Biology of major surface proteins

Neisseria porin proteins

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The porins, the predominant proteins on the surface of pathogenic *Neisseria*, form a family of structurally related proteins, whose physiological role is to allow access of nutrients into the cell. They also play an important role in pathogenesis, generating immunological specificity and interacting with host cells. Gonococci express one of two alternative classes of porin, PIA and PIB. Expression of an individual protein is stable within a strain but differences between strains are responsible for serovar differences. In meningococci the equivalent proteins are the class 2 and class 3 proteins which are responsible for serotype specificity but an additional porin, the class 1 protein, is also usually present and is responsible for subtype specificity. Since antibodies with porin specificity are protective in *in vitro* assays the proteins have been regarded as attractive candidates for the development of vaccines against gonorrhoea and meningococcal infection.

Structural and immunological studies have led to a model for the organisation of the porins within the outer membrane, in which a series of conserved regions form transmembrane β-sheets, generating eight surface exposed loops (3). Epitope mapping studies have shown that the sequence variations which are responsible for immunological specificity are located in these loop regions, although the individual loop(s) involved differ between the different porin classes. The critical regions appear to be at the apices of the surface exposed loops which represent the potential target sites for vaccine induced antibodies. Consequently the optimal immunising agents appear to be those which most closely mimic the conformation of these regions. Thus immunisation with a carrier protein conjugated to a short peptides containing the P1.16b subtype epitope from meningococcal class 1 protein induced antibodies which reacted strongly with linear peptides but poorly with the native protein and were non-bactericidal. In contrast immunisation with a larger peptide consisting of the surface exposed portion of loop 4 which was subjected to cyclisation to more accurately mimic the native loop structure, induced antibodies which recognised conformational rather than linear epitopes and which promoted complement mediated bactericidal killing of the homologous strain (1).

As an alternative approach to the synthesis of peptides with conformational stability we have recently investigated the use of multiple antigen peptides (MAPs) in which

consist of an oligomeric branching lysine core to which are attached dendritic arms of defined peptide antigens. This arrangement confers conformational stability and permits the construction of immunogens containing both B-cell and T helper cell epitopes. Immunisation with a MAP containing the P.16b subtype epitope together with a defined Th-cell epitope induced a bactericidal immune response without the use of a carrier protein, demonstrating the potential of such synthetic antigens as components of new meningococcal vaccines.

An additional problem for the development of an effective vaccine based on the gonococcal PI porin is suggested by the observation that the sialylation of LPS, which occurs *in vivo*, induces resistance not only to the bactericidal effect of human serum but also to antibodies directed against PI (2,4). We have therefore used mAbs to investigate the effect of sialylation on antibodies which react with epitopes at the apex of loop 5 on PI. The antibodies showed little difference in binding between sialylated and non-sialylated gonococci and promoted both bactericidal killing and opsonisation of the sialylated gonococci. Thus despite the presence of the sialylation it should be possible to induce protective antibodies by focusing the immune response to surface exposed epitopes on PI which are least susceptible to the potential inhibitory effect of LPS sialylation.

Further studies are currently in progress to refine the molecular models of the porin structures. Such detailed information should facilitate the production of immunogens designed to target the protective porin epitopes.

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Measurement of antibodies to variable regions of meningococcal class 1 outer membrane proteins using ELISA with synthetic peptides as coating antigens

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Neisseria meningitidis is an important cause of meningitis and septicemia in infants and adolescents worldwide. The severity of the disease requires the development of a vaccine, which can induce protection to infection in all age groups. Earlier studies have shown that the presence of serum bactericidal antibodies directed to these bacteria strongly correlate with protection against meningococcal infection. Class 1 outer membrane proteins (OMP) of *Neisseria meningitidis* are able to induce bactericidal antibodies and are therefore interesting vaccine candidates.

We developed a synthetic peptide ELISA to identify specific antibodies against class 1 OMP in sera of mice and healthy volunteers immunised with an experimental vaccine based on meningococcal OMPs. Overlapping peptides corresponding to sequences of class 1 OMP of 6 strains were synthesised by simultaneous multiple peptide synthesis (SMPS). Four variants of each peptide sequence were synthesised either by modification of the N-terminus of monomeric peptides (with or without Fmoc or biotinylated) or by multiple antigen peptide (MAP) synthesis of multimeric peptides. Biotinylated and MAP peptides were used to improve absorption of peptides to polystyrene microtiter plates. MAP peptides were also used to mimic conformational epitopes. Peptides which showed optimal binding with anti-class 1 monoclonal antibodies (mab's), were used in ELISA to examine sera from immunised mice and healthy volunteers. Binding of mouse antibodies to coated peptides is specific and could be inhibited (>80%) using an identical free unmodified peptide or purified class 1 OMP. A 100-200 times higher titre could be reached with mabs using a 5-20 times lower concentration of biotinylated peptides or MAP's compared to Fmoc or unmodified peptides. It was not possible to detect human antibodies using monomeric peptides. However, anti-class 1 OMP antibodies in human sera could be detected when multimeric (MAP's) peptides were used as a coating antigen.

Comparison of human and murine monoclonal IgGs specific for the P1.7 meningococcal porin

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There is an increasing body of evidence that human anti-PorA response is at least partly serosubtype specific (1,4). Murine MAbs to VR1 and VR2 are the basis of the current serosubtyping scheme for serological subdivision of meningococci and often are bactericidal and protective in an animal model system (3). Some of the uncertainties regarding the epitopes against which potentially protective human antibodies are directed might be elucidated by the analysis of human MAbs. Peripheral blood lymphocytes from Norwegian adolescents immunized with a meningococcal serogroup B OMV vaccine (B:15:P1.7,16) were fused with K6H6B5 human-murine heteromyeloma cells. Hybridoma SS269, established in these experiments, produces monoclonal IgG3 which reacts with *Neisseria meningitidis* expressing the P1.7 PorA protein and with linear peptides containing NGGAS, part of the VR1 sequence which accounts for the P1.7 specificity. A'dam1, a murine monoclonal antibody to P1.7, had been formerly described which reacts with linear peptides containing the partially overlapping epitope, ASGQ (2).Comparable affinity constants were determined for the human and murine antibodies but binding to bacterial cells occurred with a higher affinity than binding to linear peptides. Binding of the human antibody to bacterial cells was of low avidity and could readily be removed by treatment with 8 M urea, very high concentrations were needed before binding could be demonstrated by immunofluorescence or complement-dependent bactericidal killing. In contrast, the murine antibody bound avidly to bacterial cells and was readily detected at lower concentrations in immunofluorescence and bactericidal killing. However, efficient opsonophagocytosis was mediated even at low concentrations of the human antibody and in the absence of complement, suggesting that low avidity antibodies might also be protective against disease.

ELISA tests were used to determine whether human IgG antibodies which react with peptide D6 (AQAANGGASGQVKVTKVTKA) were formed by immunization with

the Norwegian group B vaccine in paired sera taken before vaccination and 6 weeks after 2 vaccine doses. Only 1/21 vaccinees was stimulated to synthesize antibodies which reacted with this peptide. These results indicate that the Norwegian vaccine was not generally efficient at stimulating production of antibodies resembling SS269. Such antibodies might be protective due to their opsonic activity and might therefore be a goal in future vaccine development.

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Serum opsonins induced during the course of meningococcal disease correlate with anti-outer membrane protein antibodies

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The levels of serum IgG antibodies in patients with meningococcal disease or in volunteers immunized with outer membrane vesicle vaccines as measured by an enzyme-linked immunosorbent assay (ELISA) or by immunoblotting have been shown to correlate with serum bactericidal (5-8) and opsonic (1) activity. However, the antigens used in these assays have mostly been membrane preparations or whole cell lysates (1, 5, 6, 8) and the subcapsular antigens that contribute to these findings are poorly defined (1,6,7). The development of improved anti-meningococcal component vaccines may be aided if the antigens which elicit antibodies with anti-meningococcal functional activity could be better defined. In previous studies, we have been able to purify outer membrane class 1 (3) and class 3 (2) protein and we have measured the levels of IgG antibodies reacting with these proteins in an ELISA using sera from 25 patients infected with *N. meningitidis* strains of different serogroups, serotypes, and serosubtypes.

In this study, the opsonic activity in sera from these 25 patients was measured using different meningococcal strains during the course of their disease. The serum opsonic activity was then correlated to the amounts of anti-porin in the same sera. The opsonophagocytosis was studied by measuring number of bacteria per phagocyte using a flow cytometric assay and by measuring leucocyte oxidative metabolism during phagocytosis using luminol-enhanced chemiluminescence (4). The opsonic activity of sera obtained from these 25 patients was significantly higher than of sera obtained on admission to hospital when the samples were incubated with autologous strains, or strains containing class 2 (C:2a:P1.2:L3,7,9) or class 3 (44/76-B:15:P1.17,16:L3,7,9) outer membrane protein. Thus, the convalescence sera contained a wide range of opsonins reacting with meningococci of different serogroups, serotypes and serosubtypes. The peak levels of bacteria per phagocyte and leucocyte chemiluminescence were reached during the second week of disease. About 50% of convalescence sera obtained 6 to 18 months after the acute illness contained significant amounts of opsonins, whereas the remaining sera had minor or no opsonic activity. Interestingly, acute serum from a patient with bacteremia on admission to hospital, but without septicemia and meningitis, induced pronounced phagocytosis and

leucocyte chemiluminescence production during phagocytosis of the autologous strain.

A good correlation was observed between serum opsonic activity as measured by leucocyte chemiluminescence production during phagocytosis of strain 44/76 and the levels of serum IgG antibodies recognizing purified class 1 (serosubtype P1.7,16) and class 3 (serotype 3) protein in an ELISA (2,3), suggesting that at least some of the serum opsonins react with class 1 and class 3 outer membrane proteins. Furthermore, a good correlation was also seen between serum opsonic activity and the amounts of anti-class 3 IgG antibodies for patients infected with nontypable strains or strains containing class 2 outer membrane proteins, suggesting that cross-reacting serum opsonins recognize meningococcal porins.

Since it is possible that the level of anti-class 1 and 3 antibodies in the patient sera increased in tandem with antibody levels to other outer membrane components that were responsible for the opsonic activity, the following experiments were performed. IgG levels to LOS (immunotype L3,7,9) were measured by ELISA and anti-class 1 and anti-class 3 antibodies were affinity purified and tested in functional assays. IgG levels to LOS, did not increase to as great an extent as the anti-class 1 or 3 IgG levels, and there was less correlation between the anti-LOS IgG levels and the anti-meningococcal opsonic activity in the patient sera than the correlation between the anti-porin IgG levels and the anti-meningococcal opsonic activity. The affinity purified anti-class 1 and anti-class 3 antibodies were found to contain bactericidal and opsonic activity, making it likely that the same antibodies present in the patient sera conferred the same functional activity. In conclusion, during the course of meningococcal disease antibodies are induced with anti-meningococcal opsonic activity. The levels of these opsonic antibodies peak during the second week of disease and can still be present 6-18 months after the infection has cleared. This suggests that the opsonic activity is at least, in part, due to antibodies that recognize the meningococcal class 1 and class 3 outer membrane proteins and it is doubtful that it is due to a concomitant rise in anti-LOS antibodies.

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Regulation of protein 1 porin (P1) function by host cell factors and biological implications of P1 insertion into target cell membranes

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Interaction of Opa$^+$ gonococci with human peripheral blood monocytes (PBM) induces a strong oxidative burst detectable by chemiluminescence (CL). Using purified P.1A and P.1B porins we were able to almost completely inhibit *N. gonorrhoeae* and FMLP-induced CL responses. In contrast, CL induction by PMA could not be inhibited by preincubation with P.1 porins. Our data suggest P.1 insertion into the PBM membranes selectively inhibits a distinct target cell signalling pathway involved in the generation of oxidative burst and are consistent with previous observations made by Haines and colleagues (1).

Concomitant with these studies we were able to identify a cytosolic factor (other than calmodulin) present in human target cells that specifically interacts with P.1-like porins of the pathogenic *Neisseriae* but not with those of commensal *Neisseria* species. *In vitro* studies using black lipid membranes indicate a remarkable shift in porin function when this factor is present. Patch clamp studies carried out with intact cells indicate that this shift occurs *in vivo*. Amino acid homology searches led us to identify a sequence present only in P.1-like porins of pathogenic *Neisseria* species and a human porin known to interact with the same cytosolic factor, indicating an interesting functional and evolutionary relationship. We are currently trying to demonstrate a specific function of this sequence by site-specific mutagenesis of P.1 porins.

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Pili and Opc in meningococcal virulence: Phenotypic requirements and molecular mechanisms

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Phase and antigenic variation of surface components of *Neisseria meningitidis* (Nm) grown *in vitro* give rise to phenotypes that range from non-interactive to highly invasive when examined for their virulence on cultured human cells. Phenotypic variation also occurs in vivo (1) and may be driven not only by random mutations but also by environmental signals. The importance of the latter phenomenon in the expression of virulence properties has not been investigated in detail. However, one study has suggested that capsular polysaccharide, for example, may be modulated by environmental conditions (2). The presence or absence of capsule in addition to sialic acid on LPS of diverse serogroups has profound implications for the interactive properties of meningococci with host cells (3, 4). In studies on meningococcal pathogenesis it is of interest to examine the potential of bacteria to interact with different host cells and to understand the interplay between phase variable surface structures in modulating such interactions.

Pili: Piliated meningococci have been shown to adhere to nasopharyngeal organ cultures in large numbers (5). Pili are also required for the adherence of capsulate strains to epithelial and endothelial cells and of capsule-deficient bacteria when LPS is sialylated. Pili are mediators of specific interactions between Nm and human cells and numerous strains expressing structurally variant pilus types belonging to Class I or Class II adhere to venous and capillary endothelial cells of human origin, but not to cells of bovine or porcine origins (6). In addition, variant pili readily adhere to human endothelial cells, but often fail to interact with human epithelial cells, suggesting that the mechanisms of pilus-facilitated adherence in the two cases may be distinct. Recent studies have shown that both the expression of PilC and structural variations in the pilin subunit may affect bacterial adherence to human cells (6, 7, 8). PilC is a pilus-associated protein and may be important in pilus biogenesis (9) and/or adhesion (7). Our studies show that its role in increasing bacterial adherence is also consistent with the notion that PilC increases the stability of pili or their anchorage in the outer-membrane. Whether these apparent roles of PilC can be ascribed to distinct PilC variant proteins or are coincidental with as yet unknown phenomena is unclear. In addition to their role in adherence, recent studies have shown synergistic effects of pili on LPS-mediated toxicity of bacteria for human epithelial and endothelial cells (Dunn

et. al., submitted). Meningococcal pili may also affect bacterial interactions with human erythrocytes (10) but appear not to affect interactions with human phagocytic cells (4).

Opacity proteins: Nm disease isolates express the outer-membrane opacity proteins, Opa and Opc. Meningococcal Opa proteins are related to those of gonococci and variant forms in Nm also show an ability to interact with human cells (3). The Opc protein which is distinct from Opa (both structurally and in its genetic control of expression, 12), acts as an invasin for epithelial as well as endothelial cells (11) and, in addition, increases bacterial interactions with human monocytes (4). However, neither Opa nor Opc are effective mediators of bacterial interactions in capsulate phenotypes or when capsule-deficient bacteria express sialylated LPS. Thus, if these outer-membrane proteins are primarily virulence attributes, they only become operational in the absence of sialic acids on the surface of bacteria. This may reflect on the fact that the proteins, which are basic, require a net positive charge to interact with their respective ligands. Such a phenotype may exist in vivo since nasopharyngeal isolates are often capsule-deficient and may express non-sialylated LPS (13). Whether other niches within the host favour such a phenotype is not known. The phenotype commonly isolated from disseminated infections is capsulate and possesses the L3 immunotype, which is expected to be sialylated. This is the predominant circulating phenotype and may be selected for by its resistance to humoral defences. Whether this reflects the array of phenotypes that may be adherent to endothelial cells in vivo is not known. It is probable that environmental signals and/or natural antigenic/phase variation may give rise to non-sialylated phenotypes in the adherent population. Under these circumstances, bacteria may utilise the outer-membrane proteins to invade host cells.

Bacteria expressing the Opc protein exhibited a high level of invasion for human umbilical vein endothelial cells (Huvecs) in vitro (11). In order to study the mechanisms of interaction, polarised endothelial cells were used as a model for studies on Opc interactions with apical and basal cell surfaces. Meningococci expressing Opc required serum to adhere to the apical surfaces of Huvecs. Interactions with non-polarised cells did not require serum factors. Detailed examinations have shown that Opc-facilitated interactions at the apical surface occur via bridge molecules derived from the serum. The receptors on Huvecs involved in these interactions belong to a subfamily of integrins that bind to serum and matrix proteins with the motif: Arginine-Glycine-Aspartic acid (RGD); and the vitronectin receptor (integrin "v$3) appears to be the principal receptor involved.

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Identification of pilin domains of *Neisseria meningitidis* involved in eucaryotic cell interactions

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Pili play an essential role in adhesion of *Neisseria meningitidis* (MC) to epithelial and endothelial cells. However, among piliated strains both inter and intrastrain variability exist with respect to their degree of adhesion to epithelial and endothelial cells *in vitro*. This suggests that factors other than the presence of pili *per se* are involved in this process. Antigenic variation of pilin has been shown to modulate MC adhesion and certain pilin variants are associated with a high adhesive phenotype (2, 4). In addition, a 110 kD protein, PilC1, has been identified as being a major player in pilus-mediated adhesion, PilC1$^-$ derivatives are unable to adhere to both endothelial and epithelial cells (1). Two PilC proteins, PilC1 and PilC2, are found in *N. meningitidis*. These two proteins are subject to phase variation and have a similar function in pilus biogenesis. PilC1 has an additional role on MC adhesion. However, the precise nature of the MC component, pilin or PilC1, which interacts with the eucaryotic receptor remains unknown.

In order to get insight into MC adhesion, the pilin corresponding to a low adhesive loci has been purified by fusing *pilE* with the *malE* gene of *Escherichia coli* to generate a maltose binding protein (MBP)-pilin fusion protein. This MBP-pilin hybrid protein was then coated onto a killed suspension of *Staphylococcus aureus* using anti MBP antibodies (3). Adhesion of these coated particles onto Hec-I-B cells was significantly higher (>25 bacteria per cell) than the same particles coated with MBP (< 5 bacteria per cell). In order to delineate the region of the protein involved in this binding, truncated pilin have been fused to MBP. A fusion encompassing the constant region (50 amino-acids) and the first 20 amino-acids of the variable region is required for this binding. The hypervariable region is not necessary for pilin binding. To confirm that this adhesion is solely the fact of pilin and did not involved the MBP part of the hybrid protein, pili were purified and pilin coated onto *Staphylococcus aureus* using a pilin monoclonal antibody. Such pilin-coated particles adhered to Hec-I-B cells. This binding is inhibited by preincubating the monolayer with the truncated pilin fusion, thus confirming that a cell-binding domain is located within the first 70 amino-acids of pilin. The role of this domain in MC adhesion needs to be further addressed by constructing a piliated strain mutagenized in this pilin binding site.

To assess the role of the variable region in MC adhesion, MBP-pilin fusions were generated using variants responsible for low and high adhesive phenotypes. Adhesiveness of both MBP-pilin fusions was identical. This experiment and the above results suggest that there is no direct peptidic cell-binding site in the hypervariable region of pilin. However, to further evaluate the role of the hypervariable region in MC pilus-mediated ahdesion, a hybrid pilin variant was constructed where the hypervariable region of a low adhesive variant has been replaced by the corresponding region of a high adhesive variant. A MC strain expressing such a variant has a high adhesive phenotype. This result confirms the major role of pilin hypervariable region in adhesion of *N.meningitidis*. The mechanism by which this region plays a role in MC adhesion is unclear. It has recently been shown that MC pilin was glycosylated (4), such a posttranslational modification of hyperadhesive pilins could be responsible for the occurrence of a high adhesive phenotype

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Function of PilC as the pilus adhesin and the interaction of PilC with host cells and other neisserial factors

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Pilus-mediated attachment is considered the most important event initiating neisserial infections. Both pathogenic *Neisseria* species, *N. gonorrhoeae* and *N. meningitidis*, produce at low quantities a phase-variable protein (PilC) implicated in several pathogenesis-related processes including pilus biogenesis and natural transformation competence (1,2). PilC, in conjunction with the major pilus subunit PilE, has also been implicated in epithelial cell adherence, however, the identity of the pilus adhesin has thus far remained obscure (3). Here we report on the over-production of the PilC2 protein in a *N. gonorrhoeae* context devoid of PilE, and the purification of PilC2 to homogeneity. Using antisera prepared against purified PilC2 and synthetic peptides immuno-EM reveals a location of PilC proteins primarily at the tip of gonococcal pili. Consistent with the known species specificity of neisserial infections, purified PilC binds to human ME-180 epithelial cells but interacts poorly with other mammalian cell species. Fluorescent beads coated with PilC-gonococcal pili do not bind to ME-180 epithelial cells unless complemented with purified PilC2 protein. PilC-specific antibodies inhibit pilus-mediated adherence of gonococci to epithelial cells whereas variant-specific PilE antiserum is ineffective under the same conditions. We demonstrate further that purified PilC2 completely blocks pilus-mediated attachment of both *N.gonorrhoeae* and *N.meningitidis* indicting that both pathogens interact with epithelial cells via the same mechanism and probably recognise identical or very similar receptors.

Recent results also suggest a role of PilC proteins in natural transformation competence and human epithelial cell invasion. Furthermore, we have identified a family of accessory proteins which physically interact with PilC and are probably involved in PilC secretion and/or surface presentation. Evidence supporting these conclusions and a putative model of PilC function will be presented.

The gonococcal PilT protein plays an essential role in pilus-associated phenotypes of twitching motility and natural competence for transformation

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The elaboration of pili by *Neisseria gonorrhoeae* appears to be an essential determinant of infectivity. The presence of these organelles can be correlated with expression of multiple phenotypes including competence for transformation, bacterial autoagglutination, twitching motility and adherence for cultured human epithelial cells. However, the mechanistic bases for the association between pili and these properties remain undefined. The gonococcal *pilT* gene was identified in a DNA hybridization - based screen to identify pilus assembly genes (5). The gene product is predicted to be a cytoplasmic, soluble protein containing a consensus nucleotide triphosphate (NTP)-binding motif and most closely resembles the putative PilT protein of *Pseudomonas aeruginosa* (5,7). Significant sequence homologies are also seen with a family of NTP- binding proteins involved in type IV pilus biogenesis, protein export, DNA transfer and natural competence (6).

In studies using a PilT-specific monoclonal antibody (3), it was found that expression of PilT is absolutely conserved in gonococci and that PilT is expressed at very low levels and localized to cytosolic and cytoplasmic membrane fractions. The N-terminal sequence derived by microsequencing of the protein purified from gonococci led to the correction of the previously published gene sequence. Sequencing of the gene from three strains revealed an extremely high degree of conservation at both the amino acid and DNA levels.

Gonococcal PilT mutants were constructed by allelic replacement and were found to retain piliation but fail to express twitching motility, results analogous to that found for *P. aeruginosa* PilT mutants (7). Electron microscopy of the mutants revealed qualitative as well as quantitative alterations in pilus expression. The gonococcal mutants were also incompetent for sequence- specific DNA uptake, displaying reduced frequencies of transformation equivalent to that seen for nonpiliated mutants. Mutations in *pilT* were found to account for the altered phenotypes of previously identified but genetically uncharacterized mutants deficient in competence and twitching motility. For example, we have shown that the DUD1 (DNA uptake-

deficient) mutant of Biswas, Lacks and Sparling (1) is a PilT mutant and that *dud1* is in fact an allele of *pilT*. Loss of competence in PilT mutants appears to reflect a defect in the earliest stages of transformation, that being at the level of taking up DNA into a DNase state.

It has been proposed that twitching motility (non-flagellar mediated surface translocation) associated with type IV pilus expression is a manifestation of pilus extrusion and retraction (2). In this context, PilT mutants appear to be defective in pilus retraction. This concept fits well with the fact that PilT closely resembles PilF, a NT- binding protein required for gonococcal pilus assembly (4) and it further suggests that these components may have antagonistic functions with PilF promoting extrusion (assembly) and PilT promoting retraction (disassembly).

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The structure and assembly of *N. gonorrhoeae* pilin

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The structure of pilin from *Neisseria gonorrhoeae* MS11 has been solved by multiple isomorphous replacement to 2.7 D resolution. The 18 kD protein folds into a highly asymmetric molecule, with a central 80 D alpha helix. The highly conserved hydrophobic N-terminus forms one end of this helix, and is buried by formation of a coiled-coil with a symmetry-related molecule. It seems likely that this contact is also important for fibre formation *in vivo*. As would be expected, highly variable sequences are located on the surface of the protein. An immunorecessive yet protective epitope between residues 48-60 (1) is accessible in this structure. The three-dimensional structure provides a basis for mapping epitope data from human, rabbit and mouse immune responses to pilin (obtained in collaboration with workers from the Walter Reed Army Institute for Research).

Our current efforts are focused on refining the crystal structure as well as developing a model of the pilus fibre based on the structure and published biochemical and immunological data. Ongoing parallel studies on other crystal forms will provide an independent asssessment of interactions which promote fibre assembly.

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Biology of the Opc protein from *Neisseria meningitidis*

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Class 5 proteins of meningococci include Opa proteins plus the Opc protein (formerly called 5C) (1, 3). Purified Opc possesses a trimeric or tetrameric structure and is highly basic (1). Although Opc is not strongly immunogenic in experimental animals, it is immunogenic in humans upon nasopharyngeal carriage or after immunisation with outer membrane vesicle vaccines (2,4) and both human (2) and murine (1, 4) monoclonal antibodies have been isolated which are specific for the Opc protein. Meningococci which are defective at expression of capsular polysaccharide are adhesive and invasive for various cell types when they express large amounts of the Opc protein (6) whereas colony variants expressing lesser amounts are non-invasive. Similarly, only colony variants expressing large amounts of the Opc protein are sensitive to immunoglobulin-activated complement killing (2).

The *opc* gene is only present in certain meningococci and others (especially serogroup C bacteria of the ET-37 complex and B bacteria of the A4 cluster) give no hybridization in Southern blots with a probe consisting of the 1.5 Kb segment spanning the *opc* gene nor do they yield PCR products with specific oligonucleotides. All serogroup A meningococci and numerous other bacteria, including serogroup B bacteria of the ET-5 complex, do possess an *opc* gene whose sequence varies to a limited extent in unrelated bacteria. The sequence variants are uniform within individual clonal groupings but differ between clonal groupings as if recombination events had occurred in the distant past. Because the same patterns of nucleotide variation are found within diverse alleles, the data indicate that all current alleles represent the results of recombination between 2 "Uralleles", which arose early after speciation of *N. meningitidis*. In addition, some strains possess an 240 bp insertion element upstream of the *opc* gene.

Regulation of Opc protein expression is primarily at the transcriptional level (5) and expression can vary from no detectable mRNA (and protein; Opc^{-}) synthesis to intermediate (Opc^{+}) to strong expression (Opc^{++}). In Opc^{++} bacteria, the Opc protein is one of the major outer membrane proteins. The RNA transcript begins immediately

after an unusual promoter with a -10 box but no -35 region and ends immediately after the *opc* gene. The -35 region is replaced by a poly-cytidine stretch whose length correlates with expression: Opc^{++}, 12-13 C's; Opc^{+}, 11 or 14 C's; Opc- 7-10 or 15-19 C's.

A two-dimensional structural model predicts 5 surface-exposed loops of varying sizes. The linear, continuous epitopes recognized by 3 of the murine Mab's have been mapped using synthetic peptides to a 5 amino acid stretch at the tip of the major loop, loop 2. These Mab's also inhibit adhesion and invasion, indicating that loop 2 is involved in these phenomena. The human MAb seems to recognize discontinuous epitopes. Synthetic peptides corresponding to the other loops have been used to immunize mice and murine Mab's have been isolated after immunisation with loops 4 and 5 which recognize bacteria expressing Opc. Site-directed mutagenesis is in progress to confirm the basic features of this model. 20mers spanning the *opc* sequence have been tested as human T-cell epitopes and compared with comparable peptides from *opa* and *porA* genes (7); epitopes were found in the predicted trans-membrane regions which often stimulated T-cell growth. Crystals of Opc have been obtained using the hanging drop method; they form rods which are square in cross-section and have approximate dimensions 100 x 100 x 300 microns. Preliminary diffraction data, collected at the synchrotron radiation source at Daresbury (UK), show X-ray reflections to a diffraction limit of 3.8 Angstroms. These results raise the hope that the three-dimensional structure of Opc can be resolved within the next few years.

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Genetics and function of Opa proteins: Progress and unanswered questions

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The Opa proteins of the gonococcal and meningococcal outer membrane, like a number of other surface components, demonstrate high frequency phase and antigenic variation of expression. A gonococcal or meningococcal strain can make multiple antigenically distinct Opa proteins (up to 11 or 12 in gonococci and 3 or 4 in meningococci). The reversible switching of expression of the different proteins and the capacity of the bacteria to express multiple proteins simultaneously generates a complex mosaic of Opa expression within a neisserial population. Portions of the Opa proteins are exposed on the bacterial surface, and the expression of these proteins influences the interaction of the organisms with different types of host cells. In the past few years, there has been dramatic progress in understanding the genetic basis for Opa variation, and in identifying the role of the proteins in promoting attachment to or invasion of host cells. However, a number of fundamental questions about the genetics and function of Opa proteins remain. Some (but not all) of those potentially important questions are:

1. Is the direction or rate of Opa phase variation responsive to changes in environmental conditions, particularly those encountered by the organisms *in vivo*? Phase variation of *opa* gene expression involves a RecA-independent process of insertion and deletion of CTCTT repeats in the signal peptide coding region of the gene, leading to frameshifting and the synthesis of either full-length (in-frame gene) or truncated (out-of-frame gene) Opa protein. The most likely mechanism is slipped-strand mispairing (SSM) during DNA replication (2,7,10). The rate of phase variation could potentially be affected by changes in DNA topology, transcription (2), or DNA repair. If so, the organisms might respond to their environment by increasing or decreasing the rate at which they switch, thus increasing or decreasing heterogeneity of Opa expression in the population.

2. Are all *opa* genes constitutively transcribed under all conditions? In the two gonococcal strains in which all of the *opa* genes have been sequenced (FA1090 and MS11), there are differences in the most likely promoter sequences for different *opa* loci (3,4, and our unpublished data), raising the possibility that a particular subset of Opa proteins would be more likely to be expressed under different conditions *in vivo*.

3. How large is the universe of different Opa proteins within the gonococcal or meningococcal species? The hypervariable (HV) regions of *opa* genes encode the immunodominant, antigenically variable portions of the proteins. Some HV regions appear to be shared among multiple strains, whereas others are apparently strain-specific. If HV regions contribute to the functional differences among Opa proteins, and if those functions are important in pathogenesis, then why are there no HV regions that have been shown to be common to all strains?

4. Does the strong selection for Opa expression that has been demonstrated during experimental gonococcal infection of male volunteers (5,11) indicate that the proteins are <u>essential</u> for establishment of uncomplicated gonorrhea, at least in men?

5. Do the Opa-promoted interactions of the bacteria with epithelial cells or neutrophils that have been studied *in vitro* (1,6,9,12) also occur during infection *in vivo*? If so, what is the selective advantage for the organisms of expressing a protein that mediates attachment to neutrophils, a behavior that is presumably suicidal? What is the identity of the receptor on different cell types for Opa binding? What domains of the Opa proteins are involved?

6. If Opa domains other than the HV regions are involved in Opa-mediated attachment/invasion, is it possible that antibody specific for those relatively conserved domains might protect against some or all types of gonococcal infection? A recent study showed an association between Opa-specific antibody and protection against pelvic inflammatory disease (8), emphasizing the need to understand the antigenic repertoire of Opa proteins and to identify the functional domains of the proteins involved in interactions with host cells in different types of infections.

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Genetic regulation by non-homologous recombination in *N. gonorrhoeae*

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Neisserial *opa* genes alter their expression states using an unusual form of gene regulation (5) dependent on "non-homologous" or "illegitimate" recombination (i.e. *recA* independent, 1, 3). Other neisserial genes or gene families (2, 4) use similar mechanisms to affect gene regulation. We have studied these regulatory phenomena using reporter systems in *E. coli* and *N. gonorrhoeae* with the aim of identifying cellular factors important in non-homologous recombination. We have identified a number of mutations in *E. coli* involving DNA repair and replication enzymes which increase the phase variation frequencies of our reporter systems. These mutations affect the level of phase changing in systems utilizing similar modes of non-homologous recombination. The possibility exists therefore, that similar mutations in *N. gonorrhoeae* may result in the deregulation of a number of genes or gene families controlling the expression of important surface molecules. *N. gonorrhoeae* homologs of several of these genes have been isolated and are presently being analyzed to determine their influence on phase variation frequencies in *N. gonorrhoeae*.

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Several of the gonococcal Opa proteins share a common epitope and functional features with host cell proteins such as LOS binding and 4B12 reactivity: Evidence for heterophilic bidectional adherence and host cell activation

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Gonococcal Opa proteins have been shown to be important in human infectivity studies as well as in *in vitro* invasion assays using human tissue culture cells (4-6,9). Although numerous different Opa proteins can be expressed from a single strain and have variations in antigenicity and amino acid sequence, most of these Opa proteins also display common features (1). One of these characteristics is the ability of Opa proteins to impart the opacity colony phenotype (8). Previous evidence from our laboratory had suggested that the tight intercellular adhesions between the outer membranes of gonococci displaying opacity occurred by Opa proteins expressed on one gonococci adhering to the LOS of the opposing bacterium (2). On further investigation of this activity, we have found that gonococcal Opa proteins behave functionally similar to human asialoglycoprotein receptors. We have cloned and identified the region that is responsible for this activity and found that areas of homology occur in all *opa* genes thus far sequenced. We have also synthesized a peptide representing this area which will bind to purified gonococcal LOS and inhibit the reactivity of the LOS monoclonal antibodies 3F11 and 6B4. We have also identified the amino acid sequence within the Opa proteins that is responsible for the binding of the monoclonal immunoglobulin 4B12, a monoclonal immunoglobulin which reacts with all neisserial Opa proteins.

These two common features of Opa proteins, the binding of gonococcal LOS and 4B12 reactivity, are also shared with proteins expressed on the surface of human tissue culture cells. Using HepG2 cells as a model, we have identified two cell surface proteins which will bind wildtype gonococcal LOS. One of these proteins is the well characterized major hepatic asialoglycoprotein receptor. This receptor can be found on both liver cells and sperm (3). We have been able to demonstrate that gonococci utilize this receptor on both these cell types. Interestingly, a sequence similar to that found responsible for the binding of LOS within the Opa protein occurs in the major hepatic asialoglycoprotein receptor of humans (7). The second LOS receptor on HepG2 cells was identified using ^{125}I-labeled gonococcal LOS, purified from strain 1291 wildtype. Several lines of evidence in these studies suggest that the

oligosaccharide region plays an important role in LOS binding to this p70 protein. It could also be demonstrated that this human protein would specifically bind to 4B12 as compared to other monoclonal antibodies. Once 4B12 was bound to the p70 HepG2 protein, it would significantly inhibit the interaction of this protein with gonococcal LOS.

Thus, gonococcal Opa proteins share common features with some human proteins and might suggest that the tight intercellular adhesion between gonococci and human cells is similar to that between two gonococci displaying opacity.

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Pili and Opa are required for HEC-1-B cell microvillus elongation and engulfment of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae must attach to and invade endocervical epithelial cells to cause disease in a woman. We studied the roles of pili and Opa on adherence to and invasion of genital epithelial cells and the effects of inhibitory and stimulatory compounds on the process.

Materials and Methods: Piliated (P^+) and non-piliated (P^-) variants with and without an Opa were studied for strains F62-SF, FA1090, and $MS11_{mk}$. Other P^- Opa variants of strains F62-SF and FA1090 also were studied. Pilus and Opa expression were monitored by SDS-PAGE and immunoblotting.

Gonococcal adherence and invasion were studied using HEC-1-B cells, a human endometrial adenocarcinoma cell line.

Adherence and invasion of HEC-1-B cells were studied using scanning and transmission electron microscopy. Invasion also was studied by culture following gentamicin killing of extracellular gonococci.

Selected pilus and Opa variants were studied in assays of invasion, by the culture method with inhibitory and stimulatory compounds.

Results: SEM showed the gonococci of strains F62-SF, FA1090, and $MS11_{MK}$ formed microcolonies on the surface of HEC-1-B cells. The tips of microvilli adhered to isogenic P^-Opa^+ and Opa^-, and P^+ Opa^- gonococci, but microvilli did not elongate.

The P^+ Opa^+ gonococci yielded striking microvillus elongation and full length adherence to the gonococci. On TEM the microvilli appeared to contribute to the internalization of P^+ Opa^+ gonococci. Invasion (confirmed by TEM) needed 4-6 h of exposure to P^+ Opa^+ variants except for the Opa e variant of strain FA1090 (~2 h) .

HEC-1-B cells had many intracellular P^+ Opa^+ gonococci after 10 h of incubation. The bacteria appeared to be free in the cell cytoplasm. Only an occasional P^- Opa^+

gonococcus was found within a cell, and those that were appeared to be within vacuoles.

Cytochalasins (blockers of actin polymerization) reduced engulfment; colchicine (prevents tubulin polymerization) had no effect. Cycloheximide and anisomycin (inhibit eukaryotic protein synthesis) caused dose-dependent enhancement of gonococcal invasion. Chelerythrine and phloretin (inhibit protein kinase C [PKC]) caused dose-dependent reductions in gonococcal invasion. PMA and mezerein (activate the ", $, (, *, and , PKC isozymes, but not related isozymes) nonspecifically increased invasion (including P^- Opa^- variants). Sodium dantrolene (decreases release of Ca^{2+} from the sarcoplasmic reticulum) and herbimycin A (protein tyrosine kinase inhibitor) caused dose dependent reductions in invasion. Both PKC agonists, PMA and mezerein, increased gonococcal invasion. Staurosporine (non-specific ser/thr kinase inhibitor that blocks activation of the ", $, (, *, and , PKC isozymes and protein kinases other than the epidermal and insulin-like growth factor receptors [EGFR and IGFR]) blocked only the augmentation of invasion caused by PMA or mezerein.

Discussion: Gonococci use multiple factors to induce their uptake by epithelial cells. The initial adherence is mediated by pili. This adherence appears to be augmented by engagement of receptors at the microvillar tips. Adherence to the eukaryotic membrane causes the bacteria to synthesize over the next several hours a ligand that engages expressed proximal microvillus receptors in an intimate contact that precedes bacterial invasion. Although Opa are needed for invasion, their function in the process remains unclear.

Cytochalasins inhibit gonococcal invasion of HEC-1-B cells indicating that actin polymerization and active participation by the eukaryotic cell is an essential part of the process. Invasion really is engulfment. Drugs that block eukaryotic protein synthesis caused enhanced gonococcal invasion. This suggests that HEC-1-B cells naturally resist invasion. Activation of microvillus-mediated engulfment may be a pathogenetic strategy for circumventing natural resistance to invasion. The inhibitory effects of herbimycin A indicate that P^+ Opa^+ gonococci engage HEC-1-B cell receptors that have tyrosine kinase activity. cAMP-inducible protein kinases do not appear to be involved. Gonococcal engulfment by HEC-1-B cells also requires increased cytosolic Ca^{2+} concentrations.

Staurosporine blocked only the increase in engulfment that followed stimulation of the cells by PMA and mezerein. By exclusion, the PKC isozyme activated during engulfment of P^+ Opa^+ organisms is other than those of the ", $, (, *, and , PKC families. Thus, gonococci can coordinately bind pili and Opa to HEC-1-B cells, activating microvilli through a Ca^{2+}-, tyrosine kinase-, and PKC dependent-pathway, resulting in engulfment of the gonococci.

Identification of human linear B-cell epitopes on the class 1 and 3 outer membrane proteins of *Neisseria meningitidis* using synthetic peptides

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Human humoral response against class 1 and 3 OMP of *Neisseria meningitidis* is believed to be directed both to the sero(sub)type epitopes and to other parts of these porins not recognized by murine MAb (2,3). The aim of this study was to identify the putative human linear B-cell epitopes occurring on both porins using synthetic peptides and immune sera drawn from Norwegian vaccinees. Adolescents were immunized at 0 and 6 weeks and four years with 25 ：g of meningococcal group B (15:P1.7,16) OMV vaccine (1). Sera were drawn prior to immunization, 6 weeks after the second dose and 6 weeks after the third dose. Synthetic peptides spanning the class 1 OMP (10 mers overlapping by 5 amino acids) were synthesized on pins in accordance with the amino acid sequence of the P1.7,16 strain 44/76 (4). Class 3 OMP peptides (12 mers overlapping by 6 mers) were synthesized based on the deduced amino acid sequence of the serotype 15 strain 44/76 (our unpublished data).

Prevaccination sera tended to bind to peptides corresponding to the predicted intra-membrane regions (5) of both class 1 and 3 OMP's. When paired sera from six selected vaccinees were tested with synthetic peptides spanning the class 1 OMP, we found one individual whose postvaccination serum reacted with the peptide corresponding to the P1.7 (VR1) region, two other vaccinees reacted with the P1.16 (VR2) peptide, and no significant increase against any peptide was observed for the remaining three vaccinees. All these vaccinees showed increase in the class 1 protein specific antibodies on immunoblots after vaccination and rise in bactericidal activity against the homologous strain. Paired sera from other 32 vaccinees who had received 2 doses of vaccine were tested with a synthetic peptide covering the P1.16 region (TKDTNNNLTLVPAVC) conjugated through the terminal cysteine to keyhole limpet hemocyanin, only 4 vaccinees were found significant positive. Taken together, the OMV vaccine was effective in stimulating the anti-class 1 protein bactericidal IgG response, but it could not be detected with linear synthetic peptides of 10 amino acids long for most of the vaccinees. It implies that the PorA potentially protective human response was mainly directed to conformational epitopes or larger than 10 mers.

In contrast to the class 1 protein human response, linear epitope mapping of the pre-and post immune sera from three vaccinees who had received 3 vaccine doses with class 3 OMP synthetic peptides of 12 mers long revealed a significant unanimous increase in IgG level to a single peptide located within the loop 1 of the class 3 protein (FHQNGQVTEVTT) which probably contributes to serotype specificity (6). Strong bands corresponding to the class 3 protein were observed in immunoblotting.

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Immunisation with liposomes containing recombinant meningococcal class 1 protein generates bactericidal, subtype specific antibody

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Current meningococcal vaccines based on the group-specific capsular polysaccharides provide only a limited protection against certain meningococcal serogroups and offer no protection against the serogroup B strains found in many temperate countries. Therefore, alternative sub-capsular antigens such as the immunogenic (4) class 1 outer membrane protein (omp) are being investigated as potential vaccine candidates.

Class 1 omp is a porin protein found in almost all meningococcal outer membranes, and has a predicted topology of repeating membrane spanning segments culminating in surface exposed loops (3). Antigenic diversity, or serosubtype specificity is determined by two short variable regions, termed VR1 and VR2 which are located at the apices of two cell-surface exposed loops (1). These regions are known to induce antibodies that are bactericidal to meningococci *in vitro*, and protect against infection *in vivo* (2).

To fully investigate the vaccine potential of the class 1 protein, molecular techniques were employed to generate a source of class 1 protein free from other meningococcal components. The class 1 gene (*por* A) of strain MC50 was isolated on a 1.6Kb fragment and cloned into the high expression vector pGEMEX-1^{TM} (Promega). The subsequent expression of this construct in *E.coli* produced large amounts of class 1 omp fused to a 26 kDa Gene 10 leader protein. In an attempt to remove the recombinant class 1 protein from the Gene 10 leader protein, a synthetic oligonucleotide encoding the Factor Xa recognition sequence, Ile-Glu-Gly-Arg was inserted at the N-terminus of the class 1 protein. However, the reaction of the Factor Xa protease with purified fusion protein resulted in fragmentation of the class 1 protein. Subsequent amino acid sequencing showed that Factor Xa was not specific for the tetrapeptide target sequence, but also hydrolysed any peptide bond preceded by a Gly-Arg. Since no intact class 1 protein was produced, an alternative strategy utilising unfragmented fusion protein was employed.

Rabbit antisera raised against purified fusion protein aggregates reacted with non-

protective class 1 epitopes which are inaccessible on the meningococcal surface. Therefore, in an attempt to mimic the *in vivo* conformation of the class 1 protein and thus direct the immune response towards protective, surface exposed epitopes, fusion protein was inserted into the amphipathic layers of both detergent micelles (proteosomes) (5) and artificial membrane vesicles (liposomes) (6). Liposomes were generated by the rehydration of a phosphatidylcholine/ cholesterol film and sonicated to produce small vesicles. Immunogold labelling demonstrated that the incorporation of fusion protein into these liposomes had allowed the class 1 omp to regenerate its *in vivo* conformation. The resulting exposure of a protective epitope on the liposome surface generated a rabbit sera that was specific for the P1.16 subtype both in ELISA and on Western blot. Although the proteosome induced sera also reacted specifically with the class 1 protein, the immune response was not directed against protective epitopes. The reaction of these antisera with overlapping synthetic peptides suggested that both antisera contained antibodies that were directed against class 1 specific epitopes that were non-linear in nature. An *in vitro* bactericidal activity assay demonstrated the presence of antibody in the liposome generated sera that was capable of promoting complement mediated lysis of the homologous strain.

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Immunisation with synthetic peptides containing epitopes of the class 1 outer-membrane protein of *Neisseria meningitidis*: Production of bactericidal antibodies on immunisation with cyclic and multiple antigen peptides

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Vaccines based on outer-membrane proteins have demonstrated some protection against meningococcal group B infection in recent field trials (1), and improved vaccines could be achieved by targeting the immune response to defined, protective epitopes (4). Knowledge of the conformational structure (3) and immunochemistry of the class 1 OMP, make it a particularly attractive candidate for the use of synthetic peptides for inducing antibodies which can react with the native protein and promote complement-mediated lysis of the meningococcus. In this study, synthetic peptides containing the subtype P1.16b epitope have been synthesised and investigated with respect to their immunogenicity, and the antigenic and biological activity of the antibodies induced.

Initially, linear peptides of 9 and 15 amino acids were synthesised and used for immunisation, with or without coupling to a carrier protein. These peptides induced antibodies with the desired epitope specificity, but which reacted poorly with native protein in OMs, and were non-bactericidal. Consequently, a 36mer peptide, consisting of the entire surface-exposed loop 4 of the class 1 protein was synthesised and subjected to cyclisation, in an attempt to restrict it to conformations that might more closely resemble the native loop structure. In contrast to antisera raised against linear peptides, antibodies raised by immunisation with the 36mer cyclic peptide appeared to recognise conformational determinants, and also promoted complement-mediated bactericidal killing of the homologous meningococcal P1.16b strain (2).

An alternative approach for preparing peptides with some conformational stability is the multiple antigen peptide (MAP) system (6). Initially, an octameric MAP containing the P1.16b B-cell epitope was synthesised, but found to be non-immunogenic in animals, thus highlighting the need for an effective Th-cell epitope in order to induce a humoral immune response. A MAP was then synthesised (BT-MAP) to contain the P1.16b epitope in tandem with a well-characterised Th-cell epitope from tetanus toxin (5). In contrast to B-MAP, incorporation of the Th-cell epitope into BT-MAP induced a strong humoral response towards the P1.16b B-cell epitope. Murine and

rabbit antisera showed similar reactivities in ELISA and epitope mapping with synthetic peptides containing the B-cell epitope, and also cross-reacted with meningococcal OMs from strains of subtype P1.16b and P1.16a. Antisera did not react with denatured class 1 protein in Western blotting, indicating the predominance of antibodies directed towards conformational epitopes. Rabbit antisera to BT-MAP not only promoted complement-mediated bactericidal killing of the meningococcal subtype P1.16b strain, but also of subtype P1.16a. Thus, in contrast to antisera raised to the cyclic peptide, the polyclonal response to BT-MAP is such that the protective effect is extended to a strain which shows an important variation within the subtype epitope. These studies show the potential of synthetic peptides for the production of protective immune responses against *Neisseria meningitidis*.

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Humoral immune responses to different forms of a meningococcal class 1 outer membrane protein

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Neisseria meningitidis is an encapsulated bacteria which can cause severe diseases in developing countries and in the western world. Infection with encapsulated bacteria can be circumvented by immunization with type specific polysaccharide-protein conjugate vaccines generating antibodies to the capsular polysaccharides. However, the polysaccharide from the serogroup B meningococci, a polymer of "-2,8-neuraminic acid, is a common epitope on human cells. For this reason many investigators have chosen to develop a vaccine based on outer membrane proteins.

Five classes of proteins can be identified in the outer membranes of *Neisseria meningitidis*. The Neisserial proteins are present in trimers and function by creating pores through which hydrophilic solutes can pass in a diffusion like process. All meningococcal isolates contain a class 1 protein, either a class 2 or 3 protein, a class 4 protein and several class 5 proteins. The antigenic diversity for class 1 and class 2/3 proteins forms the basis of division of meningococcal strains into subtypes and serotypes, respectively. Monoclonal antibodies with *in vitro* bactericidal activity in the presence of complement have been raised against class 1 and 2/3 proteins. Therefore, class 1 and class 2/3 proteins are thought to be of importance to generate protection against infection. In most of the recently developed vaccines, the outer membrane proteins are presented to the immune system in a vesicle formulation. It is hypothesized that the conformation of the protein is of importance for the generation of antibodies with functional activity. In this study we compare several presentation forms (outer membrane vesicles, ISCOMS, liposomes and lipid tailed cyclic synthetic peptides) of the class 1 outer membrane protein P1.7,16. Furthermore, various immunization schedules were used to investigate the priming and boosting capability of different antigen presentation forms. Humoral antibody responses (subclass distribution and bactericidal activities of generated antibodies) of these studies will be presented.

Cloning and expression of *porA*, the gene encoding the class 1 outer membrane protein from *Neisseria meningitidis*: Purification and immunological characterization of the recombinant polypeptide

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One conclusive remark from the up to date results of the experimental OMP-based vaccines is that an improved vaccine must direct the immune response specifically towards those antigenic determinants that can induce a protective immune response (3). It is not clear from the field trials which component(s) of the vaccine are responsible for the observed protective effect but evidence implicates the class 1 and class 2/3 proteins, which are the major components of the vaccines. This would be in accord with laboratory experiments which have shown the Mabs directed against these proteins, particularly the class 1 protein, are the most effective in bactericidal killing and in animal protection experiments (1). The class 1 protein is, therefore, an attractive vaccine candidate in its own right.

In the present work, we cloned and expressed in *E.coli* the *porA* gene from *Neisseria meningitidis* strain B:4:P1.15 (B385) by fusion to the first 46 amino acids of the N-terminus of P64k (2), under the control of the tryptophan promoter; yielding a 45kDa polypeptide (PM82) which accounted for 20% of the total cellular protein. After disruption, the fusion protein produced as inclusion bodies was purified by successive washing of cellular pellet, using 0.5% (v/v) NP-40 and 3 M urea. The extraction was done in CO_3/HCO_3 buffer pH 10 containing 8 M urea. Urea was then eliminated by gel filtration in PBS. A soluble protein with more than 70% of purity was obtained. To evaluate the immunogenicity and bactericidal activity of exposed epitopes of this protein, 2 groups of 8 Balb/c mice were immunized subcutaneously on at least three occasions (days 0, 20 and 35). The first group was given 20 ：g of antigen in Freund Incomplete Adjuvant. The second group was given 20 ：g of antigen aluminium-adsorbed. Sera were collected seven days after each immunization.

Data concerning the evaluation of these sera against either the recombinant protein or natural P1.15 will be discussed.

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The P1.10 subtype as a model for structural and immunological studies of meningococcal typing reagents

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Monoclonal antibodies (Mabs) form the basis of the typing of meningococci for diagnostic and epidemiological purposes (1). Recently, an increasing number of strains have been isolated which fail to react with the current set of Mabs so new typing methods are being sought. One way to assess the diversity of meningococcal antigens is to determine the nucleotide sequence of the genes which encode them. The most studied of these genes is *por*A, encoding the class 1 outer membrane protein (PorA) which is the target for serosubtyping MAbs. A new scheme for the designation of subtypes based on their amino acid sequences has been proposed (2,4) and this information, combined with a knowledge of MAb structure may ultimately lead to the engineering of reagents with desired specificities (Abstract: Feavers *et al*). In this work, the P1.10 subtype is used to illustrate the problems involved in the development of comprehensive new reagents for definition of meningococcal subtypes.

The sites of antibody binding to PorA are thought to reside in two variable, surface exposed loops; variable region (VR)1 and VR2 (3,5). Comparisons of *por*A from diverse strains have allowed specific VR sequences to be assigned to particular subtypes on the basis of their similarity in a distance matrix of aligned nucleotide sequences. The VRs from different meningococcal strains are considered to correspond to the same subtype "family" if they are greater than 80% identical by this method. However, variants of PorA occur which may or may not react with MAb but are considered to be members of the same subtype family by nucleotide (or translated amino acid) sequence. These variants are distinguished by the addition of a lowercase letter after the subtype designation; for example, the P1.10 VR occurs with seven variants, designated 10a-10g.

Surveys of meningococcal populations have revealed that the P.10 subtype is common and variable among serogroup B and C organisms. In a recent study of serogroup A meningococci (4), half of the strains analysed contained the P1.10 VR but only one isolate was a variant and 25 strains contained the "prototype" P1.10. This highlights the difference in the population structures of different serogroups (Abstract: Maiden *et al*). Two serogroup A strains contained identical class 1 genes apart from a large deletion in the gene from one strain which entirely removed the

P1.10 sequence. Together with the seven other P1.10 variants, these strains provide an ideal model for the investigation of the structural and immunological properties of meningococcal subtyping antigens.

A threefold approach was taken in this study to: 1.define the minimum P1.10 epitope; 2. determine the nucleotide sequence of P1.10 antibody genes from hybridoma cells and cloned P1.10 antibody fragments; 3. compare the immunological reactivity of P1.10 and its variants.

1.Using data from nucleotide sequencing studies a set of overlapping peptides representing the eight P1.10 variants was synthesised and reacted in ELISA with the P1.10 monoclonal antibody. The amino acid sequence QNQRP towards the end of the P1.10 VR was identified as the minimum P1.10 epitope but other peptides also showed reactivity with MAb suggesting that tertiary conformation is important in antibody binding. 2. The nucleotide sequence encoding the P1.10 antibody was determined by the amplification and cloning of the V_H and V_L genes from hybridoma cells. V_H and V_L genes were also assembled by PCR and cloned into a bacteriophage vector. Sequence data produced by each method was similar but not identical. 3. The reactivities of different serological reagents with P1.10 and its variants were investigated by ELISA and immunoblotting. Comparisons were made between ascites fluid, purified IgG and Fabs. These reagents varied greatly in their affinity for the P1.10 antigen and only the prototype P1.10 reacted strongly in any assay. The P1.10 variants reacted inconsistently, emphasising the need for improved methods for typing and providing additional evidence that structure is critical in immunological activity of meningococcal typing reagents.

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Measurement of antibodies to variable regions of meningococcal class 1 outer membrane proteins using ELISA with synthetic peptides as coating antigens

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Neisseria meningitidis is an important cause of meningitis and septicaemia in infants and adolescents worldwide. The severity of the disease requires the development of a vaccine, which can induce protection to infection in all age groups. Earlier studies have shown that the presence of serum bactericidal antibodies directed to these bacteria strongly correlate with protection against meningococcal infection. Class 1 outer membrane proteins (OMP) of *Neisseria meningitidis* are able to induce bactericidal antibodies and are therefore interesting vaccine candidates.

We developed a synthetic peptide ELISA to identify specific antibodies against class 1 OMP in sera of mice and healthy volunteers immunised with an experimental vaccine based on meningococcal OMPs. Overlapping peptides corresponding to sequences of class 1 OMP of 6 strains were synthesised by simultaneous multiple peptide synthesis (SMPS). Four variants of each peptide sequence were synthesised either by modification of the N-terminus of monomeric peptides (with or without Fmoc or biotinylated) or by multiple antigen peptide (MAP) synthesis of multimeric peptides. Biotinylated and MAP peptides were used to improve absorption of peptides to polystyrene microtiter plates. MAP peptides were also used to mimic conformational epitopes. Peptides which showed optimal binding with anti-class 1 monoclonal antibodies (mabs), were used in ELISA to examine sera from immunised mice and healthy volunteers. Binding of mouse antibodies to coated peptides is specific and could be inhibited (>80%) using an identical free unmodified peptide or purified class 1 OMP. A 100-200 times higher titre could be reached with mabs using a 5-20 times lower concentration of biotinylated peptides or MAPs compared to Fmoc or unmodified peptides. It was not possible to detect human antibodies using monomeric peptides. However, anti-class 1 OMP antibodies in human sera could be detected when multimeric (MAPs) peptides were used as a coating antigen.

Porin peptide ELISA for the serologic diagnosis of disseminated gonococcal infection

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Diagnosis of disseminated gonococcal infection (DGI) still relies on clinical signs and the isolation of *Neisseria gonorrhoeae* from systemic or local infection sites. Serologic methods using whole cells or pili as antigen were hampered by reduced sensitivity and specificity (2). The present study was undertaken to evaluate a newly developed porin protein enzyme-linked immuno-sorbent assay (ELISA) for the serologic diagnosis of DGI.

Synthetic porin A (P1-4) and porin B peptides (P5-6) (1) were coated to polystyrene plates and tested for reactivity with acute and convalescent sera of 50 DGI patients. As controls served sera of 50 dermatology and 24 HIV positive patients as well as 49 sera of children between 1 and 12 years of age. Sera were tested for anti-porin-peptide activity of the IgG and IgA class. Statistical analysis was performed using the student-t-test.

Highest optical densities (ODs) for IgG antibodies of acute and convalescent DGI sera were measured using P3 as antigen. After 12-19 days convalescent DGI sera reacted significantly ($p < 0.002$) lower with P4 than acute sera. Sera of dermatology patients reacted with all six peptides significantly ($p < 0.001$) lower than sera of acute DGI patients. Using P2 as antigen ODs for HIV positive sera were significantly lower ($p < 0.001$) than ODs for acute DGI sera. Children sera showed significantly lower reactivities with P3 ($p < 0.05$), P4 ($p < 0.05$), P1 ($p < 0.01$) and P2 ($p < 0.001$).

Highest ODs for IgA antibodies of acute and convalescent DGI sera were measured using P5 as antigen. Convalescent DGI sera and HIV positive sera showed no significantly different IgA reactivity. For dermatology patients reduced ODs were measured using P5 ($p < 0.05$) and P6 ($p < 0.01$) as antigen. Children sera showed in comparison with acute DGI sera significantly ($p < 0.001$) lower reactivities with all six peptides.

Our study demonstrates that porin A peptide 2, representing the surface exposed loop 1 of the proposed porin model of van der Ley *et al.* (3), seems to represent a useful

antigen for the serodiagnosis of DGI. Porin A peptide 4, representing loop 5 of the proposed model, might be a useful antigen to differentiate acute from convalescent DGI sera.

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Transcriptional regulation of the class 1 outer membrane protein in group B *Neisseria meningitidis*

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The *Neisseria meningitidis* class 1(PorA) outer membrane protein has been documented by several investigators to show variation in levels of expression (1, 8). Due to the high antigenicity of the PorA protein (3, 6), meningococci may down regulate the protein to low levels to enhance survival *in vivo*. *Neisseria* has several examples of regulated genes, such as pili, opa's and 5C (4, 5, 7). Regulation may be at the transcriptional or translational level. We wished to determine the mechanism that controls expression of the class 1 protein. The DNA sequence of the structural portion of *por*A has been previously reported (3), but little has been published about the gene's promoter. We cloned and sequenced the DNA upstream of the class 1 gene. The location of the promoter region was determined by identifying the start of the class 1 protein mRNA in a protection assay. A remarkable attribute of the promoter is a stretch of guanosine residues located between the -10 and -35 promoter regions. Variation in the number of G residues, due to slipped-strand mispairing (2), could result in sub-optimal spacing within the promoter. An improperly spaced promoter would reduce or prevent binding of the RNA polymerase, resulting in little or no mRNA synthesis. We have observed expression of PorA in clones that contain 10-12 G's. We are presently screening a variety of isolates in an attempt to correlate the size of this repeat unit and the quantity of protein expressed.

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Phase variation of class 1 outer membrane protein in *Neisseria meningitidis* by transcription from a variable promoter region

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The class 1 outer membrane protein coded by the *porA* gene of *Neisseria meningitidis*, is a vaccine candidate against this pathogen. The expression of class 1 outer membrane protein displays phase variation between three expression levels. Analysis of the *porA* specific transcripts by Northern hybridization showed that the phase variation is modulated at the transcriptional level. The start site for transcription is located 59 base pairs upstream of the translational initiation codon. Sequence analysis the DNA region upstream of the coding region of the *porA* gene, revealed a poly G tract in the spacer between the -10 and -35 sequence of its promoter. Comparison of promoter sequences of different phase variants showed that the length of the poly G tract can be correlated with the expression level of the class 1 outer membrane protein. These results show that the transcription of *porA* gene is modulated by slipped strand mispairing of a poly G stretch within the intervening sequence of the -35 and -10 regions of its promoter. The phase variation of class 1 outer membrane protein may provide a molecular mechanism to evade the host immune defence. Therefore the protective efficacy of a vaccine based on class 1 outer membrane protein may be questioned.

Irreversible phase variation of class 1 outer membrane protein by deletion of the complete *por*A gene

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The class 1 outer membrane protein coded by the *porA* gene of *Neisseria meningitidis*, is a vaccine candidate against meningococcal infection. From a patient with meningococcal infection a class 1 outer membrane protein positive strain (860183, C:4:P1.1) was isolated from the cerebro- spinal fluid, while the same strain isolated from the blood did not express the class 1 protein. Among 9 other clinical isolates pellicle growth of meningocci of 2 strains (H44/76, B:15:P1.7,1.16 and H335, B:15:P1.15) yielded irreversible class 1 protein negative variants. Analysis by PCR and Southern hybridization showed that the *porA* gene was completely absent in these variants. Sequence analysis of the DNA region upstream and downstream the *porA* gene revealed regions with extensive homology at either side of the gene. From these data we suggest that the *porA* gene can be deleted by homologous recombination. Appearance of *porA* deletion variants in blood and during pellicle growth *in vitro* suggests that the function of the class 1 outer membrane protein can be omitted or is substituted by other outer membrane protein(s) under these conditions. *In vivo* the class 1 negative variant might be selected because of its advantage in the evasion of the host immune defence system.

Construction and analysis of meningococcal mutants for the P64k antigen

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The P64k antigen of *Neisseria meningitidis* has been described as an outer membrane lipoamide dehydrogenase (3). This is an unusual localisation for this enzyme, since it is usually found as part of the cytoplasmic "-oxoacid dehydrogenase complexes (2). In this work we constructed and analysed meningococcal mutants for the P64k protein, as a preliminary step in the elucidation of its physiological role.

A cloned copy of the P64k gene contained within a 5.6 Kb chromosomal fragment was insertionally inactivated with a chloramphenicol acetyl transferase (CAT) cassette and used to transform *N. meningitidis* strain H355. Chloramphenicol-resistant colonies were checked for lack of P64k expression by Western blotting of whole-cell extracts employing a MAb that recognises a linear epitope upstream of the CAT insertion site (1); and proper allelic replacement of the wild-type gene was assessed by Southern blotting. A selected non-expressing mutant was designated as strain 4 and further characterised in a whole-cell dot blot format using rabbit hyperimmune sera against P64k. As expected, intact cells of strain 4 were not reactive in this assay, but surprisingly lysed controls regained reactivity. To help explain this result these samples were analysed by Western blotting with the same serum. The reactive protein in strain 4 had similar relative mobility to P64k; however, it was recognised at much lower intensity that the wild-type controls.

Finally, doubling time of strain 4 was compared to that of P11, a *porA* mutant of H355 prepared in an analogous manner and previously shown to exhibit growth characteristics identical to its wild-type parent. In our conditions, doubling time for strain 4 was 64 min and 37 min for the control P11 strain, suggesting that P64k is physiologically relevant fro growth *in vitro*.

Rescue of P64k mutants implies the existence of a second lipoamide dehydrogenase in *N.meningitidis* since this enzyme is required for aerobic growth (2). Further support for this notion arises from the results of the experiments with polyclonal serum, which is evidence for the presence of a non surface-exposed protein cross-reactive with P64k. Nevertheless, additional research is required to address this issue.

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Monoclonal antibodies specific to a 64 kDa protein from *Neisseria meningitidis*

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The lack of an effective vaccine against all *Neisseria meningitidis* serogroups is a problem in the control of meningococcal disease. Several outer membrane proteins (OMP) have been examined as vaccine candidates, the class 1 protein being the only effective one in inducing high titres of bactericidal antibodies, which are protective in an experimental model for meningococcal infection (4).
In addition to the major OMP, other surface proteins are under investigation in several laboratories (1,3). The characterization of outer membrane proteins, highly conserved in all strains of *N.meningitidis* could be an approach to confer protection against meningococcal disease in humans.

Our group has previously isolated, cloned, and expressed in *E.coli* the M-6 gene coding for a high molecular weight protein (P64k), common to many meningococcal strains (5). The protein has been purified to homogeneity by standard chromatographic methods. To continue the characterization of this meningococcal antigen, we have generated more than a dozen murine monoclonal antibodies (Mabs) against it. Balb/c mice were immunized with a recombinant antigen coded for by an expression construct (pILM-7), including the N-terminal of the Interleukin-2 and the amino acid residues 73-599 from the 64k protein (data not shown). The fusion protein was isolated by preparative SDS-PAGE and electroelution. Another set of mice received 64k protein purified chromatographically. The animals received three doses of antigens emulsified with Freund Adjuvant and then were boosted with a dose of the same antigen without adjuvant, three days before the fusion, which was performed as described (2).

Hybridomas secreting specific Mabs were selected by ELISA using as coating antigen the fusion protein or the 64k protein. Whole cell ELISA was used as secondary screening of Mabs. The plates were coated with strain B385 (B:4:P1.15). The antibodies recognised three epitopes in competition studies. Four Mabs recognised the same or at least closely related epitope. A fifth Mab recognised another epitope probably very close to the previous one, resulting in steric hindrance. A sixth Mab recognised a third epitope completely different.

In Western blotting, all Mabs bound to the protein expressed in meningococci and *E.coli*. None of the six Mabs assayed in bactericidal test in presence of human complement was positive against the strain B385. The sequence of the epitopes recognized by these antibodies are being defined.

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Microevolution of the *opc* gene: Recombinational variants of two uralleles

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An 860 bp fragment spanning the *opc* structural gene was amplified by PCR using chromosomal DNA prepared from 127 *Neisseria meningitidis* strains (serogroups A, B, C, 29E, W135, X and Y) which contain an *opc* gene. The amplified genes were analyzed by DNA-fingerprints using H*pa*II which normally yields 6 fragments. Isolates of serogroups A, W135, X and Y and most serogroups B and C strains gave a DNA-fingerprint pattern indistinguishable from the reference *opc* gene (1), whereas a few bacteria of serogroups B, C and 29E showed aberrant DNA-fingerprint patterns of three distinct classes.

Automated sequencing of the *opc* genes from 28 bacteria revealed the existence of two distinct "uralleles" of *opc*. Both "uralleles" are identical in size but differ by 8 base pair substitutions within the coding region and 6 base pair substitutions plus one base pair deletion in the regulatory region of *opc*. Most substitutions in the coding region lead to amino acid changes and result in a different migration of Opc on SDS PAGE. The changes are scattered throughout the gene, in the trans-membrane regions as well as within loops 2, 4 and 5, and might cause structural changes. Serogroups A, W135, X and Y bacteria have "urallele 1". In serogroups B, C, and 29E, either "urallele 2" or recombinational variants of both alleles forming mosaic like structures are present.

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Cloning and expression of *opc*, the gene encoding the outer membrane protein 5C from *Neisseria meningitidis*: Purification and immunological characterization of the recombinant polypeptide

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Class 5 proteins are a group of hypervariable, heat modifiable proteins in the outer membrane of *Neisseria meningitidis*. A variant of the class 5 proteins, the 5C protein, is expressed by meningococci belonging to different serogroups, with a molecular size ranging between 25-30 kDa (3). The 5C protein is biochemically, genetically and serologically different from other class 5 proteins. Unlike other class 5 proteins, the 5C protein is highly conserved and distributed among different serogroups (1,2). This protein enabled meningococci lacking capsular polysaccharide to adhere to and invade human cells when they express large amounts of 5C (5). Therefore, 5C may be an important virulence factor for meningococcal disease.

In the present work, we cloned and expressed in *E.coli* the *opc* gene from *Neisseria meningitidis* strain B:4:P1.15 (B385) by fusion to the first 46 amino acids of the N-terminus of P64k (4), under the control of tryptophan promoter; yielding a 32 kDa polypeptide (PM80) which accounted for 20% of the total cellular protein. After disruption, the fusion protein produced as inclusion bodies was purified by successive washing of cellular pellet, using 0.5% (v/v) NP-40, 1% (v/v) Triton X-100, and 4M urea. The extraction was done in $CO_3/HC0_3$ buffer pH 10.7, containing 6 M guanidium. Guanidium was then eliminated at extreme pH values. This step was performed by gel filtration and after a last step of anion-exchange chromatography, a soluble protein with more than 70% of purity was obtained. Since this protein is an integral membrane protein and contains a long stretch of hydrophobic segments interacting with the lipid bilayer, we cannot yet tell if the protein has already been renatured. Nevertheless, to evaluate the immunogenicity and bactericidal activity of exposed epitopes of this protein, 2 groups of 10 Balb/c mice were immunized subcutaneously on at least three occasions (days 0, 20 and 35). The first group received 20 ：g of antigen in Freund Complete Adjuvant and was then boosted with the same antigen in Freund Incomplete Adjuvant. The second group received 20 ：g of antigen incorporated into ISCOMS. Sera were collected seven days after each immunization.

Data concerning the evaluation of these sera against either the recombinant protein or natural P5C will be discussed.

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Neisseria gonorrhoeae Opa protein function: Structure-activity studies

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Opacity-associated outer membrane proteins (Opa's) play an important role in gonococcal pathogenesis. Each gonococcal strain possesses about 11 unlinked *opa* genes encoding for at least seven different proteins (2). All Opa's show homology among themselves. This homology is interrupted by three regions: one semi-variable (SV) and two hypervariable (HV1 and HV2), which are surface-exposed and are hypothesized to play a role in Opa's biological activities. Opa's are responsible for the non-opsonic phagocytic killing of *N. gonorrhoeae* (3). Typically Opa's mediate adherence to human polymorphonuclear neutrophils (PMN's) with subsequent triggering of the oxidative burst, and mediate adherence to and invasion of human epithelial cells (5,8). *E.coli* expressing Opa proteins mimic gonococcal adhesion to and invasion of human epithelial cells (4,7) and adhesion to and triggering of the oxidative burst in PMN's (1,4). No human cellular Opa receptor has been identified and no structure-activity studies have been completed. It remains to be elucidated how different Opas with non-homologous surface-exposed hypervariable regions mediate the same interactions with epithelial cells or PMN's. Studies involving an internal deletion and exonuclease digestions were performed to gain a better understanding of Opa's structure-function relationship.

Exonuclease III studies: To perform molecular dissection of Opa functions, we found it more convenient to use *ß-lactamase*/*opa* gene fusions expressed in *E. coli*, which mimics *N. gonorrhoeae* biological activities (7). The mature bla-opa fusion protein is 244 amino acid long and only the first seven amino acids differ from the gonococcal protein (6). The smallest deletion at the 3' end of the gene resulted in a protein 20 amino acids shorter. This minimal deletion completely abolished Opa's ability to mediate adherence to and invasion of ME-180 cervical epithelial cells; however, the same deletant completely retained its ability to interact with PMN's. This function was conserved even when up to 131 amino acids were deleted. Surprisingly, an Opa deletant possessing only 113 or 115 amino acids mediated substantially greater PMN interactions than did longer deletants possessing between 120 and 224 amino acids; adherence and the elicited oxidative burst were six times greater than that elicited by longer deletants or the complete Opa protein. When exonuclease deletions extend into HV1, all Opa protein function is lost; our longest

deletant without any activity is 102 amino acids long.

Internal deletion studies: The region immediately downstream of HV1 shows homology with the binding site of the hepatocyte asialoglycoprotein (ASGP) receptor and we hypothesized that this region was responsible for binding of Opa to human cellular glycoproteins. To test this hypothesis we deleted 42 nucleotides from this region of the *opa* gene and the two remaining parts of the gene were fused in frame. The resulting protein is 230 amino acid long with amino acids 110 to 123 missing from the original bla-opa fusion protein and is expressed on *E. coli* surface. The deletant showed a 250-300 fold reduction of invasion of ME-180 epithelial cells compared with the complete Opa (from $6x10^5$ bacteria/well to $2x10^3$ bacteria/well). However, the deletant still invaded; in two separate experiments the number of invading bacteria was ten fold above the control (*E. coli* pGEM3Z). Regarding Opa-mediated association with PMN's, we monitored the triggering of the oxidative burst by luminol-enhanced chemiluminescence (LCL). We performed two sets of experiments; in the first one we observed no difference in LCL between the deletant and the Opa control, in the second we observed a 50% reduction in LCL in the deletant compared to the Opa control. In both experiments the deletant triggered a significant LCL response.

Discussion: Our results show that Opa-mediated association with human epithelial cells and PMN's occurs by two different mechanisms. It is likely that two different classes of receptors are involved. At least two different areas of Opa protein are involved in invasion of epithelial cells: the C-terminal region and the ASGP receptor homologous region.

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Transformation and expression of Opa proteins in *Neisseria gonorrhoeae*

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Swanson's data on the apparent surface charge characteristics of gonococci suggests that DNA may preferentially coat the surface of opa expressing organisms, leading to the obvious question as to whether opa^+ transformation rates are higher than opa^-. Using an isogenic series of gonococci, either expressing no opa protein, or one of four different opa's, we find that transformation rates are modestly elevated with opa^+ cultures. However, the use of high cell density starting cultures led to considerable lethality (or switching) within the opa^+ populations, leading to the establishment of two sizeable subpopulations (i.e. opa^+ and opa^-), with the opa^- subpopulation always being transformed at a higher frequency than the isogenic opa^- control culture.

The apparent lethal phenotype associated with transformation of opa^+ populations (and also to a lesser extent with opa^- populations) could be alleviated by using low cell density starting cultures. In single colony transformation experiments, opa^- and opa^+ populations now showed differential increases in colony forming units during the course of the experiments, with opa^+ populations undergoing transformation at a decidedly higher rate than opa^- isogenic controls. Surprisingly, when transformation frequencies were assessed for two unlinked markers, opa^- gonococci proved to be the more competent for transformation. I am currently investigating as to why opa^+ populations, though less competent, seem to be more efficient at transformation than their opa^- counterparts.

Under construction: *Escherichia coli* encoding multiple gonococcal factors as a model for studying *Neisseria gonorrhoeae* salpingitis

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Understanding the pathogenesis of *Neisseria gonorrhoeae* (GC) infections has been hindered by the variability and number of GC virulence factors. Three such GC virulence factors are lipooligosaccharide (LOS), opacity-associated proteins (Opa, formerly PII), and porin proteins (Por or PI). Opa and LOS may mediate attachment to host cells, interbacterial adherence, and invasion (2,7). PI/Por is able to spontaneously insert into eucaryotic membranes making it a candidate invasion-trigger (6). GC LOS epitopes (7), Opa (1,8-10), and PIA (4) have been cloned and expressed in *Escherichia coli*. These clones allow the function of putative virulence factors to be examined in a stable background. The following plasmids in *E. coli* DH5" encode $-lactamase/Opa fusion proteins: pLPGC1 (OpaF62-SFG1 with some additional downstream GC DNA (8)), pDS002 (OpaP a.k.a. OpaF62-SFG1 minus the extra downstream DNA), pDS0031 (OpaA from FA1090), and pDS0041 (OpaB) (9,10). The 3F11 LOS epitope is expressed in *E. coli* DH5" from a 6Kb fragment (7) cloned into chloramphenicol-selectable pACYC184 (designated pYCLOS-5). *E. coli* DH5" and M16 express PIA (strain FA19) from pUNCH30 (4). Expression of each component has been documented with Western and/or dot blot.

PIA-production by *E. coli* enhanced susceptibility to penicillins and vancomycin in disc diffusion studies. The increases in zone sizes for penicillins were larger for M-16 (6 to 20mm) than for DH5" (0 to 13mm). This correlated with strain M-16 expressing more PIA than DH5". The change in antibiotic susceptibility was selective since susceptibilities to quinolone, monobactam, and carbapenem drugs were unchanged. PIA production by *E. coli* results in a functional outer membrane porin that alters bacterial susceptibility to antibiotics much like different classes of PI/Por do in an isogenic GC background (3).

E. *coli* DH5" expressing OpaP, OpaA , or no Opa were tested in the human Fallopian tube organ culture model (FTOC) by using computerized image analysis with digital confocal microscopy to measure attachment and invasion (5). *E. coli* producing OpaA (*E. coli* (*opaA*)) were more adherent (0.9 : ^{2}/cell equal to approximately 1 bacterium/cell) than *E. coli* (*opaP*) (0.017 : ^{2}/cell, about 0.02 bacteria/cell) or the non-producer (0.015 : ^{2}/cell) at 24h ($p<0.001$). There was no invasion at 24h. At 48h post-infection, *E. coli* (*opaP*) attached although to a lesser extent than *E. coli* (*opaA*). Control bacteria did not attach or invade. In comparison, quantitation of attachment and invasion by GC at 48h showed equivalent amounts of intracellular GC vs extracellular attached GC, often present in clumps (approximately 5 bacteria/cell) (5). *E. coli* (*opaA*) traversed the epithelium without clumping, as occasional single rods. By electron microscopy, *E. coli* (*opaA*) strains were attached to the epithelial surface and enclosed in membrane-bound vacuoles in the cytoplasm of non-ciliated cells similar to GC. Thus, Opa fusion proteins mediate bacterial attachment that is similar in magnitude to GC and invasion that is less than GC. This suggests that additional factors besides Opa, e.g. PI/Por, contribute to GC invasion. In parallel experiments, *E. coli* M16 producing PIA was completely unable to adhere to the epithelium, so invasion was not observed. These results led us to construct *E. coli* strains which encode more than one GC product.

We have constructed three recombinant strains of *E. coli* that encode PIA+ OpaA (strain INVOAP1), PIA + 3F11 LOS (strain INVLP1), and OpaA+ 3F11 LOS (strain INVLOA). Strain INVOAP1 was obtained by subcloning the 1kb Klenow-filled *Eco*RI/*Bam*HI fragment (*$lac/opaA* gene insert) from pDS0031 into the *Sma*I site of pUNCH30. The $-lactamase/OpaA fusion protein and PIA are both expressed in strain INVOAP1. Strains INVLOA and INVLP1 were derived via co-transformation of pYCLOS5 with pDS0031, and pYCLOS5 with pUNCH30, respectively. INVLOA (OpaA and 3F11 LOS) displays a clumping interaction. Plate-grown INVLOA was compared to control organisms that produced OpaA alone, 3F11 LOS alone, or 3F11 and PIA (strain INVLP1). Each strain was resuspended to similar optical densities in sterile PBS then Gram-stained on slides. In random microscopic fields, the size of organism clumps was measured with computerized image analysis. Strain INVLOA showed larger organism clumps than OpaA alone, 3F11 LOS alone, or 3F11 and PIA (4.508 : ^{2} vs. 2.774 : ^{2} vs. 2.831 : ^{2} vs. 2.561 : ^{2}, respectively). These differences were significant for INVLOA compared to the controls ($p<0.000008$).

Multiple GC factors can be cloned and expressed in *E.coli,* simultaneously. Each factor displays properties in an *E. coli* background that are analogous to their behavior in GC. These tools will aid investigations of GC salpingitis.

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Immunodetection of the *mtr* associated outer membrane protein in *Neisseria gonorrhoeae*

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Low level resistance to antimicrobial agents in *N.gonorrhoeae* is specified by the additive effects of several genes (1). Levels of resistance attributed to decreased membrane permeability have been associated with mutations at the *mtr* and *penB* loci, although the exact mechanisms involved have not been known. For a number of years this institution has monitored the presence of resistance determinants in clinical isolates from patients attending the STD clinic. Observations regarding the pleiotropic nature of *mtr* have prompted us to further study of the genes involved.

Acquisition of *mtr* results in increased amounts of a 44-52KDa outer membrane protein (2). We have raised a polyclonal antibody to this *mtr* associated protein by immunisation of mice with specific protein isolated from Western blots of the outer membrane protein profile. Subsequent affinity purification of the antisera has enabled us to screen a genomic library of strain FA136. Expression of selected immunoreactive clones in *E.coli* has resulted in production of a 44-46KDa protein of equivalent molecular weight to the *mtr* associated outer membrane protein. Confirmation of the nature of this protein awaits further characterisation.

The *mtr* region of *N.gonorrhoeae* has recently been cloned and found to consist of three genes designated *mtrR, mtrC,* and *mtrD* organised as an operon (3). The *mtrC* gene is predicted to encode a 44KDa outer membrane lipoprotein with significant similarity to the EnvC and AcrA lipoproteins of *E.coli.* which are components of multidrug efflux systems (4).It is suggested that the *mtrRCD* products comprise a similar system in *N.gonorrhoeae*, and may confer a selective advantage for survival of the organism in the rectum where detergent-like hydrophobic compounds are present (5). We hope to study this further, and in turn use immunological methods for the determination of the products of the *mtrR* and *mtrD* genes.

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Regulation of recombination and DNA repair in *Neisseria gonorrhoeae*

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Recombination and DNA repair are indirectly related to virulence in *Neisseria gonorrhoeae* since these systems allow phase and antigenic variation of cell surface components. Antigenic variation in pilin, the structural subunit of gonococcal pili, is thought to be a principal means by which this pathogen evades the host immune response. This process involves recombination between variant pilin gene sequences and depends on the presence of a functional *recA* gene. In *Escherichia coli*, genes involved in recombination and DNA repair are part of the SOS regulon.

In order to determine whether an SOS system exists in *N. gonorrhoeae*, experiments were designed to isolate genes that may be co-regulated with *recA*. Gonococcal equivalents of the *E. coli* excision repair genes, *uvrA* and *uvrB*, were cloned and sequenced. Both *uvrA* and *uvrB* complemented the UV sensitive phenotype of *E. coli uvrA* and *uvrB* mutants, respectively. A *uvrB* mutant of *N. gonorrhoeae* strain MS11 was constructed and shown to be extremely sensitive to UV radiation. The promoter regions of *uvrA* and *uvrB* were localised and transcription start points of the cloned genes mapped. The s^{70} promoter of *uvrB* was found to be associated with a copy of the 26 bp neisserial repeat. A putative repressor binding site, differing from the *E. coli* SOS box, was identified in the sequences upstream of *uvrA*, *uvrB* and *recA*. In addition, all three genes show the presence, within the upstream region, of an inverted repeat of the gonococcal transformation uptake sequence. The presence of the DNA uptake sequence in all three promoter regions may suggest a connection between transformation competence and regulation of these genes.

The repertoire of silent pilin gene copies and their chromosomal location is similar but not identical in gonococcal strains FA1090 and MS11

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A single strain of *Neisseria gonorrhoeae* has the ability to change the primary amino acid sequence of the pilin molecule, and thus the antigenic nature of the pili. The molecular mechanism responsible for the pilin variation involves a non-reciprocal recombination event (3,5). The *pil*E locus contains the functional pilin gene, while the *pil*S loci contain one or more promoterless pilin genes. Pilin sequences move from the *pil*S loci into the *pil*E locus generating a new pilin sequence in the *pil*E gene. The pilin molecule has a number of conserved domains that flank the six variable domains or minicassettes (mc1-mc6). In the minicassette model of antigenic variation, one or more of the minicassettes recombine as genetic units into the *pil*E locus to generate a new pilin sequence (3,6). The number of silent copies present in a strain coupled with the ability to reassort minicassettes from various *pil*S loci to generate new *pil*E sequences allows for an enormous collection of potential pilin sequences.

Meyer and co-workers have characterized the *pil*S loci of gonococcal strain MS11; it has 17 silent copies and 2 expression loci (4). MS11 is the only gonococcal strain for which the pilin repertoire is characterized, so comparison of the different *pil*S copies of multiple strains has not been possible. We have cloned and sequenced the 6 *Cla*I fragments that contain the *pil*S and *pil*E loci from gonococcal strain FA1090. Strain FA1090 has 19 silent pilin copies, one pilin copy with a significant portion of sequence deleted and 1 *pil*E gene. Most of the silent copies are clustered in two *pil*S loci, which contain 5 and 6 copies. Two other *pil*S loci each contain 3 silent copies, and the final locus contains only 1 copy. The *pil*E locus has 1 associated silent copy, and one copy that contains the conserved domains 5' of mc6 and 3' of mc2, but the sequence between these conserved domains is not typical of other pilin copies with regard to size and predicted amino acid sequence. Each of the 19 silent pilin copies contains all 6 minicassettes, although in one copy the pilin sequence begins in the middle of mc6. Four of the 19 copies are out of frame beginning at a string of guanine residues at the 5' end of mc5. These copies are otherwise in the same frame throughout the rest of the copy. Using oligos or DNA clones specific for individual copies, we located *pil*E and 4 *pil*S loci containing a total of 18 silent copies to one region no larger than 60kb of the FA1090 chromosome. The remaining locus contains only one silent copy, and is located elsewhere on the chromosome. The

arrangement of the 5 *pil*S loci of strain MS11 is similar, except that the major pilin gene cluster contains 3 *pil*S loci, and there is an additional region of the chromosome containing a *pil*S locus, relative to strain FA1090 (1,2).

We compared the FA1090 *pil*S predicted amino acid sequences to that of the sequenced *pil*S copies from strain MS11. While the sequences are very much alike, there are few variable domains that are identical in the two strains. The more N-terminal sequences are more alike between the two strains with mc5 sharing the most number of sequences, whereas within mc2 only one predicted amino acid sequence is identical in the two strains. The N-terminal portion of the pilin molecule is the most conserved, while mc2 encodes the most antigenically variable and immunodominant portion of the protein. From this comparison, we can conclude that the FA1090 repertoire of pilin sequences is related to that of MS11, but the repertoires are not identical.

Pilin variation has been demonstrated *in vitro* (3,4,6,8), *in vivo* (7,9), and in single strains of bacteria isolated from sexual cohorts (10). In the experimental human infection trials ongoing at UNC, we demonstrated *pil*E sequence variation in strain FA1090 even in the short duration of the experimental infection (7) (less than 5 days). With the available information about the pilin repertoire in strain FA1090 we will be able to address questions regarding the preferential use of individual pilin sequences at the initiation of infection.

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Generation of a novel pilin locus using CAT fusions in *Neisseria gonorrhoeae*

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Gonococcal pilus phase and antigenic variation support immune evasion and alter receptor binding specificities (2,4,5). Antigenic and some phase variants are generated when variant DNA from one of several silent pilin genes replaces sequences in an expressed pilin gene. These RecA-dependent recombination events (3) are usually non-reciprocal. Human volunteer studies have shown that many antigenic variants appear during infection (6), suggesting a high rate of *in vivo* variation. Phase variation (monitored through changes in colony morphology) has been used to estimate the rate of *in vitro* antigenic variation which suggests that the *in vitro* rate of variation is lower than the *in vivo* rate. To create a genetic means to quantitatively assay recombination between pilin genes we inserted a promoterless CAT gene cartridge into a silent pilin copy of *pil*S1 in strain MS11 The frequency of recombination between *pil*S1::CAT and *pil*E was to be monitored by selection on chloramphenicol for resistant variants (Cm^r) expressing CAT from the pilin promoter. Cm^r variants were generated at a frequency of 10^{-9} to 10^{-7} and were RecA-dependent. Analysis of both piliated and non-piliated Cm^r variants revealed a new pilin locus formed by the duplication of sequences from *pil*E and *pil*S1::CAT loci. At a lower frequency, reciprocal recombination was also detected between the loci. Simple non-reciprocal insertion of CAT into *pil*E was not detected presumably because the CAT marker, inserted in a region of *pil*E conserved at the protein and the DNA level, interfered with recombination. Cm^r variants were generated at a similar frequency when MS11 was transformed by *pil*S1::CAT and *pil*E together but transformation with *pil*S1::CAT alone did not produce Cm variants. A new pilin locus was again observed in each variant suggesting that all of these new pilin loci were created via DNA transformation.

These studies show that disruption of a conserved pilin sequence by CAT prevents simple non-reciprocal recombination of this sequence into *pil*E (i.e. prevents antigenic variation). These surprising results demonstrate that autolysis and DNA transformation can result in new pilin loci. With the mounting evidence that different neisserial strains and even different species can exchange genetic information *in vivo* (7, 1), the ability to reconfigure partially homologous sequences into new chromosomal loci allows for flexibility in the neisserial genome and evolution of these species.

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Cloning and sequence analysis of a *pil*R/*pil*S homologue from *Neisseria gonorrhoeae*

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One of the putative promoter sequences identified upstream of the *pilE* gene of *Neisseria gonorrhoeae* is a consensus -24/-12 promoter. Transcription from such a promoter is dependent on the alternative sigma factor σ^{54} and an activator protein, which typically binds to a site (upstream activator site), approximately 100 bp upstream of the promoter (1). At the appropriate distance upstream of the *pilE* σ^{54} promoter is a sequence sharing similarity (13/17 bp identical) to the binding site for PilR, the activator protein, which in association with the sensor protein, PilS, is required for transcription of the *pilA* gene of *Pseudomonas aeruginosa* (2).

We have shown that PilR is capable of activating the gonococcal σ^{54} promoter using a P*pil*E::*cat* fusion with the other promoters mutated, cloned into the *P. aeruginosa* plasmid pPAH121. On the basis of this result, we decided to search for a gonococcal homologue of the *P. aeruginosa pilR* gene, by probing MS11 chromosomal DNA digests with the *pilR* coding region. Sequence analysis of the cloned hybridising *Hin*dIII/*Bgl*II fragment revealed a region in which the derived amino acid sequence showed similarity to the carboxy terminus of PilR and upstream of this, to the central region of PilS. PCR was subsequently performed on a number of gonococcal strains including MS11 to ascertain whether a deletion may have occurred within *pilR* and/or *pilS*, or whether a single gene is present encoding a protein which can function as both a sensor and regulator. Preliminary data supports the existence of a single gene.

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Site-directed mutagenesis of the promoter region of the gonococcal *pilE* gene

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Primer extension analysis performed on RNA from *Escherichia coli* expressing the cloned *pilE* gene from *Neisseria gonorrheae* strain MS11, has revealed two major transcription start points (tsp), located 54 bp and 87 bp upstream of the ATG start codon. At the appropriate distance upstream of both these tsps are sequences showing a high degree of similarity to a -10 box of a sigma 70 promoter. In addition, a sigma 54 consensus sequence, overlapping the upstream sigma 70 promoter, acts as a binding site for RpoN, but in the absence of an activator protein, does not promote transcription. Instead, RpoN acts as a repressor of the sigma 70 promoter (1). Site-directed mutagenesis of the two -10 boxes in a P*pilE::cat* transcriptional fusion gives rise to insignificant levels of *cat* expression in *E. coli* . However, when the same construct is introduced into *Pseudomonas aeruginosa*, transcription takes place from the sigma 54 promoter, in a PilR dependent fashion. Primer extensions confirm that the tsp for this promoter is within 2 bp of the tsp mapped for the upstream sigma 70 promoter.

When primer extensions are performed on total RNA from piliated MS11, only one major tsp is observed, at the position mapped for the overlapping sigma 70 and sigma 54 promoters. A series of P*pilE::cat* constructs, with individual promoter mutations, has been introduced into the MS11 chromosome, by recombining them into the non-essential *iga* gene. Primer extensions and CAT ELISAS performed on these recombinant strains, has clearly shown that the sigma 70 promoter is responsible for transcription of *pilE* in gonococci grown under a variety of *in vitro* conditions.

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Characterization of gene products involved in gonoccocal pilus biogenesis

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The expression of pili by *Neisseria gonorrhoeae* plays an essential role in gonoccocal colonization of the human host. Recently, several *N. gonorrhoeae* gene products have been identified which bear homology to proteins involved in pilus assembly and protein export in other bacterial systems (2), including *Pseudomonas aeruginosa* (4) and *Vibrio cholerae* (3).

Experiments utilizing transposon mutagenesis have indicated that the *N. gonorrhoeae pilF* and *pilD* gene products are required for assembly of gonoccocal pilin into pili. Mutants lacking the *pilD* gene product, a prepilin peptidase, were unable to process the prepilin subunit into pilin, whereas *pilF* mutants processed pilin but did not assemble the mature subunit. Both mutants secreted S-pilin, a soluble, truncated form of the pilin subunit previously correlated with defects in pilus assembly (1). In addition, mutants containing transposon insertions in *pilD* or in a downstream open reading frame, *orfX*, had greatly reduced rates of growth. Deletion analysis of *pilD* indicated that the poor growth phenotype observed for the *pilD* transposon mutants was due to polar effects of the insertions on *orfX* expression. *orfX* encodes a predicted polypeptide of 23 kD and has apparent homologues in *P. aeruginosa*, *Pseudomonas putida*, *Thermus thermophilus*, and the eukaryote *Caenorhabditis elegans*. In *P. aeruginosa* and *P. putida*, the orientation of *orfX* downstream of *pilD* is also conserved.

Although expression of *orfX* and *pilD* appears to be transcriptionally coupled, mutants containing transposon insertions in *orfX* expressed pili. Unlike either *pilF* or *pilD* mutants, *orfX* mutants were also competent for DNA transformation. A possible relationship exists between prepilin processing or pilus assembly and OrfX function as *pilD-orfX* double mutants grew extremely poorly, and fast-growing colonies which arose from the double deletion mutants expressed greatly decreased to undetectable levels of the prepilin subunit. In addition, Western analysis using antibodies generated against purified pili has identified several polypeptide bands unique to *orfX* mutants, thus it is possible that the *orfX* gene product is associated with the expression of pilus associated proteins.

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The Product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*

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Type IV pili has been shown to play an essential role in colonization of the human host by *Neisseria gonorrhoeae* (Gc). The product of the *omc* gene, Omp-mc, has been proposed to be a highly conserved, surface exposed, outer membrane protein, but its function has not yet been characterized (4). Recent studies show that proteins essential for Gc pili biogenesis are related to a family of gene products involved in protein secretion, filamentous phage assembly and DNA transfer (1). The PilQ protein has been shown to be essential for pilus biogenesis in *Pseudomonas aeruginosa* and was found to have significant identity with Omp-mc (2).

Our studies examined if Omp-mc functions in a similar role in Gc. Using transposon shuttle mutagenesis, we have constructed defined mutations within and flanking the *omc* gene and characterized the mutants with respect to pilus assembly and pilus-associated phenotypes. The mutants exhibited a nonpiliated phenotype when cultured on clear solid media, and failed to autoagglutinate when cultured in liquid media. By electron microscopy, the mutants were devoid of cell surface-associated pili. In DNA transformation assays, the mutants exhibited a 1000-fold reduction in competence compared to wild type strains. Western blot analysis revealed that the mutants expressed wild type levels of pilin but examination of cultured supernatant of mutants revealed the presence of pilin, S-pilin, and large amounts of truncated Omp-mc protein. DNA sequencing, Northern blotting and primer extension studies suggest that *omc* is part of an operon containing other pilus assembly genes, which is similar to the results found for the *pilQ* gene of *Pseudomonas aeruginosa* (3). Collectively, these data demonstrate that Omp-mc, an abundant surface exposed Gc antigen, is essential for type IV pili biogenesis.

These data along with the homology of Omp-mc with other components of surface translocation machinery suggest that Omp-mc may function as a pore / gate channel in the extrusion of pili. In light of the results detailed here and to be consistent with preexisting pilus assembly gene nomenclature, we have redesignated the *omc* gene as *pilQ*, and its product, PilQ.

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Characterization of *omc*, gene required for type IV pili biogenesis and competence in *Neisseria gonorrhoeae*

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Type IV pili has been shown to play an essential role in colonization of the human host by *Neisseria gonorrhoeae* (Gc). The product of the *omc* gene, Omp-mc, has been proposed to be a highly conserved, surface exposed, outer membrane protein, but its function has not yet been characterized. Recent studies show that proteins essential for Gc pili biogenesis are related to a family of gene products involved in protein secretion, filamentous phage assembly, and DNA transfer. The Pil Q protein has been shown to be essential for pilus biogenesis in *Pseudomonas aeruginosa* and found to have significant identity with Omp-mc.

Our studies examined if Omp-mc functions in a similar role in Gc. Using transposon shuttle mutagenesis, we have constructed defined mutations within and flanking the *omc* gene and characterized the mutants which express truncated forms of Omp-mc. The *omc* mutants were non-piliated and failed to express pilus-associated phenotypes. These data demonstrate the Omp-mc, an abundant surface exposed Gc antigen, is essential in type IV pili biogenesis. Based on these studies and the homology of Omp-mc with components of surface translocation machinery, suggests that it may function as a pore/channel in the extrusion of pili.

The *plsC* Gene of *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The assembly of membrane phospholipids in the *Neisseria meningitidis* and *Neisseria gonorrhoeae* is not understood at a molecular level. The conversion of lysophosphatidic acid to phosphatidic acid, an integral membrane component, is mediated in *Escherichia coli* by a 1-acyl-sn-glycerol-3-phosphate acyltransferase, the product of the *plsC* gene (1).

We have identified the meningococcal and gonococcal homologues of the *plsC* gene, and describe the effects of mutation affecting these genes. A *Tn916*-generated mutant of a serogroup B meningococcal strain (2) was identified that expressed increased amounts of ("268)-linked capsular polysaccharide by immunoblots, and increased numbers of assembled pili (2-3x the number of pili/diplococcus as compared to the parent strain) by electron microscopy. The single, truncated 3.8 kilobase *Tn916* insertion in the meningococcal mutant was located in a 771 base pair open reading frame. The gonococcal equivalent of this gene was identified by transformation with the cloned meningococcal mutant gene. In *N. gonorrhoeae*, the mutation increased piliation up to five-fold. The gonococcal insertion was present in a 765 base pair open reading frame. The predicted amino acid sequence of the putative meningococcal and gonococcal gene products were homologous to the PlsC gene product of *E. coli* (>50% similarity through a 136 amino acid overlap) and other bacterial species, as well as the eukaryotic enzyme (3).

A plasmid cloned copy of the meningococcal *plsC* gene complemented a strain of *E. coli* harboring a temperature-sensitive mutation of the *plsC* gene. Complementation restored 1-acyl-sn-glycerol-3-phosphate acyltransferase activity at the non-permissive temperature. Since interruption of the neisserial *plsC* homologue was not lethal in either *N. meningitidis* or *N. gonorrhoeae*, the pathogenic *Neisseria* sap. may be able to utilize an alternative pathway to produce glycerolphospholipids in the absence of PlsC enzymatic activity. This hypothesis is supported by the observation that although the levels of mature glycerolphospholipids and acyltransferase enzymatic activity were reduced in the neisserial *plsC* mutants when compared to parental strains, they were still detected at a significant level. In pathogenic *Neisseria* sap. a

secondary pathway appears to produce glycerolphospholipids in the absence of PlsC enzymatic activity. Implementation of this alternate pathway results in alterations of glycerolphospholipid composition that lead to pleiotropic effects on outer membrane components, including capsule and piliation.

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Pilus-mediated attachment of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to host cell receptors

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Pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* facilitate binding of the bacteria to epithelial cells (4-6), and undergo both phase and antigenic variation. PilC is a 110 kD minor pilus associated protein for which two loci exists. Expression of *pilC* is controlled by frameshift mutations in a poly G tract within the signal peptide coding region that put the initiation codon in or out of frame compared to the mature protein (1). *N.meningitidis* strains carry one or two *pilC* loci containing a poly G tract similar to what is seen in gonococci. A selective mechanism for structural variation of gonococcal pili has been proposed to operate at the level of assembly due to on/off switch in PilC expression (2).

The binding to epithelial cell lines and to fixed tissue sections of *N.gonorrhoeae* MS11 derivatives and mutants has been studied. Piliated (P+) gonococcal clones carrying variant pilin gene sequences showed different binding properties to human conjunctival and corneal tissue. However, sequence changes in PilE did not effect binding to cervical and endometrial tissue. The eukaryotic receptor(s) for gonococcal pili present on a wide variety of human tissues was resistant to periodate treatment but sensitive to proteinase K, suggesting it may be a protein or a glycoprotein (3).

In order to identify a potential protein-receptor for P+ gonococci, a cDNA library was constructed from an endocervical cell line. The library was screened by a "panning procedure" using purified gonococcal pili and a clone containing a 2.4 kb insert was identified. Transfection of the cDNA-clone into a cell line that normally do not bind gonococci, resulted in cells that efficiently bound P+ but not P- gonococci. This cDNA-clone is currently being characterized.

The meningococcal PilC genes and their role in attachment and pilus biogenesis are being studied, as well as the role and function of potential carbohydrates on the meningococcal pili.

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Modulation of pilus-facilitated adherence of *Neisseria meningitidis* by pilin primary sequence changes, post-translational modification and PilC expression

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Adherence of capsulate *Neisseria meningitidis* to human endothelial and epithelial cells is only facilitated in variants that express pili. Pili produced by meningococci are either of Class I, which are very similar to those produced by *Neisseria gonorrhoeae*, or of Class II, which are antigenically and biochemically distinct (1). Both types of pili are subject to phase and antigenic variation. These processes assist in the avoidance of host immune surveillance and modulation of adhesion specificity for different tissues during the course of infection.

Variants of the serogroup B strain C311 have been obtained that express Class I pilins of reduced apparent M_r and exhibit marked increase in adherence to Chang conjunctival epithelial cells (2,3). Structural analysis of pilins from two hyper-adherent variants 311#7 and 311#16, and the parent was carried out by DNA sequencing of their *pilE* loci. Comparison of deduced amino acid sequences indicated that, although several substitutions had occurred in the variant pilins, no deletions could be observed to account for the reduced apparent M_r. Deduced MW's of pilins were also considerably lower compared with their apparent M_r on SDS-PAGE, consistent with some form of post-translational modification of the pilin polypeptide. Mature hyper-adherent pilins shared unique changes in sequence, notably substitution of Asn_{113} for Asp_{113}. This was accompanied by changes from Asn-Asp-Thr-Asp to Thr-Asp-Ala-Lys at positions 127-130. The Asn residues 113 and 127 of the "parental" C311 pilin both form part of a typical eukaryotic N-glycosylation site (Asn-Xaa-Ser/Thr). These observations invite the hypothesis that pilins may be modified by post-translational glycosylation, perhaps involving the motif Asn-Xaa-Ser/Thr (3).
Class I pilin variants derived from a separate strain MC58, 58#5 (adherent) and 58#6 (non-adherent) differed only residues 60 and 62. The glycosylation motif Asn_{60}-Asn_{61}-Thr_{62} in 58#5 was replaced by Asp_{60}-Asn_{61}-Ser_{62} in variant 58#6. Fully adherent backswitchers obtained from 58#6 always regained an Asn_{60} but retained Ser_{62}. Thus, substitution of Asn by Asp at position 60 in MC58 pilins abolishes adhesion to epithelial cells without affecting adhesion to endothelial cells (2,3). This region of the class I sequence contains two overlapping Asn-Xaa-Ser/Thr motifs.

Variants that lack the critical Asn_{60}, such as 58#6, might paradoxically be more susceptible to modification at the adjacent site. The apparent M_r of variant pilins are consistent with this notion. Functional variations in *N.meningitidis* Class I pili are thus modulated, in part, by primary amino acid sequence changes that either create (normally down modulating adherence) or ablate (normally up modulating adherence) motifs that are classic N-glycosylation sites. The precise structure of the glycosyl moieties involved are currently being investigated by fast bombardment mass spectrometry (FAB-MS) and electronspray mass spectrometry (ES-MS).

A hyper-adherent variant that exhibited no changes in primary amino acid sequence was isolated from strain MC58. MC58 elicits pilins with generally less potential glycosylation motifs than the parental C311 strain and normally produces low amounts of the phase variable pilus-associated protein PilC. In contrast, all variants isolated from strain C311 consistently produced similar but appreciable amounts of PilC. The hyper-adherent MC58 variant was found to produce substantially larger amounts of PilC than the parental strain and showed differences in kinetics of adherence to host cells. The relative increase in adherence to Chang cells was substantially greater than to HUVEC endothelial cells after 1 hr incubation. Furthermore, adherence to Hep-2 human epithelial cells or cells of non-human origin was unaffected in this variant. Thus pilus-mediated adherence to certain human cells may be modulated by PilC as well as by changes in primary amino acid sequence of pilin which is consistent with previous observations of ourselves (3) and others (4,5). Furthermore, changes in pilin and PilC appear to have differential effects on adhesion of meningococci to different human epithelial cell types.

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Anti-Gal binds to pilus of *Neisseria meningitidis*: The Iga isotype blocks complement-mediated killing

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Anti-Gal are ubiquitous natural human polyclonal antibodies that bind to terminal galactose "1,3galactose ("-galactosyl) residues (2). Gram-negative bacteria that cause sepsis are more likely to bind serum anti-Gal than are bacteria of the same species that are isolated from the stools of well persons (3). This may be because serum IgG anti-Gal can block alternative complement pathway-mediated lysis of representative bacteria that bind it to "-galactosyl LPS structures, thereby promoting survival of such bacteria in the nonimmune host (3). We now report on the binding of anti-Gal to the cell-surfaces of *Neisseria meningitidis*.

We were interested in examining whether anti-Gal binds to the LOS of *N. meningitidis*, analogous to its binding to LPS of enteric Gram-negative bacteria. Serum IgG, IgA, and IgM anti-Gal as well as colostral IgA were purified by affinity chromatography using silica-linked Gal"1,3Gal$1,4GlcNAc (3). Immunoblots of SDS-PAGE-separated outer membrane preparations from two group C strains and one group B strain revealed that all three isotypes bound the pilus but not the LOS of these organisms, suggesting an immunogenic carbohydrate pilin epitope. Binding to pilin was confirmed using pilus purified from one of the strains. The anti-Gal isotypes also bound to the pili of whole organisms as determined by immunoelectronmicroscopy. Convalescent serum from a child recovering from meningococcal disease contained antibodies that bound strongly to purified pilin from the infecting strain. Pilus-binding but not outer membrane protein-specific antibodies in normal human serum could be completely absorbed by passage of the serum over the silica-linked carbohydrate column. Mild periodate oxidation of SDS-PAGE-separated outer membranes of these strains followed by labeling of the neo-aldehyde groups resulted in labeling of bands corresponding to LOS and of the band corresponding to pilin exclusively, further suggesting that pilus contains a carbohydrate structure. This finding confirms a recent report that meningococcal pili are glycosylated (7).

We next examined the effect whole serum, serum IgG, serum IgA1 anti-Gal and colostral IgA anti-Gal on the complement mediated killing of a group C strain which was grown under conditions that caused either maximal or minimal piliation. Serum

anti-Gal containing all three isotypes blocked complement-mediated lysis of this strain whereas serum anti-Gal IgG alone had no effect on killing. Purified serum anti-Gal IgA1, however, blocked lysis indicating that IgA1 was the blocking anti-Gal isotype in serum.

Piliation of meningococci is associated with increased adherence to human nasopharyngeal cells and it also mediates initial attachment of meningococci to human nasopharyngeal tissue in organ culture (4, 6). Pili are found on meningococci and gonococci isolated from human mucosal surfaces and as well as on meningococci isolated from the bloods and cerebrospinal fluids of patients with disseminated meningococcal disease (1, 4, 5). The presence of blocking mucosal and serum anti-Gal that bind to meningococcal pili therefore may be of pathogenic importance. Our findings support a role for anti-Gal in meningococcal disease.

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