presence of minor subpopulations of Opa^+ phase variants in the inocula (Makino et al., 1991). Clusters of EGFR or panCD44 were not associated with the adherent GC under these conditions, however (Table 1). These results suggest that even if GC adhere via nonpilus adhesins, *pilE* is required for cortical plaque formation.

Two *pilC* loci are present in GC and in MC. These loci are functionally redundant in pilus biosynthesis because strains with defects in either *pilC* locus are P^+, while double mutants have few or no pili. Because mutation of MC *pilC1*results in P^+ organisms with reduced adherence, a high MOI of 200 was used in assays with the MC *pilC1* mutant. Under these conditions, all four P^+ single mutants tested (GC *pilC1* and *pilC2*; MC *pilC1* and *pilC2*) triggered clustering of EGFR and panCD44 (Table 1). In contrast, the P^- strain GC MS11 AM13.1, with mutations in both *pilC* loci, did not cause panCD44 or EGFR clustering (Table 1). This strain produces Opa30 and its adherence efficiency is equivalent to its P^+ Opa^- parent MS11A (data not shown). Although this strain did cause clustering of CD44v3, a minor CD44 population decorated with heparan sulfate (fig. 1c) it did not cause clustering of EGFR or of

the entire (pan)CD44 population (Table 1). This result demonstrates that a P^- strain with mutations in both *pilC* loci cannot trigger cortical plaque formation, even in the presence of Opa30, an outer membrane adhesin that facilitates efficient attachment *via* heparan sulfate proteoglycans.

Taken together, these results show that two different types of mutations that abolish or strongly diminish piliation (*pilE* null or *pilC* null) also eliminate the ability to trigger cortical plaques, strongly suggesting that pili are required for plaque formation. These results also indicate that the individual *pilC* loci of GC and MC are interchangeable in the induction of plaque formation. Although the *pilC1* locus in MC is specifically required for efficient adhesion to epithelial cells, plaque formation was still triggered by the MC *pilC1* mutant, provided that it was present at a high enough MOI to compensate for its low adhesion efficiency. In addition, strains carrying different *pilE* alleles (MC 8013.6 and GC FA19, MS11A, and MS11N400) all triggered cortical plaque formation, indicating that plaque formation does not depend on the expression of a unique pilin antigenic variant.

The GC *pilT* locus is required for twitching motility but not for pilus assembly, autoaggregation, or attachment to host cells (Wolfgang et al., 1998a). Two different GC *pilT* mutants were assayed, one generated by a mTnEGNS insertion and one with a nonpolar in-frame deletion that eliminates a short stretch of residues within the putative ATP-binding cassette of PilT. Both mutants caused clustering of EGFR and panCD44 (Table 1 and Fig. 4b) as well as of ezrin and ICAM-1 (not shown), however the intensity of the clusters formed was strikingly reduced in comparison to the wild type parent strain GC MS11 N400 (Fig. 4a). We were unable to quantitate this reduction in staining intensity, however. Mutation of *pilT*

therefore confers a partial defect in cortical plaque formation.

Taken together our data strongly suggest that type IV pilus-mediated adherence is required for GC and MC to form cortical plaques, and indicate that twitching motility (or a related function) strongly enhances the efficiency of plaque formation. Our results also show that Opa, Opc, glycolipid GgO_4-binding adhesin, specific lipooligosaccharide structures, and capsule are not involved in neisserial triggering of cortical plaque formation in A431 cells.

Invasion of Chang and A431 cells by piliated GC is not inhibited by heparin. The observation that P^+ GC and MC cause relatively rapid rearrangements in the host cell cortex suggested that pili might influence the course of events later in colonization. In particular, the observation that P^+ GC recruited a heparan sulfate proteoglycan to the attachment site, even in the absence of Opa expression, suggested that pili might facilitate Opa-receptor interactions. To test this prediction the well-characterized Chang cell model for GC invasion was used (see Experimental Procedures).

Chang cells are invaded efficiently by nonpiliated GC MS11 that express Opa30 (Kupsch et al., 1993; Makino et al., 1991). When Chang cells are infected with P^- Opa^- GC, spontaneously arising $Opa30^+$ phase variants are highly enriched in the gentamicin-protected fraction. (Kupsch et al., 1993; Makino et al., 1991). These and other experiments indicate that even if the GC inoculum is predominantly (>95%) Opa^-, invasion of Chang cells is mediated mainly through Opa30. $Opa30^+$ GC bind to heparan sulfate proteoglycans present on the epithelial surface, and invasion by these organisms is potently inhibited by soluble polyanions such as heparin. To address whether similar mechanisms govern the interactions of P^+ and P^-

GC with Chang cells, isogenic P^+ and P^- GC were subjected to gentamicin protection assays in the presence of increasing concentrations of heparin.

As expected, the results indicate that with P^- Opa^- GC MS11-306, heparin inhibits entry into Chang cells by 50% at $\sim 1\ \mu g\ ml^{-1}$, and by 90% at $\sim 10\ \mu g\ ml^{-1}$ (Fig. 6a). In contrast, invasion by the isogenic piliated GC strain MS11A was undiminished when heparin was present at identical concentrations (Fig. 5a). Even at 100 μg/ml of heparin, invasion by the P^+ strain was reduced by less than 50%. Identical data were obtained using A431 cells (Fig. 5a). These experiments show that pili allow GC to overcome the inhibitory effects of heparin on GC invasion of Chang and A431 cells, over a 100-fold range of heparin concentrations.

Pili could influence the invasion process in two ways. In *trans*, P^+ GC could help P^- GC to invade host cells in the presence of heparin. In *cis*, pili could confer this phenotype only upon bacteria that produce them. To discriminate between these possibilities, P^-, $Opa30^+$ GC MS11 AM13.1 were assayed for invasion of Chang cells in the presence or absence of 100 μg/ml heparin, and in the presence or absence of piliated MS11 N400 (Fig. 5b). MS11 AM13.1 carries a selectable marker, allowing the invasion of this strain to be measured in the presence of the P^+ strain N400. N400 was used because it carries the *recA6* allele, which renders it transformation defective and eliminates the possibility that marker transfer might cause the P^+ strain to be confused with the P^- strain. In both the presence and absence of piliated organisms, heparin inhibited invasion by MS11 AM13.1 by $\sim$2.3 logs. Similar results were obtained using a predominantly Opa^- inoculum in place of MS11 AM13.1 (not shown). These results demonstrate that P^+ GC do not help P^- GC to invade Chang cells in either the presence or the absence of heparin.

Discussion

Neisserial type IV pili are complex organelles that mediate bacterial adhesion, autoaggregation, twitching motility and DNA transformation (Meyer et al., 1994; Nassif and So, 1995; Seifert, 1996). Recent experiments also suggest a role for neisserial pili in the invasion and traversal of epithelial or endothelial monolayers which may be distinct from their role in adhesion *per se* (Virji et al., 1995; Merz et al., 1996; Pujol et al., , 1997). The studies reported here reveal two new phenotypes conferred by neisserial type IV pili. First, adhesion of P^+ GC and MC results in the formation of clusters, or plaques, of proteins within and immediately subjacent to the epithelial cell surface. These cortical plaques contain known signal transducers as well as molecules involved in cell adhesion and in the induction of inflammation. Cortical plaque formation depends on the presence of type IV pili but not on Opa or Opc expression. Second, neisserial type IV pili neutralize the inhibitory effect of heparin on GC invasion of two epithelial cell lines, suggesting that pilus-mediated host cell responses may promote or stabilize interactions between other neisserial adhesins and their host receptors.

Cortical actin rearrangements occur in host cells following neisserial attachment via either type IV pili or via Opa (Giardina et al., 1998; Grassme et al., 1996; Merz and So, 1997; Pujol et al., 1997). In addition, phosphotyrosine-containing proteins aggregate beneath GC and MC after attachment mediated by either type IV pili or GC Opa30 (Merz and So, 1997). In the present report these observations are extended to show that the cortical rearrangements triggered by P^+ GC and MC occur within as well as beneath the plasma membrane, and that at least some of these rearrangements occur only in the presence of type IV pili.

Experiments using genetically defined bacterial mutants establish a strong correlation

between pilus-mediated neisserial adhesion and large-scale cortical rearrangements (e.g., of EGFR and panCD44; Table 1). In contrast to the apparent requirement for type IV pili, many well-studied neisserial surface structures do not influence cortical plaque formation (Table 1). These results are consistent with the interpretation that pili are required for cortical plaque formation. However, we have been unable to induce plaque formation using pili or purified pilus preparations, either in solution or coated onto latex beads (data not shown). Therefore, we cannot formally exclude the possibility that plaque formation involves some uncharacterized function that is coupled to type IV pilus biogenesis. Consistent with this interpretation, P^+ GC strains carrying either of two defined *pilT* mutations were partially defective in cortical plaque formation (Table 1 and Fig. 4a). Although this result rules out an absolute requirement for twitching motility in cortical plaque formation, it indicates that a PilT-dependant function strongly increases the efficiency of plaque formation.

The mechanism by which PilT acts is not known, but circumstantial evidence implies that twitching motility in *Pseudomonas* and GC occurs through PilT-dependant pilus retraction (Bradley, 1974; Wolfgang et al., 1998b). Interestingly, biophysical experiments have directly demonstrated that mechanical tension increases the efficiency of adhesion plaque formation in mammalian cells (Wang et al., 1993). It is tempting to speculate that the observed PilT-dependant enhancement of cortical plaque formation occurs by analogous mechanisms, and is a consequence of mechanical tension generated by PilT-dependant pilus retraction. A different possibility is that neisserial type IV pili participate in the export of secreted effectors, as recently suggested for the type IV pili of *Pseudomonas aeruginosa* (Lu et al., 1997).

Neisseria-induced cortical rearrangements may modify interactions among host cells.

CD44 and ICAM-1 are involved the in migration and activation of immune cells, and CD44 and EGFR are involved in cellular responses to growth factors. Ezrin, another component of pilus-induced cortical plaques, binds to both CD44 and ICAM-1, and can also bind to and modulate the function of additional signaling molecules (Bretscher et al., 1997). By modifying the adhesive properties of epithelial surfaces, the signaling properties of epithelial cells, or both, the cortical plaques triggered by piliated *Neisseriae* may directly influence the course of the mucosal inflammatory response. Recent data are consistent with this interpretation. Experimental infection of male volunteers with a large dose (10^6 CFU) of a nonreverting P^- GC mutant results in colonization of the male urethra, but fails to elicit the typical inflammatory response normally associated with infection at this site (Cannon et al., 1996). Interestingly, enteropathogenic *E. coli* carrying a mutation in *bfpF*, a structural and functional homolog of *pilT*, could colonize human volunteers but was 200-fold attenuated in virulence (Bieber et al., 1998).

The observation that GC and MC attachment triggers adhesin-specific clustering of various host cell membrane proteins and cytoskeletal components is reminiscent of other eukaryotic cell adhesion processes, in which the ligation and/or crosslinking of different plasma membrane proteins results in the assembly of specific cortical structures (Monks et al., 1998; Singer, 1992; Wang et al., 1993; Yamada and Geiger, 1997). This similarity suggests that neisserial colonization of host cells may involve a similar type of multistep adhesion cascade, in which pilus-mediated attachment 'primes' the host cell surface to facilitate subsequent adhesin-receptor interactions. An observation that apparently contradicts this hypothesis is that P^- Opa^+ GC efficiently adhere to and invade certain epithelial cell lines. However, the *in vitro*

assays generally used to measure adhesion and invasion do not mimic certain aspects of attachment *in vivo*. Organisms colonizing mucosal epithelia *in vivo* are immersed in mucosal fluids. In this milieu they are surrounded by high concentrations of nucleic acids, lipids, and soluble glycoproteins including both proteoglycans and CD66 family members (Asseo et al., 1986; Briese et al., 1989; Fujii et al., 1988; Krause, 1980; Mack and Sherman, 1991; Moghissi, 1973; Nanbu et al., 1988; Neutra and Forstner, 1987; Tabak, 1995; Widdicombe, 1995). In addition, the female reproductive tract is periodically washed with menstrual blood and tissue debris. Many components present in such mixtures bind to Opas, and in a few cases these have been demonstrated to inhibit Opa-mediated adhesion or invasion (Swanson, 1992a; Swanson, 1992b; Swanson, 1994; van Putten et al., 1997).

Because numerous such components are present at high concentrations, they would be expected to compete with, and therefore inhibit, Opa-mediated binding to receptors on host cells (van Putten et al., 1997). Indeed, a major function attributed to mucus is inhibition of microbial adhesion by soluble decoys that compete with or block adhesin–receptor interactions (Mack and Sherman, 1991; Neutra and Forstner, 1987; Tabak, 1995; Widdicombe, 1995). For example, low concentrations (1-10 μg/ml) of DNA or heparin substantially inhibit adhesion or invasion by P^- GC (Chen et al., 1995; Swanson, 1992b; van Putten et al., 1997; van Putten and Paul, 1995). At the far higher concentrations of competitors present in mucus, discrimination between *bona fide* host cell receptors and soluble decoys might pose an insurmountable obstacle to adhesion or invasion mediated solely by Opa or other outer membrane adhesins. Moreover, carbohydrate structures present on GC and MC surfaces *in vivo*, including capsule and sialylated liopooligosaccharide, are known to interfere with the

function of outer membrane adhesins (van Putten et al., 1995; Virji et al., 1993; Virji et al., 1995).

We propose that pilus-mediated adhesion and the subsequent Opa-independent cell surface rearrangements documented here allow GC or MC to establish secondary adhesin–receptor interactions in the 'difficult' conditions that predominate *in vivo*. A simplified model of such a cascade is depicted in Fig. 6. In the presence of a single outer membrane adhesin such as Opa30, adhesion and invasion in tissue culture medium are efficient processes (Fig. 6a). But when decoys are present they bind to the adhesin, blocking interaction with receptors on the cell surface (Fig. 6b). Adhesion *via* a primary adhesin such as pili partially overcomes this blockade, by bringing the bacterium close to the host cell surface and raising the local concentration of receptors for the outer membrane adhesin (Fig. 6c). Moreover, if pilus-mediated attachment initiates a signal that triggers clustering of host receptors for the outer membrane adhesin, the local receptor concentration is further increased (Fig. 6d). In combination, pilus-mediated bacterial proximity and receptor clustering could increase the effective receptor concentration by several orders of magnitude. This is expected to have at least two consequences.

First, increased local receptor concentration should allow the bacterium to more effectively discriminate between host cell receptors and soluble decoys (Wickham et al., 1995). Such an effect is difficult to measure empirically, but is consistent with our data showing that pilus expression allows GC to overcome the inhibitory effect of heparin on invasion of Chang and A431 cells, and that P^+ Opa^- GC trigger clustering of at least one heparan sulfate proteoglycan (Fig. 1c).

Second, increased local receptor concentration should allow bacterial adhesins to bind more avidly to host cell receptors. Increased avidity could (a) impart greater resistance to detachment by shear stresses, such as occur in the male urethra during urination; and (b) allow low-affinity adhesin-receptor pairs to bind under conditions where they otherwise would not. Data supporting the latter prediction were recently presented. Kallstrom et al. (1998) reported that neisserial pili trigger an intracellular [Ca^{2+}] signal in ME-180 epithelial cells, and that agents which elevate intracellular [Ca^{2+}] permit ME-180 cells to bind *Neisseriae* even in the absence of Opa, Opc, or pilus expression. In addition, receptor clustering might drive the coupling of bacterial adhesins with their host receptors despite the presence of anti-adhesins such as MC capsule or sialylated lipopolysaccharide (van Putten et al., 1995; Virji et al., 1993; Virji et al., 1995).

A key prediction of our model is that additional host receptors for gonococcal adhesins, such as CD46, CD66, and asialoglycoprotein, will also be enriched in pilus-induced cortical plaques. Experiments are underway to test this prediction. Both empirical studies (Adams and Nelson, 1998; Yamada and Geiger, 1997) and theoretical analyses (Ward et al., 1994; Ward et al., 1995) show that receptor clustering and plaque formation are critical aspects of mammalian cell-cell and cell-substrate adhesion. Receptor clustering and plaque formation may also prove to be fundamental features of microbe-host interactions.

Experimental Procedures

Cell lines, bacterial strains, and infections. A431 and Chang cells were maintained in RPMI 1640 (Life Technologies) with 10% heat-inactivated fetal bovine serum at 37° C (Hyclone or Life Technologies) and 5% CO_2. HEC-1-B cells were grown as described (Waldbeser et al., 1994). For microscopy experiments, cells were usually plated at about 15% confluence onto ethanol-washed, autoclaved glass coverslips (#1.5, Fisher Scientific) 48 hours prior to infection experiments. GC and MC strains (Table 1) were grown overnight on GC agar with Kellog's supplements, and *E. coli* (Table 1) were grown on Luria-Bertani (LB) agar with carbenicillin added to 100μg/ml. Piliation and Opa phenotypes were monitored by colony morphology and with western blots. For infection experiments, bacteria were resuspended and diluted into unsupplemented DMEM (Life Technologies), then added to epithelial cells at a ratio of ~10 colony forming units per cell or as specified. Treatment with the microfilament-disrupting agent cytochalasin D (Sigma) was done either before or during infection with GC MS11A as described. Dimethyl sulfoxide (DMSO), used to dissolve CCD, was added to control cells (Merz and So, 1997).

Gentamicin protection assays. Gentamicin protection assays were done as described (Waldbeser et al., 1994) except that serum was omitted during infections, heparin (~6000 MW; Sigma) was added to the concentrations specified, and GC broth containing 0.5% saponin was used to lyse the epithelial cells. After gentamicin treatment, wash supernatents were monitored for viable counts to ensure that killing of extracellular organisms was complete. After gentamicin

treatment, wash supernatents were monitored for viable counts to ensure that killing of extracellular organisms was complete.

Antibodies and sera. rabbit sera 11507 and 8547 (Merz and So, 1997; Nassif et al., 1994) react against whole GC and MC respectively and were diluted 1:1500. mAb 4B12 recognizes a conserved epitope on Opa and was a gift from Milan Blake, North American Vaccine. Human EGFR was detected using mAb EGF(R)-(528) diluted 1:500, and affinity purified pAb EGF(R)-(1005) diluted 1:500, both from Santa Cruz Biotechnology (Santa Cruz, California). panCD44 was detected using mAbs H4C4 and Hermes-1. H4C4 and Hermes-1 supernatants were diluted 1:100 and were obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development). Human CD44v3 was detected using mAb 3C5 at 10 μg/ml. 3C5 was obtained from R&D Systems (Minneapolis, Minnesota). mAb OKT9 reacts against the human transferrin receptor (TfR). OKT9 supernatants were diluted 1:40. mAb P2A4 recognizes human ICAM-1 and was obtained from the Developmental Studies Hybridoma Bank. P2A4 supernatants were diluted 1:100. Rabbit antiserum B22 recognizes human ezrin, and was a kind gift from Anthony Bretscher. B22 was used at 1:500. Affinity-purified secondary goat antisera against rabbit or mouse immunoglobulins were conjugated to either BODIPY FL or to Texas Red X, and BODIPY 581/591-phalloidin, were obtained from Molecular Probes, Eugene, Oregon. These fluorescent reagents were diluted 1:500.

Fluorescence staining. After infection, samples were fixed for 20 minutes at room temperature in picric acid paraformaldehyde (PAPF; (Zamboni and Martino, 1967)) then blocked and detergent extracted for 30 minutes in isotonic phosphate buffered saline (PBS) containing 3% (v/v) normal goat serum (Gibco BRL), 0.02% (w/v) saponin (Aldrich), and 0.02% (w/v) NaN_3. Primary antibodies were diluted as specified above in blocking buffer, added to samples, and incubated overnight at 4° C in a moist chamber. After rinsing in PBS and reblocking, secondary antibodies and BODIPY 581/591 phalloidin (Molecular Probes) were diluted 1:250 and added to samples for 1-2 hours at 25° C. Samples were extensively rinsed in PBS prior to mounting in 50 mM Tris, pH 8.0 diluted 1:9 in glycerol, with *n*-propyl gallate (Sigma) added to a final concentration of 20 mg/ml (NPG-TBG) (Longin et al., 1993). Staining and imaging controls included the substitution of primary mAb with isotype-matched antibodies of different specificity, incubation of uninfected cells with anti-GC and -MC sera, and omission of primary antibodies to exclude artifacts caused by immunological cross-reactivity or optical bleed-through.

Microscopy. Photographs were made using a Nikon Microphot FX microscope and Kodak TMAX 400 Professional film used at exposure index 800 or 1600 and developed in XTOL (Kodak). A Leica laser scanning confocal microscope equipped with 40X and 63X Plan Apochromat objectives was used to obtain sets of optical sections. For some of the images shown, sets of optical sections were assembled into "extended focus" projections using the brightest point algorithm in NIH Image software (v. 1.62 running under MacOS v. 8.1). Color

composites, in which two fluorescence channels were merged, were made in Adobe Photoshop v. 4.0. For evaluation of cortical plaque staining, coverslips were examined on the Nikon microscope using both epifluorescence and phase contrast optics. Representative fields are depicted in figures 1b and 4. At least 50 colonies per treatment were examined (except with strains that were completely nonadherent, e.g., MC8013 *pilE*::Km) in at least two independent experiments performed on different days. Scoring of protein clustering (Table 1) was conservative, and was performed by examining a field of cells at 400X or 600X magnification and noting the positions of membrane protein clusters. Only then were phase and/or fluorescence used to determine the position of adherent GC. Colonies that were associated with the patches of fluorescent staining identified in the first step were scored as positive, and colonies not associated with distinct patches of staining were scored as negative. Isolated organisms were seldom found associated with clusters of membrane proteins, regardless of the bacterial phenotype. For this reason, only colonies of 10 or more bacteria bacteria were scored.

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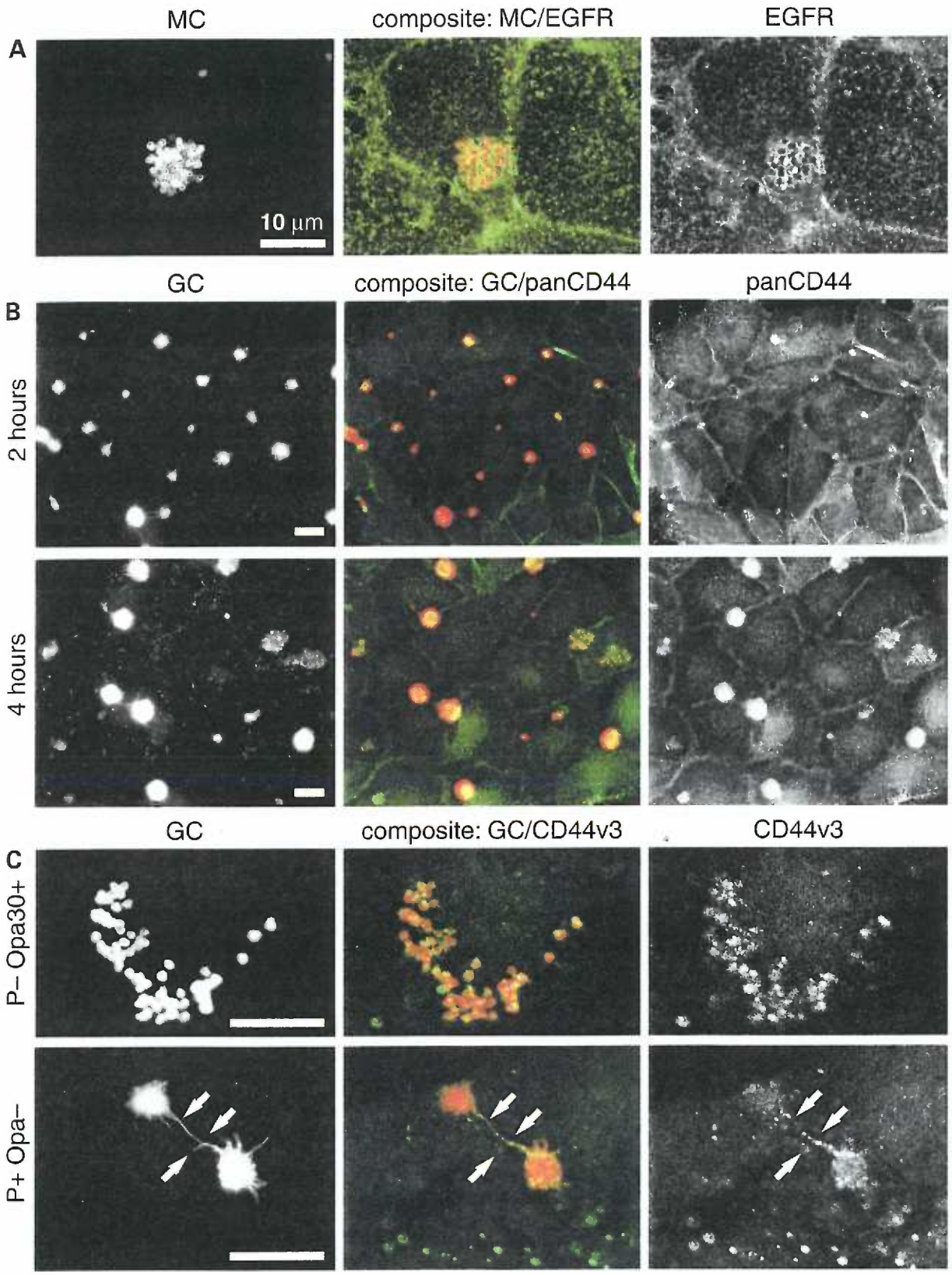
TABLE 1.

Ability of *Neisseria* strains to trigger epithelial membrane protein clustering in A431 cells. panCD44 and EGFR clustering were scored as "+" if at least 70% of adherent microcolonies were associated with clusters, and as "-" if fewer than 30% of adherent microcolonies were associated with clusters. The GC *pilT* mutants are scored as "(+)" because the panCD44 and EGFR clusters associated with these strains appeared less intense than with the parent strain (see Fig. 5). Samples were fixed and stained at 3-4 hours postinfection and a positive control (usually GC MS11A) was included in every experiment. All experiments were done at least two times on different days.

Strain	Genotype and/or phenotype	Clustering: panCD44	EGFR	Strain source or reference
N. gonorrhoeae:				
15063G	P^+ Opa^+	+	+	(Waldbeser et al., 1994)
FA1090	P^+ Opa^-	+	+	
MS11A	P^+ Opa^-	+	+	(Segal et al., 1986)
MS11A (+ chloramphenicol)	P^+ Opa^- (nonadherent)	-	-	
MS11A (heat killed)	P^+ Opa^- (nonadherent)	-	-	
MS11A (+ 50 μg/ml heparin)	P^+ Opa^-	+	+	
MS11-306	P^- (Δ*pilE1* Δ*pilE2*) Opa^- (low adherence, MOI 200)	-	-	(Merz et al., 1996)
MS11 AM1	P^+ (Δ*pilC1*::Erm) Opa^-	+	+	(Merz and So, 1997)
MS11 AM12	P^+ (Δ*pilC2*::Cat) Opa^-	+	+	(Merz and So, 1997)
MS11 AM13	P^- ($PilC^-$) Opa^- (low adherence)	-	-	(Merz and So, 1997)
MS11 AM13.1	P^- ($PilC^-$) $Opa30^+$	-	-	(Merz and So, 1997)
MS11 AM13.1R	P^- ($PilC^-$) Opa^- (low adherence)	-	-	(Merz and So, 1997)
N400	MS11 VD300 (*recA6*) P^+ Opa^-	+	+	(Wolfgang et al., 1998a)
GT103	N400 P^+ (*pilT*::mTnErm) Opa^-	(+)	(+)	(Wolfgang et al., 1998a)
GT102	N400 P^+ ($pilT_{\Delta QSL\ 318\text{-}320}$) Opa^-	(+)	(+)	(Wolfgang et al., 1998a)
N. meningitidis:				
8013.6	P^+ Opa^- *opc* (serogroup C $capsule^+$)	+	+	(Nassif et al., 1993)
8013 *pilE*::Km	P^-Opa^- (nonadherent)	-	-	(Nassif et al., 1993)
8013 *pilC1*::Km	P^+ Opa^- (low adherence, MOI 200)	+	+	(Nassif et al., 1994)
8013 *pilC2*::Km	P^+ Opa^-	+	+	(Nassif et al., 1994)


A
MC
composite: MC/EGFR
EGFR
10 µm
B
GC
composite: GC/panCD44
panCD44
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C
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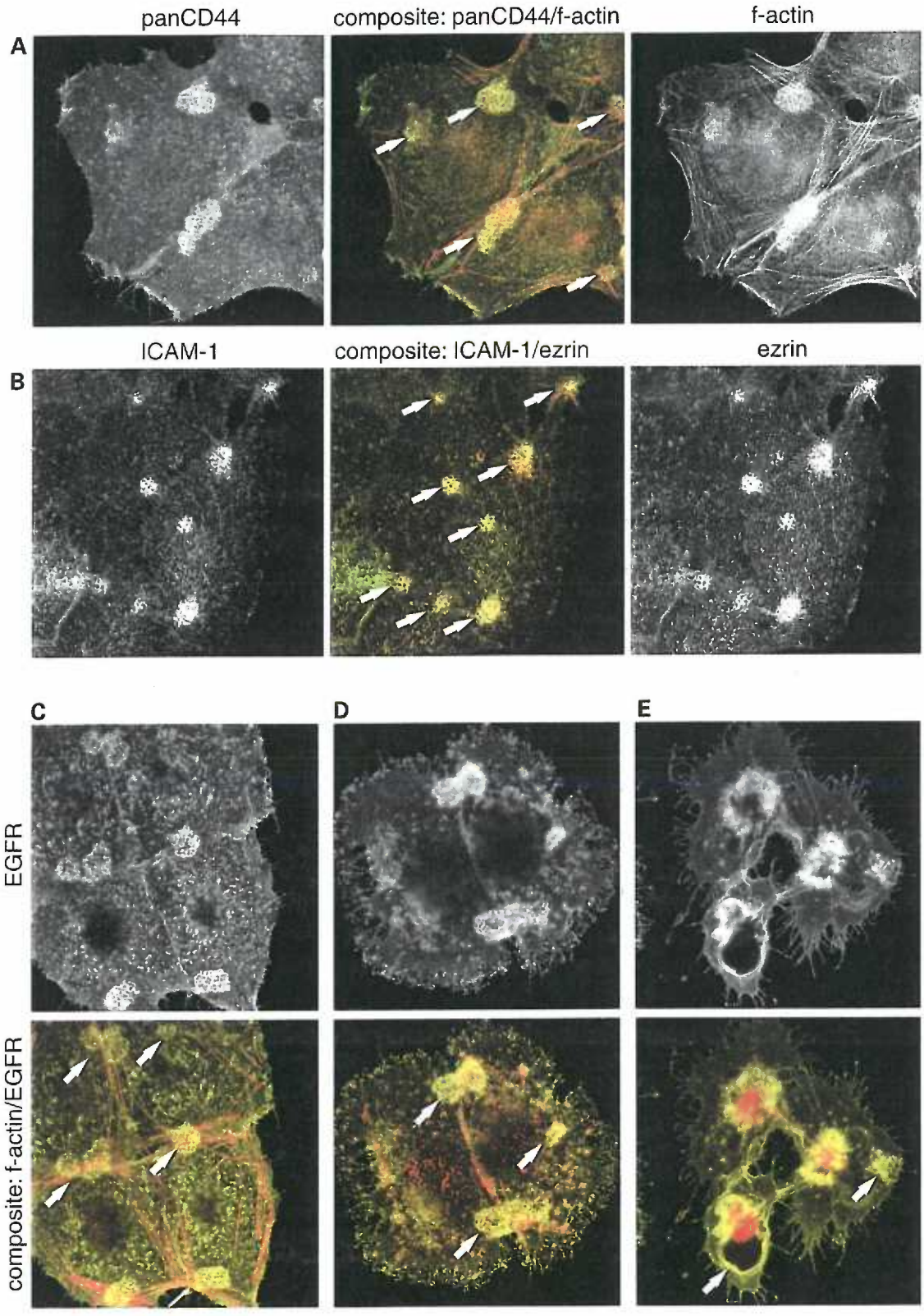
CD44v3
P– Opa30+
P+ Opa–


panCD44
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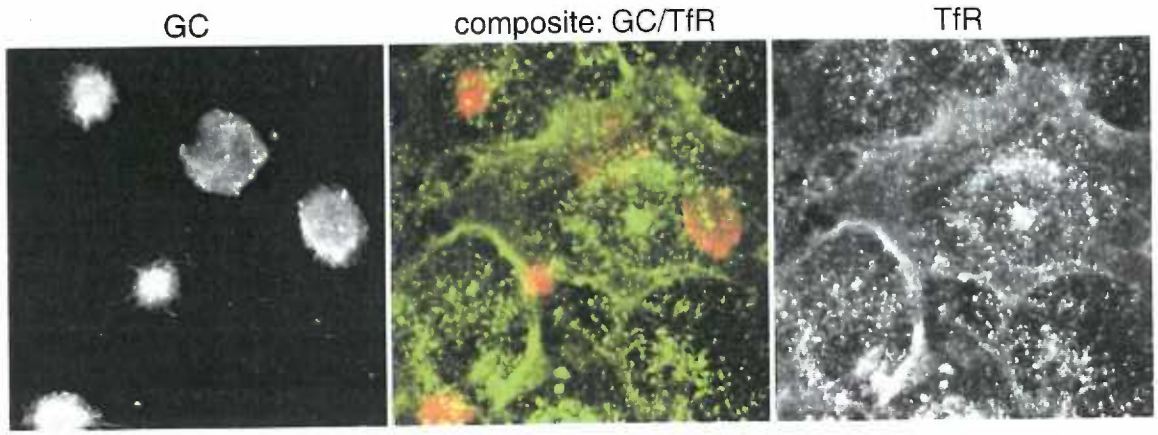


Fig. 3. Distribution of TfR in A431 cells infected with GC MS11A (P^+ Opa^-) for 4 h. Confocal optical sections were acquired at 1 μm intervals and assembled into extended focus projections as described in *Experimental procedures*. Identical results were obtained by conventional wide-field fluorescence microscopy.

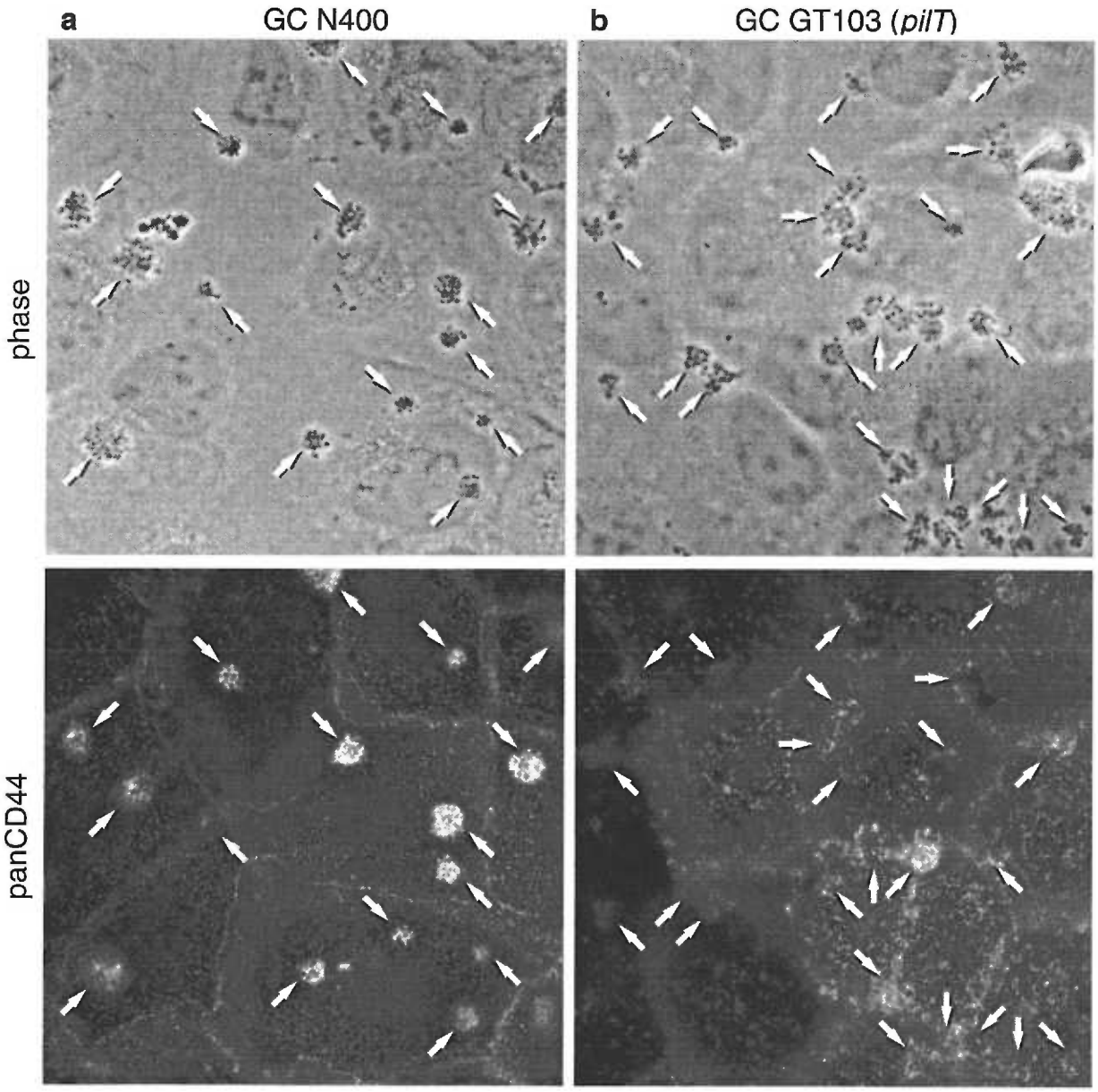


Fig. 4. pilT mutants exhibit a partial defect in the plaque formation assay

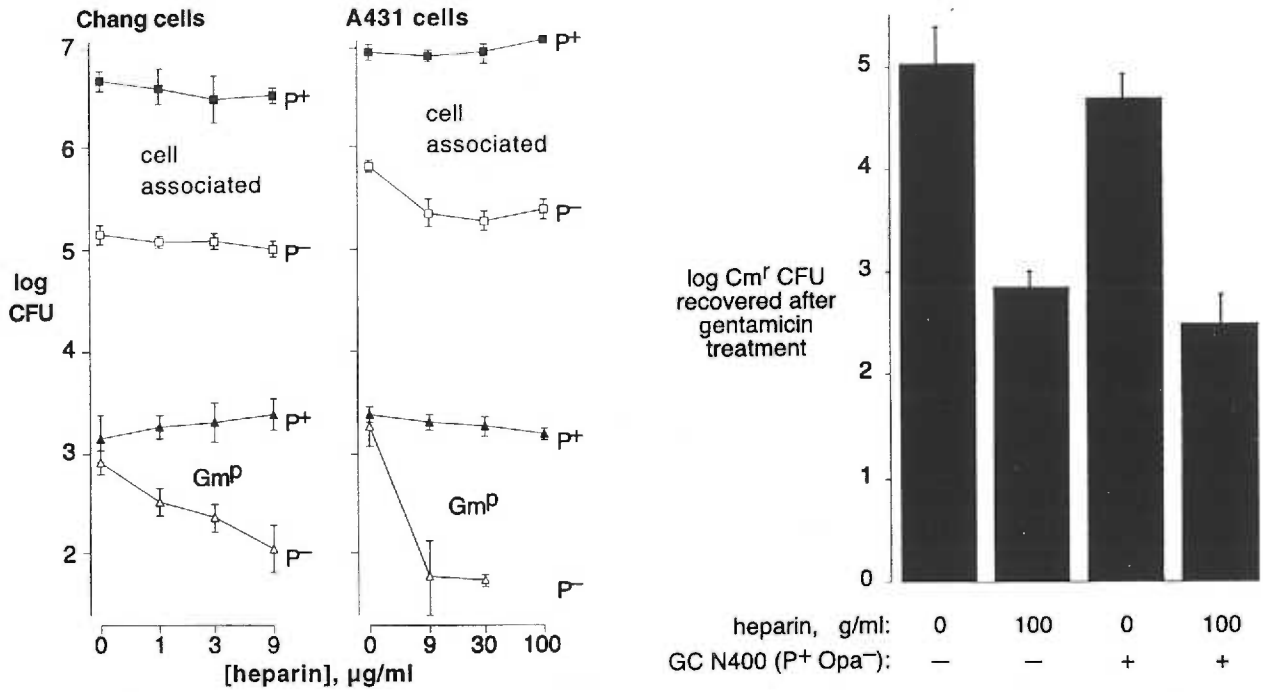


Fig. 5. Invasion of epithelial cells by P^+ and P^- GC in the presence and absence of heparin.

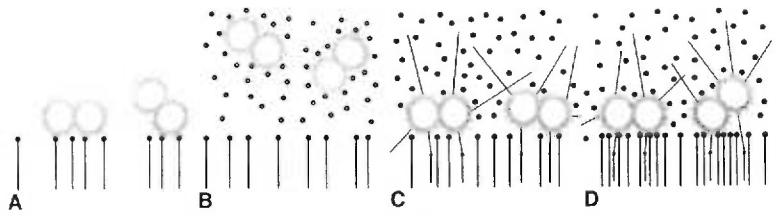


Fig. 6. Model for type IV pilus function in neisserial colonization of host cells. See text for discussion.

Figure Legends

Figure 1. Pathogenic *Neisseriae* trigger rearrangement of EGFR and CD44 variants. (a) Distribution of EGFR. HEC-1-B cells were infected for 4 hours with MC 8013.3 (P^+ Opa^- *opc*), fixed, and processed for double immunofluorescence using anti-MC antiserum and mAb EGF(R)-(528). Optical sections were acquired by confocal microscopy and assembled into extended focus projections as described in Experimental Procedures. (b) Wide-field views of distribution of panCD44 in A431 cells infected with GC MS11A (P^+ Opa^-) for 2 hours (top row) or 4 hours (bottom row). panCD44 was detected using mAb H4C4. Note that almost all microcolonies of GC are associated with panCD44 accumulations. These images were acquired by conventional wide field immunofluorescence microscopy. (c) Distribution of CD44v3 in A431 cells infected with GC MS11 AM13.1 (P^- $Opa30^+$; top row) or GC MS11A (P^+ Opa^-; bottom row). CD44v3 was detected using mAb 3C5. Note localization of CD44v3 with linear structures presumed to be type IV pili (arrows) and with microcolonies of ~10 GC cells. Individual bacterial cells are not distinctly rendered in this micrograph because the photomultiplier gain was increased to render pili clearly. These images are single optical sections obtained by confocal microscopy.

Figure 2. Colocalization of *Neisseria*-triggered transmembrane and cytoskeletal protein clusters. (a) Colocalization of panCD44 and f-actin clusters in A431 cells infected with GC MS11A (P^+ Opa^-) for 3 hours. panCD44 was detected using mAb H4C4, and f-actin was detected using BODIPY 581/591-phalloidin. (b) Colocalization of ICAM-1 and ezrin in Chang epithelial cells. ICAM-1 was detected with mAb P2A4, and ezrin was detected using antiserum

B22. Images are extended focus projections of sets of optical sections acquired by confocal microscopy. Arrows show locations of GC microcolonies as determined by phase contrast microscopy.

(c-e) Effects of CCD (cytochalasin D; 5 μg/ml final concentration) on GC MS11A-induced EGFR clusters in A431 cells. (c) Control infection (3 h) in the presence of DMSO vehicle. (d) CCD present during the final 20 minutes of a 3 hour infection. (e) CCD added 20 min prior to a 3 h infection. Images are extended focus projections of sets of optical sections acquired by confocal microscopy. Arrows indicate positions of GC microcolonies as determined by phase contrast microscopy.

Figure 3. Distribution of TfR in A431 cells infected with GC MS11A (P^+ Opa^-) for 4 hours. Confocal optical sections were acquired at 1 μm intervals and assembled into extended focus projections as described in Experimental Procedures. Identical results were obtained by conventional wide field fluorescence microscopy.

Figure 4. GC *pilT* mutants exhibit a partial loss of function in the plaque formation assay. Wide-field views of A431 cells infected for 3 hours with (a) GC N400 (*pilT*$^+$ P^+ Opa^-) or (b) GC GT 103 (*pilT*::mTnERM P^+ Opa^-). The top panels show phase contrast images and the bottom panels show the same fields of cells stained for panCD44. Arrows indicate the positions of GC microcolonies. Note that GC N400 and GC GT103 adhere in similar numbers. Identical results were obtained using GC GT 103 (*pilT*::mTnErm) or GC GT102 ($pilT_{QSL\ 318\text{-}320}$), and similar results were obtained when cells were stained for EGFR, ICAM-1,

or ezrin.

Figure 5. Invasion of epithelial cells by P^+ and P^- GC in the presence and absence of heparin. (a) Adhesion (cell associated) and invasion (Gm^R) of GCMS11A (P^+) and GC MS11-306 (P^-) in the presence of increasing concentrations of heparin. Infections were carried out for 8 hours. Plots show mean ±SD of triplicate determinations from a representative experiment. (b) Invasion of Chang cells by MS11AM13.1 (P^- Opa^+) in the presence or absence of heparin and in the presence or absence of P^+ GC MS11 N400. Infection was carried out for 4 hours. Bars indicate mean +SD of triplicate determinations from a representative experiment.

Figure 6. Model for type IV pilus function in neisserial colonization of host cells. See text for discussion.

Chapter 5: CONCLUSIONS & PROSPECTS

Neisserial adhesion to host cells as a multistep process

The central conclusion of the present work is that piliated gonococci and meningococci trigger rapid and localized rearrangements within and immediately beneath the host cell's plasma membrane. These rearrangements differ from host responses elicited by neisserial nonpilus adhesins, and depend at least partially upon the *pilT* gene that is involved in pilus-mediated twitching motility (Manuscripts II and III).

As discussed in Manuscripts II and III, pilus-induced cortical plaque formation is strikingly similar to several eukaryotic cell adhesion processes. These include the formation of focal adhesions by locomoting fibroblasts, of cell-cell adherens junctions in epithelial cells, of neuronal synapses, and of the so-called immune synapse between T cells and antigen presenting cells (APCs)(Adams and Nelson, 1998; Grakoui et al., 1999; Singer, 1992; Yamada and Geiger, 1997). In each of these cases different molecules mediate adhesion; nevertheless, all of these contact junctions share certain features. First, the local concentration (patching/capping/clustering) of the participating adhesion molecules is an early and pivotal event. Second, rearrangements of the cortical actin cytoskeleton, and the recruitment of linker molecules that attach locally concentrated adhesive receptors to the cortical actin cytoskeleton, is observed. Third, particular constellations of signaling molecules (protein and lipid kinases, small GTPases, etc.) are recruited to the contact sites.

The assembly of specialized structures (here generically called cortical plaques) at cell contact sites appears to be essential for both mechanical strengthening of adhesive junctions and for the transduction of specific signals across the plasma membrane. In each system there are two basic questions to be answered. First, "how?"- what are the components (e.g., adhesive, signaling, structural) and processes (e.g., mechanical forces, polymerization events, membrane rearrangements) required for the formation of a specialized adhesive domain or plaque? Second, "why" - what are the biological consequences of adhesive domain or plaque formation?

The present work provides a general answer to the "how" question by using genetic analyses to show that neisserial type IV pili trigger the formation of distinctive plaques, by showing that twitching motility or related functions help to drive plaque formation, and by identifying several of the host components recruited to plaques (Manuscripts II and III). It is also shown that pilus-induced cortical plaque formation at early times correlates with altered invasive behavior at later time points (Manuscripts I and III). These results are consistent with the proposal that cortical plaque formation may have important biological consequences during the colonization process.

Additional results consistent with a multistep adhesion model were recently reported by Nassif's group. Immunofluorescence experiments demonstrated that, at 8-10 hours post infection, adherent MC on T84 cells begin to lose their pili (Pujol et al., 1997). This loss of piliation correlates with both bacterial dispersal over the epithelial surface from microcolonies formed at earlier stages, and with tight association of the

bacteria with the host plasma membrane. Remarkably, both the loss of pili and dispersal from microcolonies is blocked in a *pilT* null mutant (Pujol et al., 1999). Our data indicate that dispersal from microcolonies is also blocked in GC *pilT* mutants (A. Merz, S. Lee and M. So, unpublished). Thus neisserial *pilT* mutations – although they block neither pilus biosynthesis nor adhesion to host cells – cause defects in at least four colonization-associated functions: cortical plaque formation; loss of pili upon host cell contact; tight association with host plasma membrane; and dispersal from microcolonies. Cortical plaque formation is a relatively fast process (0-3 hours post infection) compared to pilus loss, tight association, and dispersal (5-12 hours post infection). This sequence of events, and their dependence on *pilT*, is again consistent with the hypothesis that earlier adhesive events set the stage for later ones.

Further understanding of the biological function of pilus-induced cortical plaques will demand a more detailed understanding of the bacterial and host cell functions involved in plaque formation. The remainder of this essay explores some of these "how" questions, describes our current efforts to answer them, and summarizes some of the unpublished results.

Questions about PilT

PilT orthologs have recently been studied in several other bacteria that deploy type IV pili (Wall and Kaiser, 1999). In all cases reported, mutations in these genes do not prevent pilus biosynthesis but do compromise pilus-associated phenotypes. In *Myxococcus xanthus*, type IV pili and twitching motility are required for social gliding

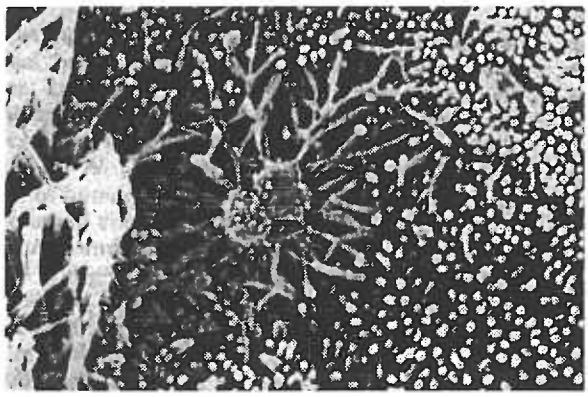
motility, one of two motility processes involved in fruiting body formation (Wall and Kaiser, 1999; Wu et al., 1997). Enteropathogenic *E. coli* (EPEC), require type IV pili for both colonization of and virulence in humans. In contrast, an EPEC mutant defective in the *pilT*-like gene *bfpF* has a large virulence defect but no colonization defect. Like *pilT*-mutant *Neisseriae*, EPEC *bfpF* mutants are trapped in microcolonies and are unable to disperse over epithelial surfaces (Anantha et al., 1998; Bieber et al., 1998). In the *Pseudomonas* family, several twitching-associated or *pilT*-dependant phenotypes have been reported. These include in vivo virulence, biofilm formation, cytotoxicity, and protein export (Comolli et al., 1999; Kang et al., 1997; Lu et al., 1997; Mattick et al., 1996). Thus twitching motility and related functions are involved in numerous important bacterial processes, most of which involve the colonization of surfaces – both biological and inert. Interestingly, many twitching-associated phenotypes can be detected only after several hours. Neisserial pilus-induced cortical plaque formation (Manuscript III) is the most rapid PilT-associated phenotype so far reported, other than twitching motility itself.

Despite intense recent interest in PilT and its homologues, the biochemical function of PilT remains obscure. As reviewed in detail in the Introduction, there is indirect evidence consistent with the hypothesis that twitching is driven by pilus retraction and disassembly, and evidence that the PilT protein is a key mediator of this process. Pilus retraction is an appealing concept because it could help to explain a number of additional observations.

First, the microcolonies formed by piliated, wild type GC and MC are relatively uniform and round, while microcolonies of *pilT* mutants have significantly more extended and variable geometries (A. Merz, S. Zimsen and M. So, unpublished data; also see fig. 4 in Manuscript III). A round, compact morphology minimizes the surface-to-volume ratio of the microcolony, and probably arises from mechanical tension dispersed over the surface of the colony (Wentworth Thompson, 1943).

Second, video microscopy of piliated GC microcolonies on inert substrates shows that within a certain radius (~ 5 μm) GC microcolonies will suddenly surge toward one another, merge, and quickly assume a rounded colony morphology. These movements are directed and linear, as opposed to the more jerky, random motion exhibited by isolated colonies. Similar behaviors by microcolonies attached to cultured epithelial cells also have been observed. *pilT* mutants are nonmotile in these experiments (A. Merz and M. So, unpublished observations).

Third, several scanning electron micrographs in the published literature depict elongated host cell microvilli reaching towards attached GC or MC microcolonies, with the appearance of the spokes on a wagon wheel (Griffiss et al., 1999; Stephens et al., 1983; Stephens et al., 1986):


(Stephens et al., 1983)

Such morphologies could arise either by directed growth of microvilli towards the bacteria, by an active contractile force exerted upon pilus-bound microvilli, or by both mechanisms. In contrast, microvilli elongate less and display a more disorganized architecture when cells are infected with MC or GC *pilT* mutants (Manuscript III; (Pujol et al., 1999)). Moreover, in recent immunofluorescence experiments we observe ezrin-labeled microvilli tipped with CD46 – the putative neisserial pilus receptor – reaching toward GC microcolonies in similar patterns (Manuscript III, Fig. 2 B, upper right corner; A. Merz, S. Lee, and M. So, unpublished observations). It will be important to determine whether these microvilli are attached to pili.

Thus several lines of evidence are at least consistent with the notion that type IV pili can exert tensile forces through retraction or disassembly processes that depend on PilT or PilT homologues. To date, however, neither retraction nor the generation of mechanical tension has been directly demonstrated for type IV pili, and other functions for PilT have not been formally excluded. PilT might serve as a sensor or signal transducer, or it might be involved in some as yet uncharacterized secretory

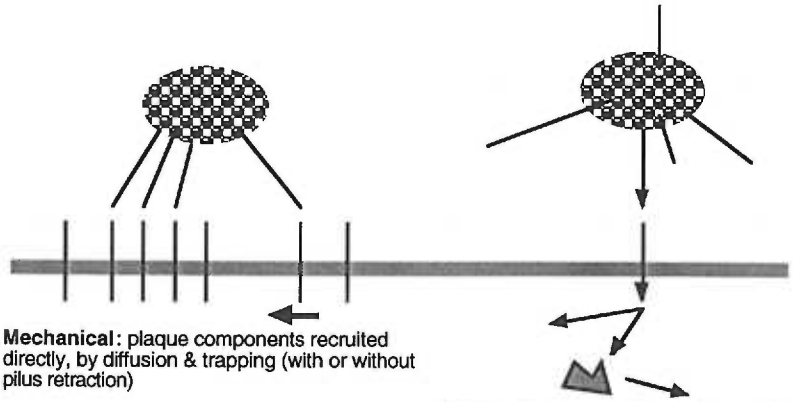
process. Nevertheless, most available evidence is consistent with the retraction and mechanical tension models.

In collaboration with Dr. Michael Sheetz (Duke University) we are attempting to use biophysical techniques including optical particle trapping (laser tweezers) to directly demonstrate that neisserial type IV pili exert contractile forces (Sheetz, 1998; Sterba and Sheetz, 1998). To facilitate these analyses I have constructed a set of GC and MC strains that express an antigenically identical pilin variant which is recognized by a mAb developed in the So Laboratory. Isogenic strains with targeted mutations in their *pilT* loci have also been constructed. In a second, alternative approach I am using the same strains and reagents to test whether piliated GC or MC can exert contractile forces on inert elastic substrates, using techniques developed for studies of mammalian cell locomotion (Pelham and Wang, 1997).

Questions about host components involved in plaque formation

Two basic models could explain plaque formation. In a *mechanical model*, physical forces from outside the cell (e.g., crosslinking of a pilus receptor) would cause the accumulation of host cell components at the contact site. This accumulation could occur through the passive trapping of laterally diffusing molecules within the plasma membrane as in classical antibody-mediated 'capping' (Singer, 1992), or through active gathering of such molecules (as might occur through pilus retraction). In a *signaling model*, a signal triggered by bacterial contact (e.g., activation of a kinase) would trigger active host-directed processes that would result in the recruitment of host components

to the contact site. In each case, the recruited molecules could arrive both through lateral mobility within the plasma membrane, and through exocytosis of cytoplasmic vesicles at the contact sites.



At this time there is evidence consistent with both models. The accumulation of cytoplasmic components such as ezrin and EBP-50 suggests that host cell signaling processes are involved in plaque formation. Furthermore, the contact-triggered accumulation of tyrosine-phosphorylated components (Manuscript II) and the discovery of pilus-triggered calcium fluxes in the host cell (Kallstrom et al., 1998) and pilus-triggered exocytosis (P. Ayala et al., in preparation) are consistent with signaling events known to be triggered during other adhesive events. At the same time, the involvement of PilT may indicate a role for mechanical force, assuming that type IV pili are in fact retractile structures.

Possible roles of CD46 and ERM proteins

In both the mechanical and signaling models an important question is whether the pilus adhesin directly mediates plaque formation by binding to pilus receptors on the host cell. At this time only one such receptor has been identified — CD46 (Kallstrom et al., 1997). As predicted in the Discussion section of Manuscript III, CD46 has now been found to be enriched in pilus-induced cortical plaques in at least two different cell lines (A. Merz, S. Lee and M. So, unpublished data). Interestingly, there are reports that CD46 associates with multiple tyrosine kinase activities and with the linker molecule moesin (Schneider-Schaulies et al., 1995; Wong et al., 1997). Moesin is highly similar to ezrin, a major plaque component (manuscript III), and radixin. Together these linker proteins are referred to as the ERM (Ezrin Radixin Moesin) family (Bretscher, 1999; Mangeat et al., 1999).

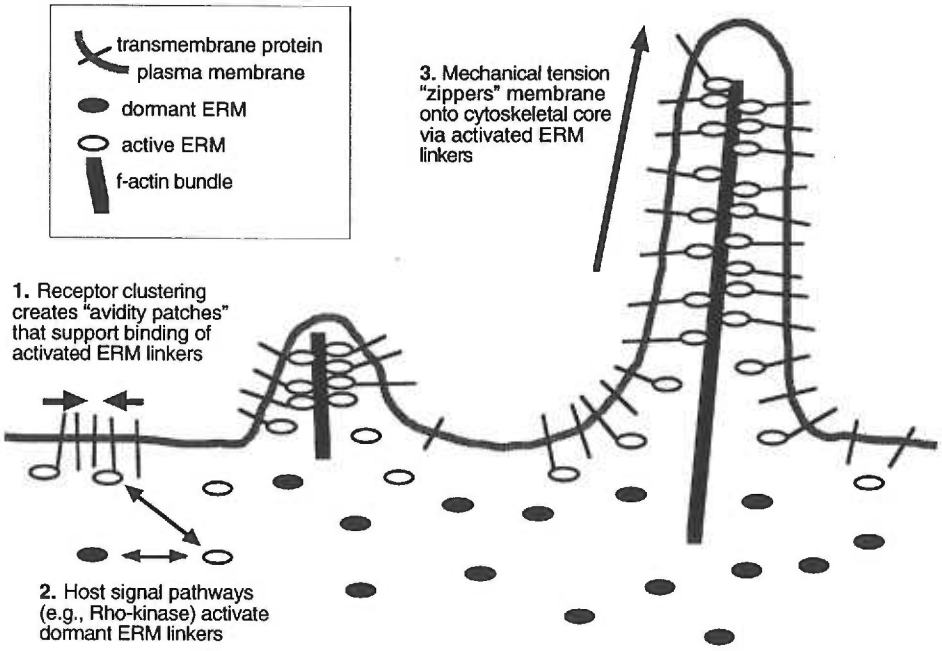
ERM proteins have a three-domain structure, with terminal globular domains linked by a predicted alpha-helical central domain. Sequences in the ERM N-termini bind to transmembrane proteins and to other linkers, such as EBP-50, and to signaling molecules such as Rho-GDI. ERM binding to these molecules is enhanced in the presence of the lipid phosphatidylinositol-1,2-bisphosphate (PIP2). The ERM N-terminal globular domains are referred to as N-terminal ERM association domains, or N-ERMADs. N-ERMADs share primary sequence homology with one of the first cortical linker molecules identified, erythrocyte band 4.1 protein. The C-terminal domains of ERM proteins bind to actin microfilaments and are referred to C-ERMADs (Bretscher, 1999; Mangeat et al., 1999).

ERM proteins have highly similar primary structures and are at least partially functionally redundant, but have divergent tissue distributions. For example, ezrin is present in many epithelial cells that lack moesin, and moesin is found in many endothelial cells, which lack ezrin. Consistent with their functional redundancy, the N-ERMADs bind to similar sequences in the juxtamembrane domains of several transmembrane proteins. These sequences are highly enriched in positively charged amino acids (Bretscher, 1999; Mangeat et al., 1999). In CD46, seven of eleven residues proximal to the plasma membrane are arginines or lysines. Moreover, at least two of the other membrane proteins enriched in cortical plaques, CD44 and ICAM-1, have been experimentally shown to bind to all three N-ERMADs, also via highly charged juxtamembrane regions (Bretscher, 1999; Mangeat et al., 1999). Thus it is probable that CD46 binds not only to moesin but to ezrin and radixin as well.

The probable interaction of ERM with the neisserial pilus receptor CD46, the strong enrichment of ezrin in pilus-induced plaques, and the well-documented interaction of ezrin with at least two additional plaque components, CD44 and ICAM-1, suggests that ezrin may play a pivotal role in plaque formation. Indeed, ezrin has been shown by several experimental approaches to be involved in microvillus formation and in the formation of other cell protrusions, and a model for ERM function in microvillus formation has been proposed by Bretscher's group (Berryman et al., 1995; Bretscher, 1999).

Very recent data indicate that overexpression of the CD44 transmembrane and N-ERMAD-binding region is sufficient to cause an increase in the length and number

of microvilli on transfected cells (Yonemura and Tsukita, 1999). Similarly, pilus-induced clustering of CD46 might be sufficient to cause the initial extension of microvilli at contact sites. In addition *pilT*-dependant pilus retraction might enhance microvillus lengthening directly, by pulling out microvilli and allowing membrane to "zipper" onto bundled actin cores via ERM linkers. This model suggests several testable predictions, and I have engineered bacterial strains and reagents that should permit some of these predictions to be tested.



Tools for studying the cell biology of cortical plaque formation

I have constructed expression vectors that encode ezrin fusion proteins with either epitope tags or green fluorescent protein tags. The constructs encode wither full-length ezrin or the ezrin N-ERMAD alone. In preliminary experiments I find that full-length ezrin-Myc or ezrin GFP colocalizes with the endogenous ezrin and rapidly (within a

few minutes of bacterial attachment) accumulates at GC contact sites in living cells. In addition I have constructed similar expression vectors encoding the pilus receptor, CD46, to epitope and GFP tags. Fusions that lack most of the CD46 cytoplasmic tail have also been constructed. Together these reagents will allow us to experimentally test the roles of the pilus receptor and its cytoplasmic domain in the formation of pilus-induced plaques, as well as to monitor plaque formation in real time using the GFP fusions. Our model predicts that not only CD46 binding but the interaction of CD46 with ERM proteins will be required for plaque formation.

In collaboration with the Sheetz group we will use these reagents to evaluate the role of mechanical tension in plaque formation by using laser tweezers to exert tensile force GC *pilT* mutants attached to cells (Chen et al., 1997; Dai and Sheetz, 1995; Schmidt et al., 1993). If *pilT*-dependant pilus retraction enhances plaque formation we may be able "complement" *pilT* mutant cells by tugging on them. Ezrin-GFP and CD46-GFP will be used as the indicators in these experiments. In combination with our other work on *pilT* described above, these experiments should allow us to determine whether (a) type IV pili exert mechanical tension on substrates; and (b) whether mechanical tension alone is sufficient to explain the enhancing role of PilT in cortical plaque formation.

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