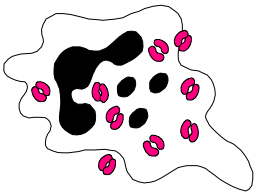

Pathogenesis

Virulence determinants of meningococci and factors that may determine between the carrier state and invasive disease

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Meningococci are isolated from the nasopharynx of up to 30% of healthy individuals and may be classified as commensals of the human respiratory tract. Meningococci elaborate adhesins such as pili and outer-membrane opacity proteins that may aid in anchorage to mucosal cells, specific nutrient acquisition factors (e.g. iron-binding proteins) and capsule that protects against desiccation. Capsule and LPS sialylation and perhaps IgA protease also aid in evasion of host immune mechanisms (1). Under some situations, meningococci cause serious conditions which can be fatal. Thus they possess attributes that give them a considerable pathogenic potential. This distinguishes meningococci from the other *Neisseriae* that colonize the human nasopharynx but are rarely associated with disease. I will explore some of the meningococcal and host factors which may allow translocation from mucosa to deeper tissues which, in some cases, results in one of the most rapidly progressing and serious infections.

Are carrier strains different from disease isolates? Studies on strains from African epidemic outbreaks show that clonal characteristics of carrier and disease isolates are similar (2). In vitro investigations on toxicity of carrier and case isolates indicate that both possess the capacity to damage human endothelial cells; multiple meningococcal components (pili and LPS) act together to destroy endothelial integrity characteristic of meningococcal septicemia (3). Also, a disease isolate has been shown to have colonized a laboratory worker without overt disease; this epitomizes the importance of host immunity (4). There are, nevertheless, characteristics that predominate in case isolates and are related to serogroups (1). Serogroups A, B and C are more often associated with disease than other serogroups or acapsulate bacteria. Serogroup A predominates in Africa and is responsible for epidemic spread whereas serogroups B and C prevail in the West and are associated with sporadic outbreaks. However, the precise bacterial factors responsible for the nature of outbreak and the reasons for geographic differences are not clear. Host, socio-economic as well as climatic factors may determine the global differences in meningococcal outbreaks (1,2).

Carrier isolates are often acapsulate but capsulate bacteria are also found in the NP (1). LPS of carrier strains also tends to be structurally different, often asialylated. Other differences may include the expression of distinct porins (class 1 / 2 / 3) which have been implicated in impairment of host cell functions (5). Putative toxic factors (e.g. RTX-like proteins) have been reported in some meningococcal strains (6). These are environmentally regulated and their expression could also increase pathogenic potential of a strain. Meningococcal phase variable opacity proteins (Opc, Opa) and pili are usually present in isolates from the throat. They are also widely expressed in case isolates (2, 7). Pili and Opa proteins are structurally heterogeneous and which structural features are selected at various sites of colonization or dissemination are not defined.

What about the host factors? Invasive disease may arise as a result of increased host susceptibility, which in turn may be governed by several factors. Nasopharyngeal colonization requires an equilibrium between the host and the microbe. Factors that limit meningococcal growth in the nasopharynx include local immunity, limitation of nutrients, shedding during flows of mucus and the presence of other oral flora (1). Disturbance of this equilibrium has the potential for overt multiplication leading to damage to mucosal tissues via toxic components such as LPS.

It has long been recognized that bactericidal antibodies are important in defense against meningococci. Infants are protected from meningococcal disease by the maternal antibodies and meningococcal infections become more common in the young as these antibodies diminish and before full immunity is acquired, possibly through exposure to *Neisseria lactamica* (1).

Epidemiological studies also suggest that factors that damage mucosa such as smoking, prior infection of the host (e.g. respiratory viral infections in winter months in the UK) or very dry atmospheric conditions (in dry seasons in Africa) may pre-dispose the host to meningococcal infection (1). An analysis of epidemic spread of serogroup A meningococci in Africa led to the speculation that epidemic dissemination may involve at least two co-pathogens and perhaps serogroup A bacterial components adhere specifically to the postulated co-pathogen (2).

Meningococci can be isolated from swabs of the posterior pharyngeal wall (1). *In vitro* studies on nasopharyngeal organ cultures have also shown specific targeting of non-ciliated cells and cellular entry was observed (8). However, it is not clear whether, *in vivo*, meningococci attach to or enter these or other epithelial cells. Cellular entry may allow bacteria to evade phagocytosis by professional phagocytes. Indeed, mucosal surfaces are monitored by phagocytic cells and meningococci of phenotypes often isolated from the nasopharynx (opaque, acapsulate, L8 LPS type) are readily phagocytosed *in vitro* by polymorphonuclear phagocytes (PMN) and monocytes (9). This phenotype also readily invades epithelial cells (10). It is not entirely clear whether meningococci can survive within host cells, either in professional or non-professional phagocytes. Many studies have addressed these questions. However, experimental problems with determining intracellular survival of meningococci are considerable since they often grow more rapidly extracellularly. Some of these studies suggest that at least *in vitro*, meningococci do not grow aggressively in phagocytic or epithelial cells. When internalized, meningococci appear to be eliminated by phagocytic cells within a relatively brief period (9). Nonetheless, the likelihood of meningococcal carriage from the nasopharynx within phagocytes cannot be ruled out. It is possible, for example that phagocytes compromised in their ability to eliminate microbes may become vehicles for transmission. It is also possible that short term survival of meningococci within phagocytes is sufficient for translocation. The ability of a phagocyte to deal with internalized bacteria may also depend on the numbers of bacteria engulfed. To this end, it would be important to know if there is overt multiplication of bacteria in the nasopharynx prior to dissemination.

Many recent studies have shown that host components targeted by bacteria include hormone, cytokine and adhesion receptors. Some host receptors are either not expressed constitutively or expressed in low numbers and may be upregulated by cells in response to inflammatory

cytokines and other factors. Some viruses, e.g. respiratory syncytial virus, affect host cells such that they down-regulate adhesion receptors (11). Other viruses such as parainfluenza virus type 2, upregulate several receptors on human tracheal epithelial cells (12). Receptor density, multiple receptor occupancy as well as affinity of microbial ligand interactions with host cell receptors may determine microbial status - commensalism or pathogenic - within the host (13, 14). Clinical observations suggest that dissemination to the central nervous system occurs via the haematogenous route. As such, bacteria must traverse the epithelial and endothelial barriers. The possible routes include direct intra- or inter-cellular translocation in addition to carriage via phagocytic cells. In considering possible molecular mechanisms of meningococcal interactions with human target cells, I will address the effects of surface polysaccharides, capsule and LPS and describe investigations on the three major adhesins/invasins pili, Opc and Opa. The roles of other outer membrane components such as porins and class 4 proteins may also be equally important but will not be discussed in this article.

Meningococcal surface polysaccharides: capsule and LPS. Capsule and sialylated lipopolysaccharides are expressed in disseminated isolates and are believed to protect the organism against antibody/complement and phagocytic killing. They are also expressed by a number of carrier isolates and may have functions that allow the organism to exist in the nasopharynx (allow avoidance of mucosal immunity) or are physically protective against extra-host environment (anti-desiccation property of capsular polysaccharide). However, possession of capsular structure that is recognized as a self antigen (group B α2-8 polysialic acid) clearly gives the organism a considerable pathogenic potential in the blood. In vitro studies on cellular adhesion have not revealed significant differences which can be assigned to capsule structure (7).

Acapsulate organisms are isolated frequently from the nasopharynx (1). In vitro studies show that adhesion and particularly invasion of epithelial cells is enhanced (aided by some opacity proteins) in the absence of capsule (10). This invites the hypothesis that loss of capsulation may help establish long term nasopharyngeal carriage where intracellular state would potentially provide protection from host's defenses. Whether factors in the nasopharynx trigger down-modulation of capsulation is not known but one study suggests that environmental factors may regulate capsule expression (15). In such cases, dissemination from the site of colonization would require upregulation of capsulation since acapsulate bacteria are unlikely to survive in the blood. Alternatively, since blood provides a rich environment in which meningococci can grow rapidly, it is possible that a small number of capsulate organisms arising as a result of natural phase variation, will be selected for in the blood. In the case of *Haemophilus influenzae*, studies on the infant rat model of haemophilus bacteraemia and meningitis have shown that bacteremia may arise as a result of survival of a single organism in the blood stream (16).

Meningococci from the nasopharynx often express the L8 LPS immunotype that resists sialylation due to the absence of lacto-N-neotetraose structure, a receptor for sialic acid (1). Sialylation of LPS has functional consequences similar to capsule and it imparts resistance to immune mechanisms of the host and in doing so, masks the functions of many outer membrane proteins (17, 10). The interplay between surface polysaccharides and various adhesins and invasins is a complex area of investigation with antigenically and phase varying components adding to the complexity. Some aspects of this interplay are addressed below.

Pili and their importance in multiple cellular targeting and in potentiation of cellular damage. Both carrier and disease isolates are usually piliated, however, pili are lost on non-selective subculture (18), which suggests that pili are selected for in vivo. Pili have been implicated in mediating epithelial interactions (8) and were shown to mediate hemagglutination (19). Our studies demonstrated that pili also mediate adhesion both to human umbilical vein and microvascular endothelial cells (7,18,20). One consequence of pilus-mediated adhesion to endothelial cells is increased cellular damage which is primarily mediated by LPS and is dependent on the presence of serum CD14 (3, 21). These *in vitro* toxic effects reflect the acute toxicity of meningococci for endothelial cells observed during vascular dissemination.

Structure / function relationships of meningococcal pili, pilus-associated adhesins. Although two structurally distinct classes of pili occur in meningococci, no discernible functional difference has been assigned to either class. Both undergo antigenic variations which alters their tissue tropism. Studies using adhesion variants (derived by single colony isolation, with or without prior selection on host cells) implied that structural variations in pilin affect epithelial interactions significantly, but have lesser effect on endothelial interactions (22). Thus the pilin subunit may contain a human cellular binding domain, or at least has influence on adhesion if mediated by an accessory protein such as PilC, which has been implicated in cellular adhesion and in biogenesis in both meningococci and gonococci (23, 24, 25, 18). At present, how pilin structural variations modulate PilC or other pilus-associated adhesion functions is not clear.

Studies on adhesion variants of meningococci have revealed that meningococcal pili are subject to post-translational modifications (20). Also, they contain unusual substitutions. A trisaccharide structure (Gal β1-4 Gal α1-3 -2,4diacetamido-2,4,6-trideoxyhexose) is present on all variant pili of strain C311 (26). Further recent studies have shown that at a distinct site, meningococcal pili contain, perhaps, a unique substitution, α-glycerophosphate (27). In addition to these, meningococcal pili may contain distinct variant-specific substitutions (26). Such additional modifications have been demonstrated by Fast Atom Bombardment- and Electro Spray- Mass Spectrometry, but the structures concerned are not yet elucidated. The functional consequences of pilin modifications are not understood at present.

Phenotypic requirements for interactions mediated via outer-membrane proteins. In fully capsulate bacteria, only pili appear to be effective in mediating cellular adhesion. Using acapsulate derivatives of a serogroup A strain (C751), the invasive potential of the meningococcal proteins Opc and Opa was demonstrated (28,10). More recently, a library of variants and mutants (varying in expression of capsule, LPS, pili, Opa and Opc) was created in strain MC58 (serogroup B). The use of these derivatives has established that Opc can act as an invasin in distinct serogroups, and that surface sialic acids inhibit Opc-mediated invasion. Also, pili may potentiate Opc-mediated invasion of some cells (29). As asialylated phenotypes occur in the nasopharynx, and Opc and Opa are expressed in many nasopharyngeal isolates, these proteins may be important in interactions with nasopharyngeal epithelial cells.

Interactions via the outer-membrane protein Opc: molecular mechanisms and identification of host cell receptors. Opc, a basic protein appears to have the capacity to bind to multiple extracellular matrix (ECM) components and serum proteins (30) giving the organism the potential to interact with several different integrins by bridging via their respective ECM or

soluble ligands. Indeed, studies using cultured human endothelial cells have shown that interactions of Opc-expressing meningococci with the apical surface of polarized host cells require serum-derived RGD-containing proteins. The integrin $\alpha v\beta 3$ (the vitronectin receptor, VNR) appears to be the major receptor involved in serum dependent apical interactions of meningococci (31).
Opc-mediated invasion of cultured epithelial and endothelial cells can be inhibited by monoclonal antibodies against Opc which appears to be the major requirement on bacteria for this interaction. However, the cloned Opc protein does not confer invasive property to the host *E. coli* even though the protein is surface expressed and is immunologically similar to that of meningococci. This suggests that additional bacterial factors may be required in host cell interactions mediated by Opc. It is also possible that the level of Opc expressed by *E. coli* is not optimum since efficient interactions of meningococci via Opc require the protein to be expressed at a high density on bacterial surface (29). Further factors may be involved in the interactions via the VNR. In analogy with the complement receptor CR3, which has been shown to interact simultaneously with C3bi-coated particles and with microbial glycolipids at distinct sites (32), VNR may require multiple ligand engagement. Indeed the vitronectin receptor also exhibits binding sites for ganglioside GD2 (33). Gangliosides and LPS share structural similarities in that both are amphipathic with strongly anionic hydrophilic groups and some manner of LPS interaction with the vitronectin receptor may be an additional factor required. This is at present a speculation and there is no evidence to support this hypothesis.

Opc targeting of extracellular matrix proteins could also enable bacteria to adhere to substrata of damaged mucosa as well as to penetrate deeper tissues after cellular invasion.

The role of Opa proteins. In contrast to gonococci that may encode up to 12 distinct Opa proteins, a single meningococcal strain encodes fewer (< 4) Opa proteins. Studies on a serogroup A strain C751, have shown that, of the three Opa proteins expressed by this strain, OpaB and OpaD are effective in epithelial adhesion and invasion whereas OpaA is ineffective (10). A receptor for a gonococcal Opa protein on some epithelial cells has been described (34). Whether OpaB and OpaD of C751 also engage this receptor is not known at present. However, on certain epithelial cells and PMN, another receptor may be targeted by meningococcal Opa proteins (see below).

Interactions of meningococci with monocytes and polymorphs: the role of surface virulence factors (Capsule, LPS, pili, Opa and Opc). Bacterial components that mediate cellular interactions in the absence of added opsonins are of importance from the point of view of phagocytes acting as potential 'Trojan Horse' carriers of bacteria. Moreover, up to 16% of the cells present in the nasal mucosa are monocytes, and inflammation increases PMN infiltration. In studies to identify bacterial factors that increase phagocyte interactions, it was shown that capsulate bacteria resist phagocytosis in the absence of opsonins, but acapsulate bacteria are internalized and opacity proteins Opa and Opc mediate bacterial uptake. Pili of distinct structural make-up, or the pilus-associated protein PilC, were ineffective in mediating interactions with phagocytic cells (9, 35).

Phagocytic receptors for opacity proteins Comparative studies on Opc and distinct Opa proteins of strain C751 have shown that Opc is more effective in monocyte interactions than Opa

proteins. The reverse is the case with PMN. The three Opa proteins of strain C751 increased PMN chemiluminescence with OpaB and OpaD being somewhat more effective than OpaA. The Opa proteins of this strain and indeed >85% of clinical isolates of meningococci and gonococci interacted with CD66a (also known as BGP, biliary glycoprotein), a member of the Immunoglobulin superfamily (36). CD66 receptors are a family of structurally related molecules and are present on epithelial, endothelial and myeloid cells. Gonococcal Opa-mediated interactions with an upregulated PMN receptor was reported by Rest et al (37) who observed that the receptor may be stored in secondary or tertiary granules. CD66a and related molecules have been shown associated with PMN secondary granules and can be upregulated by inflammatory cytokines and fMLP (36). Meningococcal and gonococcal targeting of this receptor has implications in their pathogenesis. A low level constitutive expression of the receptor, for example on epithelial cells, may favor attachment to epithelial cells without invasion. Viral infections or other conditions during which cytokines may be upregulated, could result in increased expression of CD66 and related adhesion molecules increasing the potential of meningococci to enter both phagocytic cells as well as mucosal epithelial cells. Massive epithelial cellular invasion could be injurious to the host, that of phagocytic cells could result in incomplete elimination of bacteria and possibility of transmission within phagocytic cells. Whether this represents a possible mechanism which determines host susceptibility to meningococcal invasion, and perhaps also contributes to the development of serious complications during some gonococcal infections, remains to be shown.

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Pathogenesis

The development of a primary urethral epithelial cell system for the study of adherence and invasion by *Neisseria gonorrhoeae.*

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Recent studies have demonstrated that urethral epithelial cells are invaded by *Neisseria gonorrhoeae* during gonococcal infection in men (1). Reznikoff and co-workers developed a system supporting attachment, growth, and passage of primary human ureter epithelial cell cultures (2). These cultures originated from tissue specimens collected at the time of transplantation surgery. Using modifications of their methods, our laboratory has developed a primary urethral cell system that has allowed us to study the biology of gonococcal invasion in a minimally modified cell system.

We developed a primary urethral epithelial cell system using tissue obtained from the membranous urethra of patients undergoing urologic surgery. Urethral tissue collected from surgery in sterile saline was cut into 4 mm square sections and placed epithelial side down on a rat-tail collagen matrix and covered with hormonally defined media containing DMEM/Ham's F12 (1:1) and antibiotics. After 24 hours the media was changed to Clonetics BEGM Bulletkit media. Within 3 to 5 days, epithelial cells could be seen extending from the surface of the tissue onto the collagen surface. By two weeks, organized confluent layers of epithelial cells could be found at distances up to 2 cm from the tissue section. Our primary urethral cell system allows at least two passages of viable cells to 12 mm glass cover slips coated with bovine collagen in 24 well cell culture dishes for confocal microscopy and electron microscopy. Infection studies can be performed directly in these wells after the cells become confluent and the results analyzed by removal of the glass cover slip without disrupting the integrity of the epithelial layer. Generally, within 4 days after primary passage, confluent layers of urethral epithelial cells were observed on the cover slips.

Fibroblast contamination has not been a problem. Fluorescent analysis using anti-keratin and cytokeratin antibodies demonstrated that 100 percent of the cells stained with these epithelial cell markers. Light and electron microscopic analysis indicated that the layers were formed by stratified epithelial cells arrayed in a pattern similar to that seen in urethral epithelium. Invasion studies of these cells were performed with *Neisseria gonorrhoeae* strain 1291 opa+, p+. Studies were performed comparing various exposure times of gonococcus to primary urethral epithelial cells. Immunoelectron microscopy studies showed the same pattern of invasion observed recently by our laboratory in exudates from infected patients; i.e., adherence of the organism to the epithelial cell membrane, pedestal formation with evidence of membrane fusion between the gonococcus and the epithelial cell. These events are followed by intracellular localization.

Because the studies of Schneider and co-workers (4) and that of Cannon (Abstract, this meeting) have suggested that an intact Galb1-4GlcNAcb1-3Galb1-4Glc LOS structure is important for human infection, we investigated the possibility that the asialoglycoprotein receptor was present

on human urethral epithelial cells. We have shown that the asialoglycoprotein receptor present on human hepatocytes recognizes and binds these terminal sugars of the gonococcal LOS molecule. In HepG2 cells, this receptor-ligand interaction is important for invasion. Previously, it was also found that this receptor is upregulated in HepG2 cells upon exposure to gonococcus (3). Our studies using polyclonal antiserum and *in situ* hybridization indicate the presence of ASGP-R in urethral epithelial cells both from infected patient exudates and primary tissue culture . Polyclonal antibodies to the ASGP-R used in confocal microscopy indicated an association between the gonococcus and this receptor. The level of receptors detected with the antibody increased with increasing times of gonococcal exposure from 0 to 4 to 24 hours. *In situ* hybridization using a fluorescent labeled probe specific to the ASGP-R supported this data; i.e., a significant increase in message compared to the uninfected control was observed.

Recently, Grassme and co-workers demonstrated F-actin accumulation in Chang epithelial cells associated with and as a result of exposure to opa+ *Neisseria gonorrhoeae* (5). Confocal studies of primary urethral epithelial cells and HepG2 cells showed significant actin polymerization upon exposure to 1291 opa+, p+.

In the past, the lack of an acceptable animal model and a relevant in vitro culture system in addition to the cost and inconveniences associated with human volunteer studies have limited gonococcal pathogenesis studies. The development of primary human urethral cell cultures allows studies of the biology of gonococcal adherence and invasion in a relevant primary human cell system. Studies in this system will allow the possibility of real time molecular analysis of the bacterial and cellular events involved in gonococcal invasion of the urethral epithelium.

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Pathogenesis

The role of the lutropin receptor and the cryptic plasmid in gonococcal invasion of HecIB cells.

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We have shown that gonococci grown on laboratory media cannot invade the endometrial cell line, HecIB, but will convert to an invasion proficient (inv^+) phenotype when they contact cell lines derived from the endometrium or the cervix (1). The phenotypically inv^+ gonococci express a new adhesin, as binding to HecIB cells is not inhibited by inv^- gonococci until the inv^-/inv^+ ratio exceeds 100 in competitive binding experiments. The enhanced binding of inv^+ gonococci is eliminated by human chorionic gonadotrophin (hCG), suggesting that the new adhesin binds to the lutropin (hCG/LH receptor). Induction of the inv^+ phenotype and invasion of HecIB cells by gonococci also requires the lutropin receptor, as HecIB cells down-regulated for this receptor are deficient in both inducing the inv^+ phenotype and serving as recipients for invasion.
To identify genes involved in gonococcal invasion of HecIB cells, we constructed a library of *cat* insertion mutants and screened for deficiencies in invasion. Six of 9 invasion deficient clones had inserts in the cryptic plasmid (2,3), resulting in an 8-20 fold loss of invasion ability. Several plasmidless gonococcal strains were tested for their ability to invade HecIB cells and were found to be 5-10 fold decreased in invasion proficiency. Transformation of one of these strains with a cryptic plasmid restored invasion proficiency.

Although a number of putative proteins and two putative transcripts have been proposed for the cryptic plasmid (3), attempts to demonstrate expression of either protein or mRNA from this plasmid in gonococcal strains has been unsuccessful. This suggests that expression occurs only under environmental conditions that were not tested. We used reverse transcriptase polymerase chain reactions (RT-PCR) to amplify DNA from gonococcal cryptic plasmid mRNA isolated at various times after gonococci had been added to HecIB cells. The RT-PCR revealed that the *ccpB* gene was transcribed 3.5 hr after contact with the HecIB monolayer, and no *ccpB* mRNA was made if the plasmidless RUN 5288 parental strain was used. The timing of mRNA induction suggested that the plasmid gene was transcribed only after the gonococcal strain had entered the HecIB cells.

We fused a peptide, FLAG^{TM}, to the C-terminus of the plasmid protein CppB to detect expression of the gene using anti-FLAG^{TM} monoclonal antibody. No detectable expression was found when gonococci were grown on laboratory medium, in tissue culture medium, or when gonococci were incubated with a glutaraldehyde-fixed HecIB monolayer. However, gonococci that had entered the HecIB cells expressed CppB.

Our model for gonococcal invasion of HecIB cells is as follows: When gonococci contact HecIB cells they induce a new adhesin in response to the presence of the lutropin receptor; gonococcal binding to the lutropin receptor mediated by this new adhesin induces uptake by the host cell.

Once the gonococci have entered the HecIB cells, the plasmid encoded genes are induced, allowing intracellular survival and/or escape from the vacuole.

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Pathogenesis

Host cell factors involved in opacity (Opa) protein-mediated cell adherence and cell invasion by gonococci

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Neisseria gonorrhoeae adherence to and invasion into epithelial cells involves the interaction of the invasion-associated opacity (Opa) outer membrane protein with cell surface-associated heparin sulfate proteoglycan receptors (1). We report here that various syndecans can act as cellular receptors for this Opa-mediated process. We also report on the characterization of a serum-derived host factor which is involved in Opa-mediated cell invasion rather than cell attachment.

Various syndecans can act as a cellular receptors for Opa-mediated cell attachment and cell invasion. We have used immunocytochemistry to further characterize the cell surface-associated heparin sulfate proteoglycan receptors for Opa-mediated cell attachment and cell invasion by gonococci. Various cell lines were infected with *Neisseria gonorrhoeae* strain VP1 for 2 h, fixed and double stained for bacteria and various proteoglycan receptors by indirect immunofluorescence labeling. Stained specimens were analyzed by confocal laser scanning microscopy. Labeling of cell surface-associated proteoglycans with antibodies directed against heparin sulfate revealed a patchy pattern of heparin sulfate staining associated with the periphery of adherent bacteria and a strong, ring-shaped heparin sulfate labeling around most intracellular bacteria. These findings confirm a role for cell surface-associated heparin sulfate proteoglycans as cellular receptors for gonococcal cell attachment and cell invasion as previously suggested by biochemical studies (1). Next we have tested if gonococci may specifically interact with particular proteoglycan receptors. Syndecans are the most common cell surface-associated proteoglycans. This class of transmembrane receptors is characterized by conserved short intracellular domains and highly divergent extracellular ectodomains carrying heparin sulfate side chains. Immunocytochemical labeling of syndecan-4 in Chang cells, which express elevated levels of this proteoglycan, demonstrated specific receptor recruitment to the periphery of adherent and intracellular gonococci. Staining of Me-180 cells for syndecan-1, which is highly expressed in this cell line, showed a similar receptor recruitment to the periphery of interacting gonococci. We conclude from these results that gonococci can interact with multiple syndecan receptors, suggesting that binding of the invasion-associated Opa protein is specified primarily by the heparin sulfate side chains of the syndecan receptors, rather than the core protein itself. Moreover, Western blot analysis in a variety of epithelial cell lines permissive for gonococcal invasion did not reveal a common expression pattern for any particular proteoglycan. Hence, the invasion-associated Opa protein appears to use alternative proteoglycan receptors to mediate attachment and uptake. Considering the high conservation of the intracellular domains of syndecans, the interaction of gonococci with different syndecan receptors may still lead to the

generation of a common intracellular signal, which might be involved in organizing the cytoskeletal rearrangements associated with cellular invasion.

Characterization of serum-mediated invasion of gonococci into HeLa cells.
Apart from cell surface-associated heparin-sulfate proteoglycans, other host factors may potentially be involved in Opa-mediated cell invasion by gonococci. Factors present in serum have been correlated with cell invasion by *Neisseria meningitidis* (2). We are characterizing a serum-derived factor that mediates high levels of gonococcal invasion into HeLa cells as well as other cell lines. HeLa cells were infected with *N. gonorrhoeae* strain MS11 for 6 h and the number of intracellular bacteria was determined by the gentamicin survival assay. Bacteria were able to efficiently invade HeLa cells only in the presence of fetal calf serum (FCS). The number of intracellular bacteria increased in the presence of FCS in a concentration-dependent manner. Pre-exposure of the bacteria to FCS was sufficient to promote the gonococcal invasion of HeLa cells, suggesting that a soluble serum factor was directly bond by the bacteria in order to produce this effect. Among the various Opa proteins expressed by strain MS11, only the invasion-associated Opa protein mediated this serum-dependent cell invasion. Indirect immunofluorescence labeling of bacteria revealed that the level and pattern of bacterial attachment to HeLa cells was essentially identical regardless of the FCS concentration used. TEM confirmed that in the absence of FCS bacteria were observed only on the surface of HeLa cells and intracellular bacteria were rarely seen. Following the addition of FCS to the culture media, the number of intracellular bacteria increased in a time-dependent manner. Together these data indicate that FCS affects Opa-mediated invasion but not adherence. In order to discern how FCS mediates invasion, the effect of various cellular inhibitors on bacterial invasion were tested. Colchicine and nocodazol inhibited invasion at similar rates, while taxol did not. Cytochalasin D and genistein also inhibited the FCS-mediated invasion. Bacterial entry into HeLa cells thus seems to be dependent on microtubule integrity, microfilament rearrangement, and tyrosine phosphorylation.

We could speculate that the initiation of the cytoskeletal rearrangements which facilitate bacterial entry may involve a specific interaction between the invasion-associated Opa protein, the serum factor and cell surface-associated heparin sulfate proteoglycan receptors.

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Pathogenesis

Molecular interaction of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to host cell receptors.

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Pili of *N. gonorrhoeae* and *N. meningitidis* facilitate binding of the bacteria to epithelial cells (1-3), and undergo both phase and antigenic variation. PilC is a 110 kDa minor pilus associated protein for which two loci exists. Frameshift mutations in a poly G tract within the signal peptide coding region put the initiation codon in or out of frame and thereby turn PilC expression on and off (4, 5). PilC has been implicated in pilus biogenesis (4) and has been suggested to be a pilus tip located adhesin (6).

The *pilC* genes of *N. gonorrhoeae* and *N. meningitidis* have been characterized, and contain both conserved and variable regions. We have localized PilC to the membrane of both piliated and nonpiliated bacteria. In piliated bacteria PilC was found at the base of the pilus fiber. We present a hypothetical model for the PilC protein in the outer membrane and the possible role of PilC in translocation and biogenesis of pili.

We have data showing that CD46 (MCP, membrane cofactor protein) is likely to be a pilus receptor. CD46 is a widely distributed C3b/C4b binding cell surface glycoprotein which serves as an inhibitor of complement activation on host cells (7). CD46 has previously been identified as a receptor for measles virus and group A streptococci (8).

We are examining the role of divalent ions in the attachment of pathogenic *Neisseria* to eukaryotic cells. The extracellular level of specific ions dramatically affects the bacterial attachment to both human target cells and to CHO and MDCK cells. This attachment is independent of pili, PilC and Opa. The mechanism behind this is currently being characterized.

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Pathogenesis

In vitro interaction of *Neisseria meningitidis* (MC) with a monolayer of cells forming tight junctions as a model to study the crossing of the blood-brain barrier

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The brain capillary endothelium is responsible for the existence of the blood-brain barrier (BBB). Brain endothelial cells (ECs) differ from those present in peripheral capillaries by the presence of tight junctions which limit the paracellular flux. Since a model of human brain ECs forming tight junctions *in vitro* is not available, we used epithelial T84 cells. When grown on permeable supports, T84 cells form tight junctions.

We studied the crossing of *N. meningitidis* (MC) through a monolayer of T84 cells during a 24 h period. MC cross the monolayer 8 to 9 hours after bacterial addition. Tight junctions were not disrupted, as demonstrated by (i) the persistence of a transepithelial resistance, (ii) the absence of [^{3}H] inulin penetration through the monolayer, and (iii) the absence of disruption of ZO-1 labeling. Non piliated strains passed through the monolayer 100-fold less efficiently than piliated ones. Scanning electron microscopy (EM) suggested that MC interaction with this monolayer is a two step process. During the initial interaction clumps of bacteria were seen adhering. At later time points, when bacteria start crossing the monolayer, clumps disappear and bacteria spread onto the surface of the monolayer forming small craters on the apical surface. Transmission EM confirmed this intimate attachment, identified a concentration of dense material beneath attached bacteria and localized intracellular bacteria within a vacuole. Fluorescent microscopy showed actin reorganization on the apical surface of the cell. All together our data suggest that following pilus-mediated adhesion, a cross-talk between bacteria and cells leads to a transcytosis event allowing the bacteria to cross this monolayer.

Pathogenesis

Neisseria gonorrhoeae mutants lacking outer membrane protein Rmp (PIII) are deficient in invasion of human epithelial cells

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Neisseria gonorrhoeae (GC) possesses several outer membrane proteins involved in pathogenesis. One of these - PIII or Rmp, for reduction modifiable protein - is a major, antigenically stable protein expressed only in the pathogenic *Neisseria* (1). It is intimately (but not covalently) associated with PI, or Por, the gonococcal porin. A great deal of attention has focused on PIII since, in human infection, antibody to PIII blocks serum killing of certain strains of GC (2). In addition, antibody to PIII induced by previous gonococcal infection increases ones susceptibility to gonorrhea and to gonococcal salpingitis (3).

Clemens et al, in a poster abstract from an earlier Pathogenic *Neisseria* conference, observed that PIII mutants invade human fallopian tube organ cultures much less efficiently than their parent strain. In addition, Virji et al showed that a monoclonal antibody directed against the exposed sulfhydryl loop of PIII inhibits the ability of gonococci to damage Chang conjunctival epithelial cells in culture (4).

Gonococci (gc) expressing various surface components, including Opacity associated (Opa) outer membrane proteins, adhere to and invade human epithelial cells, and induce an oxidative burst in human neutrophils (PMNs) in an opsonin-independent manner. We report here that Opa^+ gc lacking Rmp exhibit dramatically reduced invasion. Two Opa^+/Rmp^+ gc strains, F62 and Pgh 3.2, and their respective *rmp* deletants were tested for their ability to adhere to and invade monolayers of the human cervical epithelial cell line, ME-180. Invasion was measured by resistance to extracellular gentamicin (50 µg/ml, 1 hr). Opa^+/Rmp^+ strains and their Opa^+/Rmp^- mutants adhered to ME-180 cells to similar degrees; 15.9 - 28.8 bacteria/cell (range, n = 4). However, the Rmp^- mutants invaded these cells ~50-fold less efficiently than Rmp^+; only 1 to 4 X 10^3 vs 1.0 to 4.1 X 10^5 (n = 4) per monolayer (in 24 well plates). Invasion background for Opa^- gc is 2 X 10^2. In experiments with human PMNs, Opa^+/Rmp^+ and Opa^+/Rmp^- strain pairs adhered to human PMNs to similar extents, and stimulated similar oxidative responses. These data suggest that Rmp mediates gc internalization into epithelial cells, but not stimulation of PMNs.

E. coli expressing an Opa protein on their surface adhere to and invade ME-180 cells in culture (5). This indicates that Opa mediates *E. coli(opa)* invasion. However, there is considerable homology between the C-termini of gonococcal Rmp and *E. coli* OmpA (6). An alternative interpretation of the *E. coli(opa)* invasion results is that Opa brings *E. coli* in close proximity to the host cell, and that OmpA mediates invasion. With this in mind, we transformed an *E. coli* lacking OmpA (BREΔ51), and its parent strain (MC4100), with plasmid pDS002 containing *opaP* from gonococcal strain F62SF. We performed adhesion and invasion assays with these

transformants. *E. coli* OmpA mutant BREΔ51(OpaP) and its parent MC4100(OpaP) both adhere to and invade ME-180 cells to similar degrees -- Adhesion: MC4100(OpaP) 10.6/cell; BREΔ51(OpaP) 9.8/cell. Invasion: MC4100(OpaP) 0.51/cell; BREΔ51(OpaP) 0.34/cell. Thus, OmpA is not involved in *E. coli(opa)* invasion of ME-180 epithelial cells.

Finally, we have begun structure-activity studies by observing epithelial cell invasion of some well-defined Rmp mutants (7). These preliminary experiments indicate that the C-terminal dozen amino acids of Rmp are not required for Rmp-mediated invasion, whereas the first disulfide loop of Rmp confers significant structural integrity or functional activity, since this mutant invades about 10-times less than the parent F62.

We conclude that: 1) Rmp is required by Opa+ gonococci for optimal invasion into, but not adhesion to, human ME-180 (cervical epithelial) cells in culture. 2) PIII is not required by Opa+ gonococci for adhesion to human neutrophils or subsequent stimulation of the oxidative burst. 3) Although there is significant homology between the C-terminus of *E. coli* OmpA outer membrane protein and GC PIII, the ability of *E. coli(opa)* i.e., *E. coli* expressing gonococcal Opa outer membrane proteins, to invade ME-180 cells is not OmpA-dependent. 4) The carboxyl terminus of PIII is not required for PIII's invasion-potentiating ability.

At least three hypotheses exist to explain our observations: 1) PIII directly mediates gonococcal invasion into epithelial cells. 2) PIII associates with PI (aka Por), which is involved in membrane perturbation of epithelial cells, and subsequent invasion. 3) PIII stabilizes the gonococcal outer membrane. Thus, in its absence, the membrane is perturbed to such a degree that Opa, or other putative invasins, can no longer mediate invasion. We are working to determine which of these is most likely.

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CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins for phagocytosis.

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Neisseria gonorrhoeae (GC), the etiologic agent of gonorrhea, can adhere to and penetrate mucosal epithelial cells and attain access to submucosal sites. Accumulating observations suggest that a family of outer membrane proteins, the phase-variable opacity (Opa) proteins, play a role in gonococcal pathogenesis (8). In gonococcal strain MS11, this family consists of 11 unlinked opa genes whose sequences are known. One distinct Opa protein, the OpaA, correlated with adherence and subsequent internalization of GC by Chang conjunctival epithelial cell lines (6). Moreover, studies (2,9) have demonstrated that the interaction of the $OpaA^+$ GC to epithelial cells involves binding to heparan sulfate on the cell surface.

One major property of Opa proteins is to stimulate adherence of the Opa^+ bacteria and their phagocytosis by polymorphonuclear leukocytes (PMN). Characteristically, some Opa proteins promot strong PMN phagocytosis such as OpaI in MS11, and other Opa proteins elicit intermediate interaction. However, OpaA strains do not stimulate PMN adherence and phagocytosis (1,5). Moreover, Opa^+ GC do not adhere to human monocytes, lymphocytes and HL-60 cells (promyelocytic cell lines). Farrell et. al. also noticed that the adherence and phagocytosis of Opa^+ GC by neutrophils could be enhanced dramatically if the PMN were pre-activated, suggesting that the receptors for Opa proteins may be located in granules (4).

Because OpaA mediated epithelial cell interactions were heparan sulfate dependent, we postulated that the binding component of other Opa^+ bacteria to PMN might be to proteins or glycoproteins. We examined whether $OpaI^+$ *E. coli* (pEXI) bind to a specific protein from PMN lysed with Triton X-100. Our date showed that pEXI bound a 30 kDa band, however, the control strains Opa^- *E. coli* (pGEM) did not.

CGM1a antigen, a CD66-related member of the carcinoembryonic antigen (CEA) family, is exclusively expressed in the granulocytic lineage, not in human monocytes, lymphocytes and HL-60 cells (7). This protein is ~30 kDa in size. Immunological studies using CD66 antibodies have described that CD66 reactive antigens are stored in granules and are surface exposed upon activation (3).

Based on these observations, we speculated that the component responsible for PMN and pEXI interaction might be CGM1a. A stably transfected CGM1a HeLa cell line (HeLa-CGM1a) was used to test this hypothesis, and the HeLa cell line transfected only with the vectors (HeLa-Neo) was employed as a control. These HeLa cell lines were kindly provided by Dr. Fritz Grunert, Institute for Immunobiology, Albert-Ludwigs University, Freiburg, Germany. We showed there was no adherence of pGEM to both cell lines, but pEXA adhered to both cell lines. pEXI attached to HeLa-CGM1a cells only. OpaA protein-mediated adherence could be blocked by soluble heparin in both cell lines, but the adherence of pEXI to HeLa-CGM1 was not influenced

by heparin. Furthermore, the interaction of pEXI to HeLa-CGM1a cells was blocked by anti CGM1 monoclonal antibody. When we studied the phagocytic ability of HeLa-CGM1a, pGEM (Opa^-) could not adhere to, or enter the HeLa-CGM1a. In contrast, HeLa-CGM1a bound and strongly engulfed pEXI ($OpaI^+$). Almost 30% of HeLa cell associated bacteria were gentamicin resistant. This result was confirmed by electron microscopy, which showed evidence for a classic phagocytic process; bacteria first attached to the cell surface and this was followed by appearance of phagocytic processes and membrane fusion. These results demonstrate that the non-phagocytic HeLa cells when expressing the CGM1a antigens mimic the functions of PMN; they bind and phagocytose $OpaI^+$ bacteria.

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Role of the gonococcal cryptic plasmid in epithelial cell invasion

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Strains of *Neisseria gonorrhoeae* differ in the number and type of plasmids harbored, however, a large portion carry a small 4.2 kb plasmid which was first identified in 1972 (1). The fact that this 2.6 MDa phenotypically cryptic plasmid appears in 96% of all clinical isolates is strong evidence for its possible selective advantage (2). Despite the construction of an isogenic strain with and without this plasmid, no difference in phenotype, such as auxotype, antibiotic sensitivity, membrane proteins, lipooligosaccharide structure, or phase variation frequencies of outer membrane protein PIIb or pilin could be detected (3). Attempts to isolate RNA in gonococci corresponding to the two putative cryptic plasmid transcripts identified by Korch et al.(4) have been unsuccessful. We report here that the cryptic plasmid is transcribed upon interaction with a Hec1B monolayer and plays a role in invasion of human epithelial cells.

To identify genes involved in invasion, gonococcal insertion mutants were made by transforming the parent strain, F62, with chromosomal DNA ligated to a *cat* cassette under the control of the *tac* promoter. A number of invasion-deficient chloramphenicol-resistant transformants were isolated by their inability to disrupt a Hec1B monolayer after an eighteen hour incubation. Nine clones were chosen for further study and their invasion indices were established via conventional gentamicin resistance assays (5), in which gentamicin-resistant intracellular and cell-associated gonococci are counted, and these indices were ranked relative to the parent strain. Of the nine studied, six were found to have a single copy of the P*tac cat* cassette inserted at various locations in the cryptic plasmid, which was verified by Southern blotting and sequencing data. This was strong evidence that the cryptic plasmid played a role in invasion.

To investigate this phenomenon, a derivative of the cryptic plasmid, pLES96, was created. Due to the putative overlap of the cryptic plasmid's two divergent promoters and termini, there was no naturally-occurring location in which to ligate a selection cassette which did not interfere with transcription. To rectify this, the termini of both transcripts were replicated via PCR and a copy of the P*tac cat* cassette was ligated between them. This allowed pLES96 to be replicated and transcribed as a wild-type cryptic plasmid yet also furnished a selection marker. Strain F62 was transformed with pLES96 and passaged on chloramphenicol plates to ensure complete replacement of the wild-type cryptic plasmid and this was confirmed by PCR. F62(pLES96) had its new invasion index established and it was found to be indistinguishable from the parent strain's index, indicating that pLES96 functioned in a similar manner to the wild-type cryptic plasmid. Finally, RUN (Rochester University *Neisseria*) 5288, a plasmidless isolate, had its unique invasion index established and was then transformed with pLES96, creating an isogenic strain with and without the plasmid. The invasion index for RUN 5288(pLES96) was found to be approximately five-fold higher than RUN 5288, confirming a role for the cryptic plasmid in human epithelial cell invasion.

To identify when the plasmid was transcribed, total RNA from RUN 5288 and RUN 5288(pLES96) was taken at various times after a) overnight growth on GC media plates plus Kellogg's supplement, b) growth in RPMI 1640 tissue culture media with 5% fetal bovine serum, ferric chloride and sodium pyruvate, and c) interaction with a confluent Hec1B monolayer. RT-PCR was performed on the RNA using a primer (B2) which annealed to the *cppB* transcript. Subsequent PCR of the resulting cDNA with B2 and a primer 5' to B2 (primer B1) amplified a 400 bp product with RUN 5288(pLES96) total RNA isolated at 3.5 hours after contact with the monolayer, but not with RUN 5288 RNA under any conditions or with RUN 5288(pLES96) RNA under any conditions other than after contact with the monolayer for at least 3.5 hours. Comparisons of the protein profiles of the gonococci recovered from invasion assays suggested that one or more new proteins were initially being synthesized approximately 3.5 hours after contact. These proteins reached a peak at 5 hours and decreased thereafter.

Successful invasion by *N. gonorrhoeae* can be divided into four parts: initial adherence to the cell, entry, intracellular survival, and growth. In this study, the temporal appearance of the cryptic plasmid transcript provides evidence that the plasmid's role is in intracellular survival and/or growth rather than in adherence or entry into the cell.

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Internalization of *Neisseria gonorrhoeae* by Chinese Hamster Ovary cells

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Gonococci producing a distinct opacity protein (OpaA in strain MS11) adhere to and are efficiently internalized by cultured epithelial cells of human origin. Both adherence and uptake require interactions between OpaA and heparan sulfate proteoglycans on the mammalian cell surface. Nonhuman cell lines such as Chinese Hamster Ovary (CHO) cells also support adherence of gonococci, which also occurs through interaction of OpaA with cell surface heparan sulfate proteoglycans. However, despite this similarity in the requirements for adherence, CHO cells are not capable of internalizing gonococci. In this report, we characterize this apparent deficiency, and identify a factor in fetal calf serum which is capable of mediating uptake of gonococci by CHO cells. In the absence of FCS, OpaA$^+$ gonococci adhered to, but were not internalized by CHO cells, whereas in the presence of up to 15% FCS, efficient uptake was observed. This effect was specific for OpaA producing gonococci, since uptake was not observed for bacteria producing either no Opa, or one of the other ten Opa proteins. Preincubation of bacteria, but not cells, with FCS also stimulated internalization, suggesting that a factor present in FCS was binding to the surface of gonococci and subsequently stimulating entry. Using a combination of chromatographic isolation procedures, we have identified the serum factor which mediates the internalization of OpaA$^+$ gonococci by CHO cells. Defining the molecular requirements for gonococcal uptake in CHO cells may provide useful information concerning internalization of gonococci by human epithelial cells.

Neisseria meningitidis toxicity for cultured human endothelial cells requires soluble CD14

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Vascular lesions, signifying endothelial damage, are commonly associated with meningococcal disease. Virji *et al* [1] demonstrated that *N. meningitidis* (Nm) caused cytopathic damage to human umbilical vein endothelial cells (Huvecs) *in vitro*. Using this model LPS was identified as the major toxic factor of Nm for Huvecs[2]. Several studies have shown that endothelial damage in the presence of low concentrations of LPS is dependent on serum factors, including the toxic effect of *H. influenzae* LPS for bovine endothelial cells[3,4]; although, at high concentrations of LPS, the serum requirement may be partially overcome. CD14 is a 55KDa myeloid cell receptor for LPS which is absent from endothelial cells, but is found in a soluble form in serum (sCD14). It has been demonstrated that sCD14 is necessary for endothelial cells to respond fully to LPS. In this study, we have investigated the dependency of meningococcal toxicity on serum and sCD14.

Cytotoxicity was assessed by inhibition of Huvec ^{3}H-thymidine uptake following a 5-hour incubation period and by phase contrast microscopic examination of monolayers following a prolonged incubation period. Cytotoxicity of a dose of 10^{7} Nm observed in the presence of 5% serum was reduced by approximately 50% if the serum was first heated to 70^{o}C for 30 minutes, suggesting that a heat-sensitive serum component was involved in the cytotoxic mechanism. Further experiments, in which Huvecs were incubated in the presence or absence of serum, revealed that the cytotoxicity caused by either Nm or purified meningococcal LPS was serum-dependent at low doses although at high doses of LPS (10mg/monolayer) or bacteria (>10^{6} per monolayer), the dependency on serum could be partially overcome. Greater than 0.5% serum was required for manifestation of meningococcal toxicity in the *in vitro* assays.

The role of CD14 was further investigated using neutralising (60bca) and non-neutralising (26ic) anti-human CD14 antibodies. Both ^{3}H-thymidine uptake assay and microscopic examination of monolayers showed that 10mg/ml 60bca was able to inhibit Nm toxicity for Huvecs in the presence of 1% human serum, whereas 26ic was without effect even at a concentration of 50mg/ml. In further experiments, recombinant human sCD14 was used in place of serum to investigate directly its role in cytotoxicity. In these experiments, 3mg/ml recombinant sCD14 was sufficient to support the cytotoxic activity of Nm.

In summary, at low doses of bacteria or purified LPS, the major pathway of LPS-induced cytotoxic damage appears to be serum-dependent, whilst at high doses a serum-independent pathway may operate. These findings are consistent with reports on other systems.[4-7] Inhibition of serum-dependent toxicity by anti-CD14 antibodies and the ability of sCD14 to substitute for serum in supporting toxicity suggests that the presence of CD14 is a critical requirement for meningococcal LPS-dependent cytopathic effect on human endothelial cells. However it is

likely that additional accessory factors in serum, such as lipopolysaccharide-binding protein, may also play a role by enhancing sCD14-supported cytotoxic damage.

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Inhibitory effects of a monoclonal antibodies against *Neisseria meningitidis* on bacterial adhesion and invasion of HeLa cells

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Adherence has been considered an important factor for the maintenance of bacteria on the mucosal surface of the host organism. Little is known about the mechanism of adherence of *Neisseria meningitidis* even though a brief period of pharyngeal mucosa colonization is known to to precede bacteremia and meningitis.

We investigated the effect of the cross reactive monoclonal antibody (MAb 8C7Br1), directed against the 50 kDa peptide of *N. meningitidis* and the 65 kDa peptide of *Escherichia coli*, on the capacity of adherence and invasion in HeLa cells.

Adherence assays were carried according to the technique described by Jones (1) and *E. coli* invasion assay as Miliotis (2). *N. meningitidis* of different serotypes and subtypes isolated from blood of patients with meningococcal meningitis were supplied by the Section of Bacteriology (Adolfo Lutz Institute). *N. meningitidis* strains were cultured according to Sacchi (3). *E. coli* (EPEC and EIEC) strains were grown in 3 ml of Trypticase soybroth for 16 to 18 h at 37°C. HeLa cells (ATCCCL2) were from Section of cells Culture (IAL). The cells were maintained in Dulbecco›s modified Eagle Medium (DMEM).

The MAb inhibition effect was determined by calculating the percent of invasion or adherence on HeLa cells. The inhibitory capacity of adherence on HeLa cells was compared with the controls carried out under the same conditions with PBS.

The inhibitory effect on Adherence on HeLa cells of *N. meningitidis* and *E. coli* is related to the dilution of the monoclonal antibody used in the assay. At dilution of 1:1000 the MAb 8C7Br1 showed inhibition of 50% in the adherence of *N. meningitidis* and *E. coli*. The inhibition of penetration into HeLa cells was 80% in *E. coli* assay.

This monoclonal can be very useful to selecting peptides for further studies on development of a polyvalent oral vaccine.

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Functionality of Por, Opa, and the 3F11 LOS epitope expressed simultaneously in *Escherichia coli* (EC): A gonococcal surrogate in the human Fallopian Tube model?

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Understanding the pathogenesis of *Neisseria gonorrhoeae* (GC) salpingitis has been hindered by the variability of GC virulence factors. GC Opa and 3F11 LOS can mediate inter-GC adherence and GC attachment/invasion to host cells. GC Por (a porin) may trigger invasion, as it spontaneously inserts into host cell membranes and subsequently interacts with cytoplasmic components (1-5). The multiple actions and interactions of these factors can make experimental interpretation difficult, but expression in EC eliminates the complications of GC phase and antigenic variation (4,6,7). By using blunt-ligation subcloning and co-transformation strategies, we co-expressed all three factors in various combinations in EC strain DH5a. This was documented by Western blot analysis. Co-expression of Opa and the 3F11 LOS epitope enhanced bacterial clumping, but simultaneous expression of Por and the 3F11 LOS epitope did not ($p < 0.01$). Dot blot analysis with whole organisms showed Por to be surface-exposed in EC. Por selectively enhanced vancomycin sensitivity (control MIC > 256 mg/ml vs 4 mg/ml for Por-producer), but did not change imipenem or ciprofloxacin MICs. Thus Por is present in the outer membrane of EC. Opa, Por, and 3F11 LOS are functional in EC.

Multiple cell culture models have been used to study GC pathogenesis, but results have sometimes conflicted. Because cell lines are several steps removed from natural GC target tissues, extrapolation to infections *in vivo* must be done cautiously. Many experimental infections of natural GC target tissue have utilized the human Fallopian tube organ culture model (FTOC). Quantitative studies in this model have been limited by technical difficulty. To circumvent these pitfalls, we developed a method that utilizes digital confocal microscopy with computerized image analysis to quantitate bacterial attachment and invasion in the FTOC model (8). The measurement strategy follows: Fluorescein-labelled antibodies (a green fluorochrome) stain the bacteria, rhodamine-phalloidin (a red fluorochrome) stains filamentous actin and defines the eucaryotic cell intracellular area, and Hoechst 33342 (a blue fluorochrome) identifies cell nuclei. For a given epithelial region, serial registered images are acquired in the z-axis for each fluorochrome. Next, out-of-focus haze is removed by digital confocal microscopy. By using computerized image analysis, cellular regions of interest are defined on digital confocal images of rhodamine-stained actin with a mouse-driven cursor, and the area of this region is measured in square microns. This region of interest template is superimposed on the corresponding fluorescein-stained bacterial image. Fluorescent objects are distinguished from background by choosing a grayscale “threshold” on a scale of 0 to 255. Total cell-associated bacterial areas are measured by including any threshold-defined fluorescent objects that are

inside or touching the template. In a second measurement, intracellular bacteria are quantitated by including only those bacteria that are inside but not touching the template. Extracellular attached bacteria are represented by the difference between these two measurements. An invasion ratio (IR) for each region of interest is calculated as "area of invasive bacteria/ total area occupied by cell-associated bacteria." One must assume that bacterial attachment is a precursor to any alleged invasion-triggering by Por. EC which express Por alone show little interaction with the epithelium, presumably because Por conveys no attachment advantage (unpublished observation). We compared EC expressing Por only (IR = 0.4265), to the following experimental groups: Opa alone (IR = 0.3271), 3F11 LOS alone (IR = 0.5344), Opa plus Por (IR = 0.616), 3F11 LOS plus Por (IR = 0.5166). The IR for the Opa plus Por group was significantly higher than the Opa alone group. Thus, Por caused a higher proportion of cell-associated bacteria to be intracellular or invasive ($p = 0.0012$). In addition, the area of attached bacteria corrected for the area of epithelium measured was significantly higher for the Opa-producing variants compared to 3F11 variants ($p < 0.002$). 3F11 variants showed a relatively low overall level of attachment or invasion in absolute terms, although the invasion ratio was intermediate. These data show that Opa is an attachment and invasion factor for GC in the FTOC and suggests that Por is an invasion factor when the bacteria first have a means to attach to the epithelium.

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Construction, characterization, and analysis of chimeric Opa proteins in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae MS11 contains 11 genes encoding opacity proteins (Opa, 1), a family of outer membrane proteins, which contribute to colony opacity and are believed to be involved in a number of functions including interaction with the host cells. Opa proteins are very similar to each other with the exception of one semi-variable (SV) and two hypervariable (HV) regions. Particular Opa proteins confer on *N. gonorrhoeae* the ability to adhere to tissue culture cells of human or non-human origin (2) and to be taken up by human conjunctiva (Chang) cells (3, 4). Other Opa proteins appear to be involved in the interaction of *N. gonorrhoeae* with human polymorphonuclear leukocytes (PMN's, 5).

In order to determine functional domains of the Opa proteins of *N. gonorrhoeae,* chimeric *opa*A, B, and C genes of *N. gonorrhoeae* MS11 were constructed using DNA amplification techniques. Three *opa* genes (*opa*A, B, and C) were selected from the repertoire of strain MS11 for the following reasons: OpaA mediates the adherence and uptake of *N. gonorrhoeae* by heparan sulfate-expressing epithelial cells (2, 6), but this Opa renders *N. gonorrhoeae* non-reactive with PMN's *in vitro* (7); OpaC expressing *N. gonorrhoeae* adhere to both Chang cells and PMN's but are only internalized by PMN's and not Chang cells (4); OpaB expressing *N. gonorrhoeae* do not interact with Chang cells but do adhere to and are internalized by PMN's (7), thereby resembling *N. gonorrhoeae* that express the other Opa proteins (D, E, F, etc.) of this strain's repertoire. That portion of each *opa* gene that encodes its HV_2 hypervariable region was replaced by the analogous region of the other two *opa* genes. In this way, six chimeric recombinant *opa* genes were constructed (AB_2, AC_2, BA_2, BC_2, CA_2, and CB_2).

Chimeric *opa* genes were expressed from a plasmid in *N. gonorrhoeae* MS11 as described by Kupsch et al. 1996 (8). The chimeric *opa* genes were cloned onto plasmid pH6a such that their expression was under control of the same synthetic *opa* promoter. The phase variable signal peptide encoding portion of the *opa* genes was modified to encode a leader peptide of orthodox amino acid sequence, but lacking the CTTCT repeat element as described by Kupsch et al. 1993 (4).

N. gonorrhoeae MS11 expressing mosaic *opa* genes were examined in several assays for Opa structure-function correlations. These include adherence to, and internalization by tissue culture cells, phagocytosis by PMN's, protection by heparin against serum bactericidal killing, susceptibility to polysulfated compounds, growth and colony characteristics and influence on whole cell (gonococcal) electrophoretic mobility.

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Deletion of *porA* by site specific recombination in clinical *Neisseria meningitidis* isolates.

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Class 1 outer membrane protein coded by *porA* of *Neisseria meningitidis*, is candidate to be a constituent in a vaccine against meningococcal infection. However, success of this vaccine may be hampered by the antigenic and phase variability of this protein. Recently, phase variation at the transcriptional level, which is mediated by a variable polyguanidine stretch between the -10 and -35 domains of the *porA* promoter has been described (1). This study describes phase variation by complete deletion of *porA*. From 1 patient with meningococcal disease a *porA* positive strain was isolated from the cerebro-spinal fluid while the same strain isolated from the blood was *porA* negative. From 2 out of 57 patients, which were infected with non subtypeable meningococci, *porA* negative meningococci were cultured from the blood as well as the cerebro-spinal fluid. In addition, two (H44/76, B:15:P1.7,16 and H355, B:15:P1.15) out of 9 isolates yielded *porA* deletion variants after pellicle growth. The sequence upstream from *porA* appears to be polymorphic and comprises sequences similar to a region upstream of *opc* (2). Homology was found with Neisserial repetitive sequences described by Correia et al (3) and with RS3 (4), which were also found downstream of *porA* (5). Sequence analysis of a *porA* deletion variant indicates site specific recombination causing the *porA* deletion.

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Gonococcal entry into Chang conjunctiva epithelial cells

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Gonococci that express a distinct member of the opacity (Opa) outer membrane protein family adhere to and are internalized by cultured human Chang conjunctiva epithelial cells. Binding of Opa to cell surface heparan sulfate proteoglycan receptor is a key element in the adherence and internalization process (1). Opa proteins that bind proteoglycans and confer entry into Chang epithelial cells have been identified for all (more than 20) clinical isolates that have been examined. To further unravel the molecular events that result in the internalization of the bacteria, we characterized the phagocytic event in more detail (2). Blockage of the clathrin-dependent receptor-mediated endocytosis process by chemical inhibitors (monodansylcadaverine, ouabaine) or potassium depletion of the host cells in combination with confocal laser microscopy suggested that the uptake of the bacteria was a clathrin-independent event. Experiments with the actin-filament disrupting agent cytochalasin D and microtubule inhibitors (nocodazole, colchicine) indicated an absolute requirement of an intact host cell cytoskeleton for bacterial internalization. Confocal microscopy staining for F-actin and gonococci showed a rearrangement of actin filaments during infection and accumulation of F-actin at the sites of bacterial entry. This bacteria-induced recruitment of actin was observed only for gonococci that expressed the Opa protein that binds the proteoglycan receptor. *E. coli* expressing this Opa protein however, did not induce accumulation of F-actin and were not internalized by the host cells though they adhered to the cells in a proteoglycan-dependent fashion. Co-infection experiments using *E. coli*-Opa strains and gonococci showed specific entry of gonococci only, suggesting that the gonococcal uptake process involves a classical phagocytic event rather than macropinocytosis reported for *Salmonella* and *Shigella* species. Further infection experiments using inhibitors of protein phosphorylation showed that the gonococcal entry process requires phosphorylation on phosphotyrosine residues. In the presence of genistein, large numbers of bacteria were arrested at the stage of adherence. Removal of the compound, resulted in rapid internalization of the adherent bacteria. Confocal microscopy on infected cells stained for bacteria and phosphotyrosine indicated intense phosphotyrosine signals at the sites of bacterial entry, that disappeared once the bacteria were completely internalized. These data suggest that Opa-protein expressing gonococci are able to induce their own uptake into epithelial cells by a phagocytosis-like process involving stimulation of tyrosine phosphorylation and recruitment of F-actin at the site of entry.

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Complement component C1q is required for IgA1-initiated killing of *Neisseria meningitidis*

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Resistance to disseminated meningococcal disease correlates with the presence in serum of strain-specific bactericidal activity (1). The principal mechanism by which this process is effected is antibody- dependent, complement-mediated immune lysis. We previously reported that human IgA1 can initiate lysis of group C meningococci via the classical complement pathway when bound to specific outer membrane proteins (2). We now report that the function of lytic IgA1 is dependent on an interaction with C1q of the classical pathway.

Our earlier studies had suggested a role for C1q in IgA1-initiated lysis based on the results of bactericidal experiments done in the absence of calcium which is required for C1q-dependent activation of the classical pathway. Using C1q-immunodepleted serum and purified C1q, we now examined whether C1q is required for IgA1-initiated lysis of group C strain MC19. IgA1 initiated complete lysis of stain MC19 in 20% normal human serum but was unable to effect killing in either 20 or 40% serum depleted of C1q. Reconstitution of the C1q- depleted serum with increasing concentrations of purified C1q resulted in a dose-dependent increase in IgA1-initiated lysis of strain MC19. C1q added at final concentrations of 1, 5, and 25 μg/ml resulted in killing of 63.3%, 89.3% and 86.0% of the organisms, respectively.

Since C1q has been shown to bind directly to porin proteins of certain Gram-negative enteric bacteria (3), we next investigated the binding of purified C1q to group C meningococci by using a whole bacteria ELISA assay. Nine strains which differed in their susceptibility to IgA1-initiated lysis were tested. All nine strains bound C1q, but to different degrees. The effect of IgA1 on C1q binding was studied by incubating the bacteria with IgA1 for 30 minutes before adding the C1q. In the presence of IgA1, six of the strains bound increased amounts of C1q suggesting that bound IgA1 exposes additional binding sites for C1q on the bacterial surface or that IgA1 per se contains binding sites for C1q. Western immunoblots of the outer membrane complex of strain MC19 showed that the primary site of direct binding for purified C1q was a 72 kDa protein. This protein may be identical to the 74 kDa protein expressed by multiple group C strains to which lytic IgA1 binds.

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Characterization of a eukaryotic pilus receptor for *Neisseria gonorrhoeae* and *Neisseria meningitidis*.

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Pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* facilitate binding of the bacteria to epithelial cells (1-3), and undergo both phase and antigenic variation. PilC is a 110 kDa minor pilus associated protein which has been implicated in pilus biogenesis and has been suggested to be a pilus tip located adhesin (4-6). In this study we aimed to identify a pilus receptor in the eukaryotic cell membrane.

We have data showing that CD46 (MCP, membrane cofactor protein) is a pilus binding protein on the eukaryotic cell surface. CD46 is a widely distributed C3b/C4b binding cell surface glycoprotein which serves as an inhibitor of complement activation on host cells (7). CD46 has previously been identified as a receptor for measles virus and group A streptococci (8). Typical for CD46 is that it migrates as a double band of approximately 55-60 kDa on SDS-PAGE.

We are examining the role of divalent ions in the attachment of pathogenic *Neisseria* to eukaryotic cells. Small changes in the extracellular concentration of specific ions affect the bacterial attachment to both human target cells and to CHO and MDCK cells. The mechanism behind this "ion-induced" attachment, which is independent of pili, PilC and Opa, is currently being characterized.

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High levels of interleukin 10 are associated with fatality in meningococcal disease

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Interleukin 10 (IL-10) suppresses the production of proinflammatory cytokines *in vitro* (1-5) and in murine models of endotoxemia (6-8) and has been suggested as a candidate for treatment of bacterial septicemia (6-8).

To investigate the role of IL-10 in meningococcal disease, a sandwich IL-10 enzyme amplified sensitivity immunoassay (IL-10 EASIA) was used to quantitate IL-10 in serum and cerebrospinal fluid (CSF) samples from 41 patients with serogroup B meningococcal bacteremia or meningitis with or without septic shock. High levels of IL-10 were demonstrated in sera from patients with meningococcal septic shock (mean 21,221 pg/ml, range 25 to 64,500 pg/ml). All cases involving fatalities had IL-10 concentrations of ≥1,000 pg/ml in serum (mean 23,058 pg/ml, range 1,000 to 64,500 pg/ml). The highest levels of circulating IL-10 were encountered in samples taken on admission and before initiation of therapy. Concentrations of IL-10 were positively correlated with previously reported levels of TNF- , IL-6 and IL-8 in serum in the same patients (9).

Patients with meningococcal meningitis without septic shock had comparably low concentrations of IL-10 in serum (mean 119 pg/ml, range 0 to 1,050 pg/ml), but exhibited compartmentalized release of IL-10 in CSF.

We conclude that IL-10 is extensively activated along with proinflammatory cytokines during the initial phase of meningococcal septic shock and that high levels of IL-10 in serum are associated with prognosis in meningococcal disease.

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The IgA1 protease of pathogenic Neisseriae increases LAMP1 turnover and promotes survival of bacteria in epithelial cells

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The ability of the pathogenic Neisseriae to avoid lysosome killing in epithelial cells was examined. Intracellular pathogenic Neisseriae decrease acid phosphatase activity and reduce LAMP1 (1) levels in infected cells, suggesting that infection of epithelial cells by pathogenic Neisseria leads to modification of late endosomes and lysosomes. The decrease in LAMP1 is due to an increase in the turnover rate of this protein. Several lines of evidence indicate that the Neisserial IgA1 protease plays a role in LAMP1 turnover. LAMP1 contains an IgA1-like hinge region with potential cleavage sites for the Neisserial IgA1 proteases (2,3). Unlike its wildtype otherwise isogenic parent (4), the iga- mutant cannot affect LAMP1 turnover, is coloalized with LAMP1 and fails to survive in epithelial cells. Many mucosal pathogens secrete IgA1 proteases and these enzymes are proposed to function in protecting colonizing bacteria from inactivation by mucosal immunoglobulins (5). Our results show that the Neisserial IgA1 protease plays an important role in promoting intracellular survival of pathogenic Neisseriae.

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Gonococci and meningococci traverse a polarized epithelium: maintenance of epithelial barrier function and importance of GC type IV pili

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We adapted the polarized T_{84} human colonic epithelial cell system to study *N. gonorrhoeae* (GC) and *N. meningitidis* (MC) infection of epithelial tissues. In these studies, all pathogenic Neisseriae examined crossed the monolayers. The traversal times are species-specific and identical to times established previously in organ culture studies : MC strains usually traversed the monolayers within 12-20 hours postinfection, and GC strains took ~40 hours (1, 2).

Barrier function of the polarized monolayers was assessed by measurement of transepithelial resistance (TER) and by measurement of [^{3}H]mannitol flux. In contrast to results obtained with some enteric pathogens (3-6), transmigration by GC and MC was not accompanied by a loss of integrity to the polarized monolayers until high numbers of bacteria were present in the subepithelial compartment.

Consistent with results from human volunteer and organ culture studies (7, 8), GC *pilE* mutants lacking type IV pili were compromised not only in adhesion, but in invasion and traversal of T_{84} cells as well. Our studies indicate that polarized T_{84} cells will be a useful tool for studying the biology of epithelial infections by GC and MC.

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Carbon metabolism of *Neisseria meningitidis* serogroup B with a view to potential pathogenic links.

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A vast amount is known about many aspects of the pathogenesis of *Neisseria meningitidis* but very little work has been carried out on its physiology and metabolism for the past thirty years. Providing a complete study on various aspects of metabolism will provide valuable information that may lead to the development and discovery of new and novel means of approaching the prevention and treatment of the infection.

Initially directional studies of the TCA cycle enzymes via both direct and indirect enzyme assays were carried out along with the determination of subsequent amino acid production patterns and labelling via NMR as described using other bacteria (1).

Using established enzyme assays the following enzymes relating to the TCA cycle were detected: Malate dehydrogenase, Fumarase, Succinate dehydrogenase, Succinate thiokinase, 2-Oxoglutarate dehydrogenase, Isocitrate dehydrogenase, Aconitase, Citrate synthase and Pyruvate dehydrogenase. . Neither Isocitrate lyase or Malate synthase were detectable indicating the absence of a glyoxylic bypass system. This result seems logical when considering the bypass is primarily used for the metabolism and growth on acetate as the sole Carbon source and the fact that *N. meningitidis* is unable to grow on acetate as sole Carbon source. Of the enzymes involved in Carbon dioxide fixation only PEP Carboxylase has so far been detected.

The NMR study results established that amino acid production was essentially derived, as indicated from Carbon-13 enrichments of individual Carbon atoms, from a cycle that operates oxidatively. The slight scrambling of signals that occurred results from the turnover and re-synthesis of end-products which in itself signifies that an oxidative cycle is in operation.

Perhaps of most interest was the fact that Malate dehydrogenase (MDH) was not detectable via the conventional NAD-dependent Malate oxidation or the NADH-dependent Oxaloacetate assays; in fact the enzyme was only detectable via the reaction used for Succinate dehydrogenase (SDH) with Malate instead of Succinate as the substrate. In order to determine the cellular location of this enzyme the cell-free extract was ultracentrifuged and the membrane and cytoplasmic portions where assayed for MDH activity. Enzyme activity for MDH was detected only in the membrane fraction. Enzymes, such as this MDH, that are dye-linked *in vitro* are often found to be linked to the Electron transport chain *in vivo*. This type of enzyme is present in other bacteria and, on purification, has been found to be a Flavoprotein (2,3). At present this protein is in the process of being purified and consequently the exact characteristics and its targeting potential can be defined.

Conclusion. Overall the TCA cycle operates in an oxidative direction; although reversible reactions occur there is a strong tendency for the cycle to occur oxidatively. The NMR work also showed that amino acids were formed from precursors that predominantly originated from an oxidative TCA cycle. This is distinguishable by the fact that different and distinct 13C labelling of TCA intermediates and consequently amino acids occurs depending on whether the cycle is oxidative or reductive. Also the glyoxylic bypass is inoperative due to the lack of activity or absence of the two enzymes involved.

A novel protein in the form of membrane-bound MDH was found. This protein is linked to the electron transport chain and is most likely a flavoprotein. This sort of membrane-bound enzyme is referred to as dye-linked. The potential of this MDH is as a possible target for a metabolic inhibitor, but that would require the purification and characterisation of the enzyme.

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Activation of NK-kB and cytokine gene expression in *Neisseria gonorrhoeae* infected epithelial cells

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Epithelial cells represent the first barrier for infecting organisms and have a supporting role in the immune response against parasites. Within the course of microbial adhesion, inflammation or invasion in eukaryotic epithelial cells, small amounts of induced cytokines and chemokines exert a protective role in host defense. Activation of these immune response genes is mediated by immediate early response factors.

We studied whether human epithelial cells transcribe genes encoding cytokines and chemokines after infection with *Neisseria gonorrhoeae* (Ngo) using duplex reverse transcriptase polymerase chain reaction (RT-PCR) assays. Furthermore, we analyzed the activation of potential transcription factors involved in the regulation of a variety of target genes (e.g. immediate early immune response genes) by using electrophoretic mobility shift assays (EMSA) and Western blots.

Epithelial cells infected with Ngo strain F3, which shows adherence at the epithelial cell surface, but no invasive behavior, exert *de novo* synthesis of cytokine specific mRNAs within 15 min after infection for IL-8, TNFα, TGFβ, GM-CSF and Rantes, while intracellular levels of IL-1α, Il-1β, and IL-12 levels are not effected. Cytokine and chemokine mRNAs of IL-3, IL-4, IL-5, IL-6, IL-10, MPC-1 and I-309 were not detected. The transcription factor NF-κB, involved in the activation of several cytokine gene transcripts, becomes strongly activated both in adherent and invasive Ngo strains F3 and VP1, whereas weak activation can be observed at transcription factor AP1.

Ngo infection results in NF-kB activation, new gene transcription, and induces mRNA accumulation of proinflammatory cytokines (TNFα, and IL-8), of monocyte and memory TH cell attractant Rantes, a cytokine involved in growth and differentiation (GM-CSF), and a growth repressive compound, (TGFβ) in epithelial cells. Therefore, with respoct to our *in vitro* data, we suggest that epithelial cells infected with Ngo have the ability to recruit and activate PMNs and monocytes by the release of cytokines/chemokines. These results emphasize an important role of epithelial cells in the defense against Ngo infection.

A recombinant molecule of *Neisseria gonorrhoeae* that confers C1q dependent virulence in experimental animals.

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An experimental gonococcal infection (1) was conducted to investigate the importance of a recombinant molecule of pRP350, as a virulence determinant of *N. gonorrhoeae*. A recombinant molecule pRP350 sufficient to transform strain F62 to serum resistance was prepared from *sac-4* of *N. gonorrhoeae*, a serum resistant clinical isolate. Deletion of the 100 bp DNA segment resulting in pRP240, abolished the ability of the plasmid to confer serum resistance. Inocula of both recombinants of F62, serum resistant SN350 and serum sensitive SN240 transformed with pRP350 and pRP240 respectively, were prepared and standardized by colony count. Rat pups were randomized to receive either the ser^R or ser^S recombinant strains with or without C1q. Seven separated experiments were conducted using progressively increasing doses of C1q (between 5 µl to 100 µl/ml) with constant concentration of bacteria (10^6). Suspension of 100 µl was injected intraperitoneally and animals were monitored for symptoms of infection. Blood, peritoneal fluid and tissue samples were collected for culture. The results show that the rat pups injected with ser^R recombinant SN350 but not with ser^S SN240 in the presence of C1q developed bacteremia. None of the animals injected with *N. gonorrhoeae* SN350 and SN240 without C1q developed bacteremia. Quantitative colony counts in blood and tissues showed significant increase proportional to the concentration of C1q/ml in the inoculum. Current study indicates that the recombinant molecule pRP350 appears to be an essential determinant for C1q dependent virulence in an animal model. Deletion of 100 bp segment resulting in pRP240, abolished both the ability of the plasmid to confer serum resistance to human serum *in vitro* and virulence in animal model.

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Neisseria meningitidis infections and terminal complement component deficiency in Ireland. Diagnosis and management, and investigations of C6*/C7* DNA haplotypes.

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Investigations of complement function in patients presenting with recurrent meningococcal disease (1) in Ireland has led to the diagnosis of two families with C7 deficiency, one family with C6 deficiency and one family with C8 deficiency. In addition one patient in his sixties, with no history of meningococcal infection, was found to have C9 deficiency.

The diagnosis of C6 or C7 deficiency was made using a double diffusion functional haemolytic assay that screens for C5, C6, C7 or C8β deficiency (2). C9 deficiency was diagnosed because of abnormal total complement activity and absence of antigenic C9.

Management of all cases except the C9 deficient individual was counseling and administration of the tetravalent polysaccharide meningococcal vaccine (3). Patients were given prophylactic penicillin during times of particular risk (4).The index case in the C6D family was approximately forty years old, and at diagnosis of C6 deficiency she had suffered three episodes of meningococcal infection. At that time she was not given penicillin, but it was prescribed in order to be available should symptoms recur. In fact, she forgot to take medication with her on holiday,. Within two weeks of receiving the vaccine, and while on holiday, she again suffered a meningococcal group C infection Pre- and post-vaccination anti-group C antibodies showed that she had adequate pre-vaccination antibodies but that there was a slight, but definite, drop in antibody level two weeks post vaccination, followed by rise in IgG antibodies a month later. It is possible that vaccine produced yet another "window of susceptibility" and these post vaccination periods should be covered by penicillin.

The C6 deficient family comprises six sibs and a study of DNA marker haplotypes showed the affected sibs were homozygous for the C6* DNA markers but heterozygous for several C7* DNA markers (5). It was not possible to determine whether there were two different C6 defects or one defect that has become associated with two different C7 genes. The C6*Q0 haplotype was different to that reported for a cohort of 18 South African C6Q0 cases (5) suggesting that when the DNA defects are identified they will be different. DNA haplotypes were studied in one of the C7D families and again heterozygous haplotypes were found in the affected subjects indicating that the two molecular defects in the C7 genes will also differ.

Thus the terminal complement deficiencies in Ireland are heterogeneous in the components affected and probably in the genetic defects within the genes. Although there are reports of mild disease in some terminal complement deficient individuals (6) the index case in the C8 deficient family and a C7 deficient member of one of the families had suffered extremely severe disease

in which they nearly lost their lives. Thus in complement deficient individuals, as well as sufficient individuals, there is a wide spectrum of disease severity although there may be more mild cases in complement deficient individuals. The differences in disease severity may depend on the *N..meningitidis* strains (7) as much as on differences in host factors in deficient subjects.. Counseling and prophylaxis against meningococcal disease is very important in these patients and they remain at risk even at times when there is a low incidence of disease in the population at large.

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The problem of septic shock in patients with meningococcal disease

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The management of patients with meningococcal septic shock is the actual problem for intensive care medicine. Despite large clinical experience, modern equipment for intensive care, effective antibiotics and improving of treatment based on the recent principles it does not lead to increasing survival of patients with septic shock. We have studied clinical data of 3391 patients with meningococcal disease admitted to the Hospital in 1984-93 and especially the records of 1271 patients admitted to the ICU. There were 352 patients with septic shock: 201 (57%) patients developed multiple organ failure (MOF) and 151 (43%) patients were without MOF. The lethality for patients with meningococcal disease was 6.6% (221/3391) in all, 17.4% (221/1271) for patients treated in ICU, 49.4% (174/352) for patients with septic shock, 83% (167/201) for patients with septic shock and MOF. In 50% patients with MOF the fatal outcome occurred during less than 4 hours; 77% patients died during 24 hours after admission at Hospital. The main cause of fatal outcome in patients with septic shock was MOF. The endotoxin level had prognostic value for the fatal outcome of patients with septic shock. The median endotoxin level (12200 ng/ml) in plasma of septic shock patients with MOF was approximately ten times greater than that for patients without MOF (1300 ng/ml). We had never observed the increase of endotoxin level in patients' plasma after start of antibiotic treatment (penicillin). On the other hand, we could not demonstrate the usefulness of phasmapheresis for endotoxin elimination from blood, because high rate of natural endotoxin clearance was observed in different patients including those with MOF. The importance of tumor necrosis factor-alpha (TNF) in patients with meningococcal disease was estimated carefully in different groups of patients with septic shock. We showed the correlation on admission of patients between TNF and LPS ($p<0.02$), TNF and some shock signs, such as a low urine output ($p<0.05$), an increase of respiratory rate ($p<0.05$), a low level of platelets ($p<0.05$), and a low mean blood pressure ($p<0.05$). However, we did not find the significant difference of TNF levels in patients without shock (715+153 pg/ml), patients with shock (929+134 pg/ml) and patients with shock and MOF (1311+262 pg/ml), although significantly different lethality was registered in these patients' groups. Our data suggested that the TNF level was not correlated with the MOF development and lethal outcome in studied patients' groups. The hereditary deficiency of terminal component complement predisposes for meningococcal disease, plasma bactericidal activity is absent. However, septic shock develops rarely in these patients. On the other hand, for complement sufficient patients with septic shock and MOF, the low level of complement activity correlated with fatal outcome ($p<0.02$). High level of complement activation and consumption occur in patients having high blood endotoxin level; endotoxin binding to the endothelial surface would result in forming of membrane attack complexes at these areas. Complement dependent endothelial cell damage might be one of the specific mechanism of MOF development in patients with meningococcal disease. The secondary activation of DIC at the areas of endothelial damage leads to microcircular disturbances and skin lesion. Our data suggested that the high level of endotoxin and complement consumption were

the obligatory conditions for MOF development in patients with meningococcal disease. TNF serum levels were elevated in different patients with shock, but not correlated with MOF development and fatal outcome. So anti-TNF strategy would not be promising for the treatment of patients with meningococcal septic shock.

Characterization and surface translocation of pilus associated protein PilC of *Neisseria gonorrhoeae* and *Neisseria meningitidis*.

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PilC, a pilus associated protein of *Neisseria gonorrhoeae* and *Neisseria meningitidis* is essential for pilus biogenesis and adhesion (1-5). Insertional inactivation of *pilC*1, *pilC*2 or both in *Neisseria gonorrhoeae* and *Neisseria meningitidis* indicated that PilC1 and PilC2 have a similar function in *Neisseria gonorrhoeae* MS11. In *Neisseria meningitidis* only PilC1 is essential for adhesion while either PilC1 or PilC2 can carry out the pilus biogenesis (4).

Sequencing of *pilC*1 and *pilC*2 of *Neisseria meningitidis* revealed two open reading frames encoding proteins of 1063 and 1046 amino acids with 84.7% homology and 74.5% identity. Sequence comparison of PilC1 and PilC2 of meningococci and gonococci showed the presence of variable and conserved regions.

PilC has been suggested to be a tip located adhesin of type IV pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* (3). In order to characterize the receptor binding domain of PilC, fusion proteins were generated against different overlapping regions of PilC and antibodies were produced against these fragments. None of the fusion proteins or the antibodies against them could inhibit the binding of gonococci or meningococci to epithelial or endometrial cell lines.

In order to identify the surface location of PilC immunogold electron microscopy was performed on whole cells, crude pili extracts and cryosections of different *pilC*, *pilE* mutants and their wild type strains. PilC was identified on the membrane of the bacteria in cryosection, or at one end of shadded pili but never on the tip of a intact pilus. Agglutination experiments using *S. aureus* coated with affinity purified anti-PilC antibody were in agreement with electron microscopy data.

These results indicates that PilC is probably located on the surface of the bacteria and helps in translocation of an unidentified pilus adhesin or modulates the pilus dependent adhesion by in an unknown mechanism.

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Experimental coinfection of human rhinopharyngeal mucosa with influenza virus and Group B *Neisseria meningitidis*

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There is an epidemiological association between meningococcal disease and both Influenza A (1) and B (2) virus infection. We tested the hypothesis that influenza virus influences meningococcal interaction with upper airway epithelium using experimental coinfection of explants of human rhinopharyngeal mucosa with influenza B virus and a clinical isolate of BI5P1.7,16,L3,7,9 (NmB). Explants were inoculated with Singapore B/222/79 for 48h. Infection was verified by serial quantitative ELISA, and by immunoperoxidase staining which demonstrated viral antigen within 5-20% of sustentacular cells. After 48h, virus-infected and -uninfected explants were inoculated with 5 x 10^5 cfu of NmB. Over a further 24h incubation NmB replicated by 2-3 logs with no significant difference between virus-infected and -uninfected explants.

Virus infection modified epithelia, but by morphometric analysis of transmission electron micrographs (3) no increase in subsequent bacterial association with virus-infected epithelium was observed. Internalization of NmB by epithelial cells and penetration of basement membrane by NmB occurred in virus-uninfected explants only (n = 5).

To test whether virus infection results in novel expression of epithelial receptors for NmB, explants were solubilized, electrophoresed and Western blotted onto nitrocellulose (NC) filters. NmB were labelled by culture in leucine-free MEM containing ^{14}C leucine and incubated with NC filters for 2h. By autoradiography NmB was shown to associate with NC filters in regions corresponding to 60-70 kDa, but there was no difference between bands derived from virus-infected and -uninfected explants.

Conclusion: Influenza B virus did not alter NmB interactions with rhinopharyngeal mucosa in vitro.

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Intracellular *Neisseria gonorrhoeae* bind host pyruvate kinase via their Opa proteins

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Gonococci bind to and invade human epithelial cells and likely reside within the cytoplasm (1). One family of outer membrane proteins involved in gonococcal adhesion and invasion are the Opa (Opacity-associated) proteins (2). In these studies, we looked for host cell proteins that could interact with gonococcal Opa proteins, and determined if such interactions occurred in vivo. Using CLONTECH's MATCHMAKER yeast two-hybrid system, we identified 5 plasmids containing HeLa cell cDNAs coding for potential Opa-Interacting Proteins (OIPs). One of these, OIP3, is human Pyruvate Kinase (PK) subtype M2.

PK is both a glycolytic enzyme, converting phosphoenolpyruvate (PEP) to pyruvate, and a cytoplasmic thyroid hormone (triiodothyronine, T3) binding protein (3). PK exists in two forms within a cell: monomers and homotetramers. PK monomers are relatively inactive as glycolytic enzymes, but can avidly bind T3, serving as cytoplasmic receptors for T3 (3). PK homotetramers are formed from PK monomers and are active as glycolytic enzymes, but cannot bind T3. The in vivo monomer-homotetramer interconversion is regulated through glucose metabolism via intracellular fructose 1,6- bisphosphate (F1,6P2) concentrations (4). T3 also stimulates transcription of the PK subtype M1 gene (5). Thus, PK is a key enzyme in regulating cellular ADP, ATP, and pyruvate, and mediates cellular metabolic effects induced by T3 (6).

In an attempt to confirm the yeast two-hybrid results, we investigated the ability of Opa(+) and Opa(-) gonococci, and of Opa(+) and Opa(-) *E. coli*, to bind commercially available rabbit muscle PK subtype M1 in vitro by employing a standard pyruvate kinase assay. Rabbit PK subtype M1 is 96% similar and 93% identical to human PK subtype M2 at the amino acid level over the entire length of the protein, and 90% similar and 84% identical at the amino acids corresponding with OIP3 (A366-P531). Opa(+) bacteria bound substantially more PK subtype M1 than did Opa(-) bacteria. Observations were dose-dependent for bacteria and PK concentrations. These in vitro binding studies indicate that bound PK retains its enzymatic activity.

To determine if Opa binds PK in vivo, rabbit antiserum was raised against recombinant human PK subtype M2 and used to determine if PK surrounds *N. gonorrhoeae* within host cells. Opa-expressing gonococci were allowed to invade ME-180 human cervical epithelial cells for 4 hours, the cells were fixed and probed with anti-PK antiserum followed by fluoresceinated anti-rabbit antibody, and visualized via confocal fluorescent microscopy. These in vivo studies revealed that intracellular, but not extracellular, gonococci bind PK.

Gonococci are thought to use only three carbon sources - glucose, pyruvate, and lactate (7). Intracellularly, there is little available glucose, since glucose is present mainly as glucose-6-phosphate. Pyruvate, on the other hand, can be continuously produced intracellularly. It appears

that intracellular gonococci bind active PK, and use it to supply themselves with pyruvate. Additionally, this interaction may enable intracellular gonococci to interact with the T3 metabolic pathway.

These results suggest that (a) the yeast two-hybrid system can be used successfully to investigate host-parasite protein-protein interactions, and (b) gonococci can bind a metabolic enzyme (PK subtype M2) via their Opa proteins for the purpose of gaining access to a carbon source or growth substrate (pyruvate), and/or to alter host cell metabolism to their advantage.

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The role of the lutropin receptor in gonococcal invasion of Hec1B cells: A preliminary report.

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Pelvic inflammatory disease (PID) is a major health issue, affecting more than one million women in the U.S. annually, and the most costly consequence of sexually transmitted disease. The total annual cost of PID is estimated to reach 10 billion dollars by the year 2000. The distribution of gonococcal PID is not random, but is clustered at menses. The reasons for this distribution are unknown. Hypotheses include the effects of hormonal influences, the milieu of the menstrual flow, or both. The two major infectious agents responsible for the majority of PID are *Neisseria gonorrhoeae* (GC) and the obligate intracellular parasite,*Chlamydia trachomatis* . It is known that gonococci can exist as facultative intracellular parasites in the human endometrial cell line Hec1B (1). We are interested in determining what environmental conditions influence the development of invasive GC and whether these factors are involved in gonococcal PID.

Invasion is an important virulence factor for many bacterial pathogens. Gonococcal invasion is known to be a multifactorial process involving pilin, opa proteins and LOS**.** In addition,we have reported a contact-inducible invasion mechanism in gonococcal strain F62 (2). The contact-inducible invasive phenotype (Inv^{+}) requires protein synthesis as its development is blocked by the addition of chloramphenicol. This conversion is also host cell specific, as only human cells from the reproductive tract are capable of converting F62 to the Inv^{+} phenotype (3). Since gluteraldhyde-fixed cells are capable of inducing the Inv^{+} phenotype, the initiating host cell factor(s) must be constitutively present on the cell surface, i.e. not secreted in response to the gonococcus, and unique to human reproductive cells. Previous studies of the effect of human chorionic gonadotropin (hCG) on GC invasion in the fallopian tube organ culture (FTOC) system demonstrated a time-dependent effect (4). Long term exposure of the FTOC cells to hCG resulted in less invasion while pre-treatment alone resulted in higher gonococcal invasion as compared to untreated controls. The Hec1B in vitro system also shows time-dependent effects of hCG treatment on both adherence and invasion .

A putative adhesin induced in the Inv^{+} GC results in enhanced binding to Hec1B cells, a human endometrial cell line, both in terms of rate and final level of adherence, exceeding Inv^{-} GC adherence by two-fold. Addition of hCG simultaneously with GC in adherence assays leads to a dose-dependent decrease in the adherence of both Inv^{+} and Inv^{-} GC. Although the magnitude of the effect is different, the relative loss of binding is equivalent for both phenotypes. Competitive binding assays showed that Inv^{+} GC interact with Hec1B targets via a unique mechanism that is not blocked by addition of 100x excess of Inv^{-} GC. Addition of hCG to this competitive assay eliminates the enhanced binding of Inv^{+} GC to the Hec1B targets. This implies that the Inv^{+}-specific adherence to Hec1B cells is mediated by the lutropin receptor (common receptor for hCG and luteinizing hormone). Addition of hCG to invasion assays decreases only Inv^{+} GC uptake, with minimal effect on Inv^{-} GC invasion. These data suggest that the access to the

lutropin receptor is critical for gonococcal adherence that leads to subsequent uptake of Inv^{+} GC. Overnight exposure of either Hec1B induction monolayers or invasion targets to hCG, conditions known to stimulate down-regulation of the lutropin receptor (5), affects gonococcal invasion in two ways. First is the loss of the ability of treated Hec1B cells to convert GC to the Inv^{+} phenotype. The second is a decrease in the susceptibility of hCG treated Hec1B targets to invasion by previously induced Inv^{+} GC.

This evidence strongly suggests a dual role for the lutropin receptor in gonococcal invasion both as the induction signal recognized by Inv^{-} GC and as the specific uptake mechanism utilized by Inv^{+} GC. Lutropin receptors have recently been identified in the fallopian tubes (6) and many of the tissues lining the female reproductive tract. The level of lutropin receptors is not constant but varies with the menstrual cycle, rising from mid-cycle to peak levels at the end of the cycle (6). The observed clustering of gonococcal PID with the onset of menses (7,8) may be due to a lutropin receptor mediated increase in conversion of GC to the Inv^{+} phenotype.

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High erythromycin prescribing during an outbreak of meningococcal disease

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During a prolonged outbreak of meningococcal disease caused by serogroup B serotype 15 sulphonamide resistant strains in one British health district, there was considerable variation in attack rates by town. General practitioner (GP) antibiotic prescribing rates were compared in high and low incidence towns. The only significant difference found was that erythromycin prescribing was more frequent in the high incidence towns (risk ratio 4.0, 95%CI 3.2-4.8 in March 1987 and 3.0, 95%CI 2.4-3.7 in November 1987). This was probably due to increased GP consultation rates for upper respiratory tract infection, but higher erythromycin usage may have increased meningococcal acquisition rates or susceptibility to meningococcal disease.

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Respiratory syncytial virus infection and meningococcal disease

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Although viral respiratory tract infections may predispose to meningococcal disease, strong evidence that they do so exists only for influenza. Data on laboratory reported cases of respiratory syncytial virus (RSV) infections and meningococcal disease in England and Wales were examined from mid-1989 to mid-1994. Although the rise in RSV cases preceded the rise in meningococcal disease cases each winter, the interval between the rise and fall of the two diseases was inconsistent, no association was found between time series after removal of the seasonal component, and there was no evidence that more cases of meningococcal disease occurred in winters with more RSV disease. RSV may have less effect on the two most likely mechanisms whereby influenza predisposes to meningococcal disease, namely lowered immunity and impaired pharyngeal defenses.

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Gc susceptibility to heparin-like compounds

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Gonococci (Gc) resemble a number of other sexually-transmitted infection-causing microbes (chlamydiae, HIV, herpes virus) in utilizing heparan sulfate-bearing glycosaminoglycan molecules of host epithelial cells as ligands for adherence. Such interactions with tissue culture cells are restricted to Gc variants expressing certain Opa proteins and can be inhibited by several sulfated compounds. Accordingly, highly sulfated compounds may be useful for nonimmunological prophylaxis against gonorrhea and other sexually-transmitted infections.

Heparin, highly-sulfated forms of heparan sulfate or dermatan sulfate, dextran sulfate of differing molecular sizes, and two sulfated poly/vinyl alcohol-acrylic acid/copolymers (PAVAS, PAVS, ref. 1) all induce colony opacity and some inhibit Gc growth at high concentrations. Light plus scanning and transmission electron microscopies were used to define attendant morphological changes in Gc. After studying a large collection of spontaneous and recombinant phenotype variants, we conclude that:

1) Opa expression correlates with enhanced susceptibility to heparin, PAVAS, etc.; only variants that express certain Opa proteins are more susceptible than their respective Opa$^-$ variants.
2) Opa$^-$ Gc expressing porin IA are more susceptible to PAVAS and heparin than otherwise identical porin IB Gc
3) Outer membranes of Opa$^+$ organisms exposed to PAVAS form many small, spherical blebs whereas Opa- cells form larger blebs and elongated structures.
4) LOS phenotype of Gc has little apparent influence on susceptibility.
5) There is an apparent inverse correlation between susceptibility to PAVAS and presence of a silver-staining protein of M_r = 36 kDa (p36) whose structure is undefined.

The last-noted relationship is especially evident for Opa$^-$ *rfa*F organisms whose colony opacities vary with p36 amount. But p36 may also be relevant also to PAVAS susceptibilities of Opa$^+$ versus Opa$^-$ Gc; p36 is absent when some, but not all, Opa proteins are expressed, and PAVAS susceptibility is highest for Opa$^+$ variants devoid of p36 and lowest for Opa$^+$ variants with unchanged amounts of p36.

Opa$^+$ Gc typically show enhanced positive charge whose magnitude varies according to which Opa is expressed. Those Opa proteins conferring greatest positivity have the greatest net excesses of cationic amino acids (arginine, lysine) in their surface-exposed domains and have highest (most alkaline) pI's. PorIA Gc are also more positively charged than Gc with PorIB and PorIA has a higher pI than PorIB. Taken all together, these observations suggest that polysulfated compounds interact electrostatically with arginine/lysine-rich surface-exposed portions of Opa and Por polypeptides. We suspect that polysulfated compounds cause conformational alterations of the outer membrane proteins *in situ*, analogous to those seen in

apolipoprotein E after its interaction with heparin (2). Such conformation changes likely induce distort outer membrane geometry such that vesiculation (blebbing) ensues (3). In this way, the expression of both PorIA and certain Opa proteins "destabilizes" the outer membrane of Gc when they are exposed to polysulfated compounds. Understanding the apparent relationships among p36, Opa phenotype, and PAVAS susceptibility awaits elucidation of p36's structure and function.

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The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis*

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We have used COS cells transfected with cloned cDNA encoding several distinct surface molecules which include constitutively expressed or inducible human cellular adhesion molecules (1,2) in order to identify receptors for meningococcal adhesins. These studies showed that some meningococci adhered only to transfected cells that expressed the CD66a molecule at high levels. CD66a (BGP, biliary glycoprotein) is a member of the Immunoglobulin superfamily. It belongs to carcinoembryonic antigen (CEA or CD66) family, which comprises numerous structurally related, secreted, GPI-anchored or trans-membrane proteins (2,3).

The meningococcal ligand that targeted CD66a was identified by the use of stably transfected CHO cells expressing the receptor (CHO-66a) (2). Starting from an inoculum of a capsulate serogroup B strain expressing L3 LPS immunotype, Opa, Opc and pili, a highly adherent phenotype was obtained after 9 cycles of selection on CHO-66a. This phenotype had lost capsule and pilus expression and was >25 fold more effective than the parental phenotype in adhesion to CHO-66a and did not adhere to non-transfected CHO cells or CHO cells transfected with CD33. The hyper-adherent phenotype also produced an altered LPS type (L8 immunotype). The expression of Opa and Opc proteins was retained. The interactions of the hyper-adherent phenotype with CH0-66a were inhibited by a monoclonal anti-CD66 antibody. These results suggested that either one or both of the outer membrane opacity proteins may be responsible for observed adhesion to the host cells tested. The loss of surface sialic acids (capsule and L3 LPS) also favors maximal interaction via outer membrane proteins as has been reported previously (4).

The involvement of meningococcal Opa proteins was demonstrated in overlay experiments in which chimeric CD66a-Fc soluble receptor molecules were applied to electrophoretically separated bacterial proteins. The receptor-ligand interactions were confirmed by co-precipitation experiments. Three soluble Fc constructs were used which spanned either the N-terminal immunoglobulin V-like domain, three distal domains (N,A1,B1) or the complete extracellular structure of CD66a (N,A1,B1,A2 where A1, B1 and A2 are immunoglobulin C2-like domains) (5). In addition, CD31-Fc and Muc18-Fc were used as controls. The three CD66a-derived Fc constructs co-precipitated with Opa proteins but no other proteins from whole cell lysates of meningococci. Control Fc chimeras did not precipitate any proteins. These data indicate that CD66a interacts specifically with Opa proteins of meningococci and the N-terminal domain is sufficient for this interaction.

CD66 comprises a large number of related CEA receptors, normally implicated in inter-cellular interactions via homotypic or heterotypic binding which may involve the terminal domain/s (3). These molecules are expressed on numerous cells, including epithelial, endothelial and myeloid

cells (2,3). The surface expression of these receptors may be regulated by inflammatory cytokines (6). Targeting of these receptors by meningococcal Opa proteins suggests that factors such as prior viral or other infections, during which inflammatory cytokines are upregulated and which may increase the surface expression of CD66 molecules, may determine host susceptibility to meningococcal infection.

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CD66 adhesion molecules on polymorphonuclear phagocytes and epithelial cells are targets for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The carcinoembryonic antigen (CEA) family (also known as CD66) includes clinically important tumor markers, such as the CEA, which is upregulated in epithelial carcinomas. The CEA family also includes cross-reacting antigens, e.g., non-specific cross-reacting antigens (NCAs), biliary glycoproteins (BGPs) and pregnancy-specific glycoproteins (PSGs). The members of the CEA family share antigenic determinants and show high levels of similarity in amino-acid sequences. Their predicted secondary structures indicate that they have immunoglobulin-like domains and are therefore classified within the immunoglobulin superfamily (1).

We have demonstrated that CD66a (BGP) is targeted by meningococcal Opa proteins. CD66a was found to bind to Opa proteins of a number of distinct strains when applied to electrophoretically separated bacterial proteins on nitrocellulose. CD66a adherence to distinct Opa proteins of a single strain was demonstrated in an ELISA using defined derivatives of a serogroup A strain C751 and soluble chimeric receptor molecule CD66a-Fc (2). These results showed that all three Opa proteins of C751 interacted with CD66a but OpaB and OpaD bound more CD66a-Fc than OpaA. In our previous studies, we have shown that the Opa proteins of strain C751 increased non-opsonic interactions with polymorphonuclear phagocytes (PMN) and that OpaB and OpaD are marginally more effective than OpaA in these interactions (3). PMN express several members of the CD66 family including BGP (1,2). Accordingly, we investigated the roles of PMN CD66 in meningococcal interactions. Monoclonal antibody against the N-terminal immunoglobulin V-like domain of CD66a and soluble chimeric receptors inhibited interactions of several Opa-expressing meningococci. Other antibodies and soluble receptor constructs, used as controls, were not inhibitory.

Since HT29 a colonic carcinoma cell line is known to express CD66a (4), we used this cell line to investigate adhesion of CD66a by Opa-expressing meningococci. A monoclonal antibody against the N-terminal domain of CD66a as well as soluble CD66a-Fc chimeric constructs inhibited binding of meningococci to HT29. In contrast, control reagents had no effect. The epitope recognized by the anti-N terminal domain antibody is present on several other members of CEA family expressed by human cells of epithelial origin. We extended our investigations to A549, a lung carcinoma cell line. These cells were shown to express CD66 and bacterial adhesion was inhibited by anti-CD66 antibody. These data together with data on PMN indicate that BGP-like CD66 surface molecules are a target for meningococcal Opa proteins on both PMN and certain epithelial cells.

In a dot blot overlay experiment using human mucosal and disease isolates (including 50 strains each of gonococci and meningococci), we examined the interactions of soluble CD66a-Fc. This

study provided a striking demonstration of the specific adherence of the N-terminal domain of CD66a to> 95% of Opa-expressing pathogenic *Neisseriae*. All isolates that did not produce Opa did not bind CD66a-Fc. None of the strains adhered to CD34-Fc. No commensal *Neisseriae* (14 strains) or other human commensals or pathogens (including *E. coli*, *Pseudomonas* and *Haemophilus*: a total of 16 strains) adhered significantly to CD66a-Fc. These studies show that CD66a is a target for a conserved epitope present on the majority of Opa proteins of gonococci and meningococci.

Targeting of the CEA members of cell adhesion molecules which are up-regulated during inflammation (5) may be critical to pathogenesis of meningococcal infection and may shift the balance from carrier state to dissemination. The identification of the CD66 family as a target for pathogenic neisserial Opa proteins and the demonstration that Opa interactions with the receptor can be inhibited by antibodies to the N-terminal domain as well as receptor analogs may have implications for intervention in neisserial diseases.

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Meningococcal interactions with human phagocytic cells: a study on defined phenotypic variants

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The mechanisms involved in bacterial dissemination from the nasopharynx to the blood and cerebrospinal fluid (csf) during infection with *Neisseria meningitidis* (Nm), are not fully understood. Several variable surface components such as pili, LPS, and outer membrane opacity proteins have been implicated as potential determinants of virulence. Meningococcal isolates from the nasopharynx are often acapsulate whereas those from blood and csf are usually capsulate and have sialylated LPS (1). Our previous studies have examined the roles of meningococcal surface structures (capsule, LPS, pili and opacity proteins: Opa and Opc) in bacterial interactions with human epithelial, endothelial and mononuclear phagocytic cells. In the current studies, using defined meningococcal derivatives we examined the roles of these surface structures in bacterial interactions primarily with human polymorphonuclear phagocytes (PMN) and also with monocytes.

Interactions of phenotypic derivatives of strain MC58 with PMN. In order to examine the effects of pili, capsule, Opc expression and LPS phenotype on bacterial interactions with PMN, a panel of variants with altered expression of these structures derived from MC58 were used (2). Using a chemiluminescence (CL) assay, comparison of acapsulate Opc$^-$, Opa$^-$ variants expressing or lacking pili demonstrated little effect of pili in interactions with PMN. Acapsulate Opc$^-$ bacteria induced at least two-fold higher levels of PMN CL than acapsulate Opc$^-$ variants demonstrating some effect of Opc in promoting association with PMN. In contrast, Opc mediated a five-fold increase in monocyte association in the same experiment. The effect of Opc on PMN interactions was reduced when LPS was sialylated (L3 immunotype) and completely inhibited when capsule was present.

Effects of PilC expression and pilin structural variations on PMN interactions. A pilus-associated protein of *E. coli* has been shown to affect bacterial interactions with PMN(3). As such, we decided to examine the effect of PilC expression and that of pilin structural variations on meningococcal interactions with PMN. A high PilC-expressing variant of strain MC58, designated 58#18.18 was compared to the low PilC-expressing parental phenotype, MC58, with identical pilin sequence. In addition, pilin structural variants of strain C311, with distinct post-translational modifications, but consistent expression of PilC were investigated (4). All piliated capsulate phenotypes were comparable and failed to induce a significant PMN CL response. These studies show that pilin structural variations or PilC-expression, that affect epithelial and endothelial adherence, do not affect bacterial interactions with phagocytic cells.

Roles of distinct Opa proteins and Opc of strain C751. In order to examine the effects of distinct Opa proteins compared with Opc, we used a panel of defined acapsulate variants derived from strain C751 which expressed one of three distinct Opa proteins or Opc (5). The highest CL response was induced by bacteria expressing Opa proteins B and D, and a slightly lower

response induced by Opa A-expressing variants. Opc, previously shown to be the most effective protein in increasing interactions with monocytes (6) was the least effective protein in inducing CL responses in PMN. Bacteria expressing undetectable levels of opacity proteins failed to interact with PMN. Results from a phagocytic killing assay correlated with the CL assay. There was no reduction in viability of Opa^- , Opc^- bacteria and all capsulate organisms were resistant to phagocytic killing.

Strain C751 produces LPS of immunotype L9, but is not sialylated intrinsically. We investigated the effect of LPS sialylation on OpaB- and Opc-mediated interactions with PMN and monocytes using bacteria grown on 50 μg/ml CMP-NANA. Although induced CL values were significantly different for $OpaB^+$ and Opc^+ bacteria, sialylation of both phenotypes resulted in decreased interactions with PMN and monocytes.

Conclusions. As with monocytes, the expression of opacity proteins by Nm affects bacterial interactions with PMN. This requires meningococcal surface sialic acids (of capsule and LPS) to be down-modulated. Also, in contrast to their roles in human epithelial and endothelial adherence, neither pili nor PilC expression had any effect on phagocytic cell interactions. Examination of the relative influence of Opa and Opc indicated that Opa proteins were more effective than Opc in PMN interactions whereas the reverse was the case with monocytes. Differential effects of Opa and Opc on human PMN and monocytes suggest the presence of distinct receptors for these proteins on phagocytic cells.

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Investigations on the glycosylation status of pilins of carriage and clinical isolates of *Neisseria meningitidis*

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Following the identification of pilus glycosylation in *Neisseria meningitidis*(1,2), several studies have reported the presence of glycans on neisserial pili (3,4). Our previous studies have defined the structure of a pilus-associated trisaccharide by genetic and biochemical means. The structure found on Class I pili of the serogroup B strain C311 is Gal (β1-4) Gal (α 1-3) 2,4-diacetamido-2,4,6-trideoxyhexose (2, 3). X-ray crystallographic studies have suggested the presence of a disaccharide on *N. gonorrhoeae* MS11 pili (4). Whereas anti-Gal, a human antibody with primary affinity for Gal (α1-3) Gal structure, was found to bind to some meningococcal pili (5). Collectively, these studies suggest that neisserial pili may contain diverse glycan structures. However, galactose appears to be commonly present.

In recent studies, we have investigated several clinical isolates of meningococci in order to determine the extent to which pilins are modified in strains expressing Class I and Class II pili and in nasopharyngeal as opposed to disseminated isolates. We have introduced *galE* mutations in Class I and Class II strains. The majority of GalE mutants produced pili and LPS which were truncated compared to those of the parent strain - as determined by their migration on SDS-PAGE. This phenomenon was reproducible each time *galE* mutation was introduced in a strain suggesting strongly that like LPS, both Class I and Class II pilins of numerous strains contain galactose substitutions.

In order to define further the structures of glycans present in distinct isolates, we have used synthetic disaccharides, Gal (β1-4)Gal and Gal (α1-3) Gal, conjugated to KLH and have raised antisera against these structures. Affinity purified antisera are being used to determine the extent of variation in the expression and in the structure of glycans on pili in isolates from distinct sites.

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Rapid detection of blood contamination in the cerebro-spinal fluid of infant rats by Sangur™ test strips

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The pathogenesis of meningitis caused by a variety of Gram positive and Gram negative bacteria is frequently studied by use of the infant rat model [1]. Meningitis in this model occurs as a sequelae of the systemic spread of the bacteria in the bloodstream. The interpretation of published results concerning meningitis is made difficult in many records due to the disregard of the fact that blood contamination of the cerebrospinal fluid (CSF) cannot be avoided during the traumatic puncture procedure leading to a falsification of the CSF bacterial counts. We here present the evaluation of a rapid and quantitative test for CSF blood contamination using Sangur™ test strips (Boehringer, Germany) which is available for the detection of hematuria. Each CSF and blood from an individual infant rat were diluted appropriately and inoculated onto the test strips. Colored spots were counted using a magnifying glass. The procedure required minimal amounts of CSF and allowed direct calculation of the CSF bacterial load due to blood contamination and thus provided refined criteria for the presence of bacterial meningitis in the infant rat model. The results obtained from Sangur™ test strips correlated well with erythrocyte counts using a Neubauer´s chamber and were much less time-consuming. Bacteria did not influence the test results. Using an infective dose of $2x10^7$ CFU of *Neisseria meningitidis* strain B1940 we could demonstrate by Sangur™ strip testing that bacterial counts in the CSF of up to $10^{4.5}$ /ml in several animals predominantly derived from blood contamination after 9 h of infection, suggesting that no meningitis had occured despite of high CSF bacterial counts. However, true meningitis could be detected after 17 h of infection. Less than 0.01% of the CFU isolated from the CSF originated from the bloodstream due to blood contamination during the puncture procedure. Evaluation of blood contamination of the CSF using Sangur ™ test strips is a rapid and reliable method to precisely judge bacterial meningitis in the infant rat model.

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Antimicrobial polypeptides of human polymorphonuclear leucocytes increase interaction of *Neisseria meningitidis* with epithelial cells in an opacity protein independent way.

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Upon stimulation/degranulation of polymorphonuclear leukocytes, defensins among other granule polypeptides are translocated to phagolysosomes. Small amounts of the defensins however are released in the extracellular compartment in estimated concentrations up to 6-8 μg/ml (1). In the initial phase of meningococcal disease, defensin concentrations in the plasma of patients are significantly higher than those in healthy blood donors. These high concentrations of released defensins most probably reflect the number and activity of neutrophils at the site of infection and inflammation (2). Defensins are strongly cationic peptides, that avidly bind to eukaryotic cells, amongst others to epithelial cells (3). In addition, pathogenic *Neisseria*, at least gonococci, are highly resistant to the antimicrobial actions of human defensins (4).

In order to test the hypothesis that defensins can affect meningococcal interaction with epithelial cells, we tested adherence and invasion of meningococci in the presence of purified defensins (HNP 1-3). Low concentrations of these defensins (2-6 μg/ml) added to our in vitro invasion assays strongly increased the interaction of non-capsulated meningococci (Serogroup B strain H44/76) with human (Chang and Hec-1B) epithelial cells and resulted in a 5-6 times increase in the number of internalized meningococci. Moreover, an increase in invasive abilities was also observed with meningococcal phenotypes that lacked appropriate Opc and/or Opa proteins and which are not or only marginally invasive in the absence of defensins. These results point to the possibility that upon stimulation and degranulation of PMN's on the mucosa of the airways (nasopharynx), meningococci (of different phenotypes) in the vicinity of such phagocytes gain the ability to adhere to and enter epithelial cells of the mucosal barrier in vast numbers.

Since invasion of meningococci lacking Opc occurred, further experiment were done, using meningococcal variants of strain H44/76 (cps^-, P^-, Opc^{++}), expressing a not endogenously sialylated L3 type of LPS. These variants invade Chang epithelial cells, but after addition of CMP-NANA, a 60% reduction of adherence was noted and invasion was abolished, probably because the exogenously sialylated LPS hampered opacity protein (Opc) function. Addition of defensins restored adherence, but the number of internalized bacteria was still low. Selective enzymatic removal of the host cell receptor for meningococcal Opc protein from Chang epithelial cells, also abolished bacterial invasion and reduced adherence to 10%. In the presence of defensins, adherence was restored but invasion was still marginal. These results indicate that meningococcal adherence may be sustained by defensins, through interaction with surface structures on the eukaryotic cells other than the Opc receptor, whereas for internalization of meningococci by Chang epithelial cells, this host cell receptor appears to be required.

To our knowledge this is the first report on the adherence/ invasion enhancing effect of antimicrobial defensins. This finding may be a new and potentially important mechanism in the pathogenesis and onset of meningococcal disease.

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The potential of [Cu,Zn]-superoxide dismutase to contribute to survival of *Neisseria meningitidis* in the presence of human polymorphonuclear leukocytes.

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Superoxide dismutases (SODs) are metalloenzymes involved in the dismutation of the highly reactive superoxide free radical (O_{2^-}) to hydrogen peroxide and molecular oxygen (1). The removal of O_{2^-} effectively aborts further reactions including the formation of the highly toxic hydroxyl radical, which once generated would readily attack proteins, lipids, carbohydrates and nucleic acids.

Two groups of SOD have been identified in bacteria; those containing Fe or Mn, which have very similar primary structures and generally protect against endogenously generated O_{2^-}, and more recently, enzymes with Cu and Zn as their cofactors. [Cu, Zn]-SOD (encoded by *sodC*), once considered a solely eukaryotic protein, has now been identified in a considerable number of micro-organisms, including some important human and animal pathogens. Localization studies have revealed [Cu, Zn]-SOD to be situated in the periplasm (2,3,4,5) and a role in defense against a periplasmically or exogenously located source of O_{2^-} has been proposed (3,6). This might operate during the respiratory burst of polymorphonuclear leukocytes (PMNLs) - a first line defense against invading micro-organisms.

We have reported the cloning, expression and mutagenesis of a periplasmic [Cu, Zn]SOD from a serogroup B strain of *Neisseria meningitidis* MC58 (7). In an attempt to define the role(s) this might play in meningococcal biology, we have investigated survival of the wildtype and its *sodC* mutant in the presence of human PMNLs. To facilitate optimal uptake by PMNLs as described by McNeill et al. (8), non-capsulate isogenic *sodC*+/- strains were constructed and characterized. Their relative survival was then measured in killing assays. PMNL activation was monitored by elastase production. We did not observe any differences in survival. Benov and Fridovich (9) have shown that in a Fe/Mn-SOD deficient background, [Cu, Zn]-SOD is produced by *Escherichia coli* towards the end of exponential growth, accumulating during early stationary phase. These authors and St John and Steinman (10) have suggested that the biological function is correspondingly to protect periplasmic contents during stationary phase. We were concerned that our failure to detect a difference between wildtype and *sodC* mutant might simply reflect our selection of growth conditions under which [Cu, Zn]-SOD production was minimal. MC58 was grown in liquid culture and aliquots withdrawn at 2, 4 and 6 hours, during early to mid exponential phase. In contrast to the situation found with *E. coli*, [Cu, Zn]-SOD was clearly detectable from the earliest sampling time, suggesting that the failure to differentiate wildtype and mutant in the PMNL killing experiments could not be ascribed simply to failure of [Cu, Zn]-SOD production.

Our results so far do not suggest that bacterial periplasmic [Cu, Zn]-SOD is crucial for protection of meningococci against killing by phagocytic cells. However, a contribution earlier in the

interaction between host cell and microbe, perhaps during phagocytosis, has not been ruled out, and this is being examined further.

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