PLENARY SESSIONS

SESSION I

Pathogenesis

Signaling functions of the *Neisseria* pilus

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The pathogenic Neisseria, like a number of other bacteria, express type IV pili. This surface structure promotes neisserial adherence to epithelial cells, motility, and DNA uptake. Recent observations strongly suggest that many biological activities of the pilus result from its ability to induce or modify signaling cascades in host epithelial cells. The pilus triggers a series of calcium fluxes in the epithelial cell cytosol, leading to the exocytosis of subcellular compartments, modification of lysosomes, and the promotion of intracellular bacterial replication. Infection of cells by piliated Neisseria also triggers tyrosine phosphorylation of epithelial cell CD46, to which the pilus binds, and the formation of adhesion-promoting cortical plaques. A model will be presented for the role the type IV pilus plays in initiating the first stages of bacterial attachment to epithelial cells.

Infection-induced immunosuppression: *Neisseria gonorrhoeae* OPA_{52} protein binding to CEACAM1 (CD66a) arrests the activation and proliferation of human CD4+T lymphocytes

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Introduction: *Neisseria gonorrhoeae* causes ~78 million infections globally per annum and the persistence of this pathogen results, in part, from the fact that up to 80% of infected women remain asymptomatic. Infection with *Neisseria gonorrhoeae* typically results in an intense inflammatory response; however, protective immunity and immunological memory are absent and consequently, repeat infections are common. While gonococci are most often localized within the urogenital tract, infection correlates with a transient reduction in T lymphocyte populations present in blood. *Neisseria gonorrhoeae* can expresses opacity (Opa) proteins some of which bind to members of the carcinoembrionic antigen cell adhesion molecule (CEACAM) receptor family. CEACAM1 contains an immunoreceptor tyrosine associate inhibitory motif (ITIM), and we speculated that CEACAM1-Opa interactions might mediate immunosuppression thereby enabling gonococcal persistence both in individuals and the population. Herein we present data supporting this hypothesis, and further, propose a mechanism consistent with such effects.
Materials and Methods: Gonococcal strains constitutively expressing single Opa variants or pilus were used to infect primary human $CD4^+$ T lymphocytes *in vitro.* Lymphocytes were prestimulated using either recombinant human IL2 and/or anti-human CD3ε IgG +/- anti-human CD28. Following incubation for up to 144 h (post infection) lymphocyte proliferation was determined, and cellular activation was quantified by flow cytometric analysis of CD69 expression. Association of the tyrosine phosphatases SHP-1 and SHP-2 with CEACAM1 was determined by bacterial precipitation and subsequent Western blotting. Cell death was quantified by incorporation of Annexin-V versus propodium iodide, allowing differentiation of apoptosis and necrosis.
Results: *N. gonorrhoeae* that do not express CEACAM1-binding Opa proteins augment $CD4^+$ T cell activation and proliferation in response to various stimuli. In constrast, exposure of lymphocytes to either *N. gonorrhoeae* expressing the CEACAM-specific Opa_{52} protein or CEACAM specific polyclonal serum inhibited both the proliferation and activation of these cells in response to the stimuli. In both instances proliferation was inhibited in a dose dependent manner and, critically, occurred without induction of either apoptosis or necrosis. CEACAM1 bound by Opa_{52}-expressing gonococci was associated with the tyrosine phosphatases SHP-1 and SHP-2, suggesting that these enzymes play a role in the immunosuppressive effects associated with Opa-CEACAM interactions.
Discussion: We conclude that the Opa_{52} protein-mediated binding of *N. gonorrhoeae* to CEACAM1 suppresses the activation and proliferation of primary $CD4^+$ T lymphocytes, and that CEACAM1 association with SHP-1 and SHP-2 implicates the receptor's immunoreceptor tyrosine-based inhibitory motif (ITIM) in this effect. These observations constitute the first demonstration of specific immunosuppression mediated by bacterial infection, and have considerable significance in the understanding of gonococcal infection and associated infections including HIV.

The interaction of *Neisseria gonorrhoeae* with CEACAM1 (CD66a)-expressing B cells inhibits antibody production

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Neisseria gonorrhoeae (GC) can cause repeated infections. Hedges et al. examined gonorrhea patients with a history of prior infections for local and systemic anti-gonococcal antibody levels, and found that the levels were extremely modest (2), indicating that GC may suppress immune responses during infections. Several GC opacity (Opa) proteins mediate adherence and phagocytosis by interacting with members of the carcinoembryonic antigen family (CEA, CD66 antigen family), such as CEACAM1 (CD66a), which was, however, identified as an inhibitory receptor able to mediate negative signals in DT40 B cells (1). One of the biological functions of inhibitory receptors is to inhibit B cell proliferation and antibody production (4), and human peripheral B cell express CEACAM1 after activation by IL-2 (3). We asked whether the interaction of GC with CEACAM1-expressing B cells inhibits antibody production. B cells, obtained from human peripheral blood lymphoid (PBL) cells, were treated with IL-2 to express CEACAM1. CEACAM1-expressing B cells were plated in 96-well plate and co-incubated with Opa$^-$, OpaA and OpaI GC. Ig secretion (IgG, IgM, and IgA) was determined in culture supernatants by ELISA. The results showed that OpaI GC, but not Opa$^-$ GC, have the ability to inhibit the antibody production. GC may use the same molecule, CEACAM1, not only for their binding to host, but also for the subversion of regulatory immune pathways.

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Fibronectin mediates Opc dependent internalisation of *Neisseria meningitidis* in human brain microvascular endothelial cells

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A central step in the pathogenesis of bacterial meningitis caused by *Neisseria meningitidis* (the meningococcus) is the interaction of the bacteria with cells of the blood-brain barrier (BBB). An *in vitro* model of the BBB using human brain derived endothelial cells (HBMEC) has been used to study the invasive potential of hypervirulent meningococcal lineages of the ET-5 and ET-37 complex.

In contrast to previous observations made with epithelial cells and human umbilical vein derived endothelial cells (HUVEC), significant internalisation of encapsulated meningococci by HBMEC was observed. However, this uptake was found only for the ET-5 complex isolate MC58, not for an ET-37 complex strain. Furthermore, the uptake of meningococci by HBMEC depended on the presence of human serum, while serum of bovine origin did not promote the internalisation of meningococci in HBMEC.

By mutagenesis experiments we could demonstrate that internalisation depended on the expression of the *opc* gene, which is present in meningococci of the ET-5 complex, but absent in ET-37 complex meningococci. Chromatographic separation of human serum proteins revealed fibronectin as the uptake-promoting serum factor. Fibronectin-depletion of separated human serum did not support meningococcal entry, and the deficiency could be partially restored by the addition of purified fibronectin. Furthermore, the role of fibronectin-binding integrin receptors in bacterial uptake event was analysed. Blocking integrin function by peptides containing the amino acid sequence arginin-glycin-aspartic acid (RGD) and by antibodies specific to alpha5-beta1 integrins resulted in abrogation of fibronectin-triggered bacterial internalization.

These data provide evidence for unique molecular mechanisms of the interaction of meningococci with endothelial cells of the blood-brain barrier and contribute to our understanding of the pathogenesis of meningitis caused by meningococci of different clonal lineages.

Neisseria-host cell interactions: cell-contact induced protein expression in bacteria and infection of CD46 transgenic mice

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In order to characterize molecular changes that occur upon bacteria-cell contact, we used a proteomics approach. By using two-dimensional gel electrophoresis and silver/coomassie blue staining, we have done a comparison in protein expression pattern between *Neisseria* adhering to a human epithelial cell line, ME180, and non-adherent bacteria. Several proteins showed an increased staining when the bacteria were attached to the cells, indicating that expression of these proteins were upregulated. Some other bacterial proteins stained less intense after attachment, which might reflect either a downregulation of expression or secretion of the proteins. Both upregulated and downregulated proteins were subjected to mass spectrometry in order to determine molecular mass and identity of the proteins. A number of novel proteins involved in the bacteria-host cell interaction have been identified, confirmed and characterized. This approach has lead to identification of novel adherence-associated proteins expressed during contact with human target epithelial cells.

An important interaction occurs between pili of *Neisseria* and its host cell receptor CD46. CD46, a human cell surface protein for which no murine homologue is available, is produced ubiquitously as four major isoforms and protects host cells from complement activation. In order to develop experimental infection models that mimic the human host we evaluated transgenic mice expressing human CD46. Such studies will be important and necessary to rapidly experimentally consider vaccine candidates as well as to study *Neisseria* pathogenesis. Our results show that piliated, but not nonpiliated, *Neisseria* adheres to mouse tissue expressing human CD46. Also, piliated *Neisseria*, but not nonpiliated, binds to primary cell lines of CD46 mice. Intranasal infection of CD46 mice with piliated wild-type *N. meningitidis* lead to rapid lethal disease, but only if the mice were pre-treated with antibiotics to diminish the normal flora. Infection of CD46 mice with nonpiliated bacteria was never lethal, and nontransgenic mice always survived infection. Intraperitoneal infection of CD46 transgenic mice with 10^8 *N. meningitidis* resulted in 100% mortality and infection with 10^6 resulted in 15% mortality. In contrast, bacterial infection of nontransgenic mice resulted in 100% survival with both challenge doses. All affected animals showed clinical signs of neurologic disease. Analysis of cellular host responses as well as infection with meningococcal mutants was performed and will be discussed.

The pathogenesis of gonococcal cervicitis: pilus and porin bind directly to the I-domain of complement receptor 3 by a non-opsonic interaction

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The use of primary, human, ecto- and endocervical epithelial cell cultures has increased our understanding of the pathogenesis of gonococcal infection in women. We recently described the distribution of complement receptor 3 (CR3) within epithelia derived from the female reproductive tract. CR3-mediated endocytosis serves as a primary mechanism by which *Neisseria gonorrhoeae* elicits membrane ruffling and cellular invasion of primary, human, cervical epithelial cells. We now extend these studies to describe the nature of the gonococcus-CR3 interaction. Opsonic and non-opsonic interactions contribute to the adherence of the gonococcus to CR3. Complement protein C3 is produced by the cervical epithelium, deposited upon the gonococcus surface, and rapidly inactivated to iC3b. iC3b covalently bound to the gonococcus serves as a primary ligand for CR3 adherence to primary ecto- and endocervical cells. In addition to iC3b-mediated adherence, non-opsonic, direct, interactions are also required for the gonococcus to bind to CR3. We have demonstrated by Far-Western Blot analysis and by ELISA that gonococcal porin and pili can bind to the I-domain of CR3 in a non-opsonic manner. The association of the gonococcus with CR3 on primary cervical cells required porin and pilin outer membrane proteins. We were unable to detect a direct interaction of LOS with CR3, which may be reflective of the ability of LOS to act as a C3 acceptor molecule. Similarly, Opa proteins are not required for the initiation of gonococcal cervicitis. Using quantitative inhibition assays, we have demonstrated that the association of the gonococcus with primary ecto- and endocervical epithelial cells is dependent upon the availability of the I-domain of CR3. These data suggest that opsonic and non-opsonic gonococcal adherence to CR3 occurs in a cooperative manner that facilitates targeting to and successful invasion of the cervical epithelium. This is the first demonstration of the direct adherence of a microorganism to the I-domain of CR3. These data further indicate that CR3 is a receptor for gonococcal porin and pilus on primary, human, ecto- and endocervical epithelial cells.

Phosphoethanolamine substitutions on neisserial lipooligosaccharide are the preferred acceptors for C4

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The classical complement pathway is essential to mediate bactericidal killing of *Neisseria gonorrhoeae* and *N. meningitidis*. Early events in complement activation, such as bacterial targets for C4b, have not been defined. We identified an unencapsulated meningococcal mutant strain (derived from serogroup Y strain 2220; ST-172) that bound 3- to 4-fold more C4 when incubated with normal human serum than the corresponding mutant of strain MC58, despite binding similar amounts of antibody (Ab). Western blotting identified a low M_r acceptor as the main C4 target. The target was identified as lipooligosaccharide (LOS) by co-localization with anti-LOS monoclonal (mAb) L8. The C4b-LOS bond was not susceptible to cleavage by 1M methylamine; pH 11, suggesting the bond was not ester, but likely amide in nature. The only known primary amine on neisserial LOS available for forming an amide linkage is phosphoethanolamine (PEA). Consistent with the preponderance of amide-linked C4b to LOS, mass spectrometric analysis of the LOS of this serogroup Y meningococcal strain revealed 2 PEA residues (simultaneously at the 3- and 6-positions of the distal ß-chain heptose [HepII] residue) on HepII (in contrast to most meningococcal strains that have a PEA residue at either the 3- or 6-position of HepII). The role of PEA at the 3- position of HepII in binding C4b was studied using an MC58 mutant (MC58::*lpt3*)[1] that lacked HepII PEA. This mutant did not form appreciable C4b-LOS adducts, in contrast to an amide-linked C4b-LOS adduct seen with the parent strain (PEA substitution at the 3-position of HepII). Studies with serum-sensitive strains of *N. gonorrhoeae* also showed amide-linked C4b-LOS adducts. A naturally occurring gonococcal strain (PID2) lacking LOS PEA failed to demonstrate amide-linked C4b-LOS adducts. Consistent with the amide nature of the C4b-LOS linkage, C4 in serum containing only the C4B isoform (that preferentially forms ester linkages) formed small amounts of ester-linked C4b-LOS adducts with the serogroup Y mutant strain, while serum containing only the C4A isoform (that forms exclusively amide linkages) bound LOS exclusively via methylamine-resistant (likely amide) linkages. Hexose substitutions on neisserial LOS mimic host glycosphingolipids, and utilization of PEA (which is not normally exposed on host cell membranes) might provide the host a means to selectively activate complement on the meningococci. Supporting this hypothesis is the observation that enhanced complement activation occurs in certain pathological states such as sickle cell disease, which is thought to be because of abnormally exposed PEA on the membranes of dense sickle erythrocytes[2]. To our knowledge, this study is the first demonstration of a target for C4b on a microbe, and identifies LOS PEA as an important acceptor for this complement component.

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Components of DNA binding and uptake during genetic transformation linked to type IV pilus expression

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Introduction: Natural genetic transformation is critically important to genetic diversity in *N. gonorrhoeae* and *N. meningitidis* since it accounts for the extensive polymorphism underlying their panmictic population structure. As in many other Gram negative species, Type IV pili (Tfp) play a crucial but undefined role in the early steps of the transformation process. Despite its biological significance, the processes related to competence remain one of the most poorly understood forms of DNA metabolism.

Results and Discussion: Although related components essential to the uptake of DNA during transformation have been defined in many systems, it remains unclear if binding and uptake are discrete or dissociable events. The inability to define a distinct binding step in these studies may reflect the rapidity with which DNA uptake is achieved or that binding and uptake are intrinsically coupled, either temporally or mechanistically. Our earlier studies have identified three components essential to DNA uptake: Tfp; ComP, a pilin subunit-like molecule, and PilT, a cytoplasmic protein involved in pilus retraction and twitching motility. The latter two components are dispensable for Tfp expression but can under certain circumstances inlfluence Tfp expression. Utilizing a genetic approach in which the level and timing of ComP and PilT expression was regulated, we have been able to show that DNA binding and uptake are distinct events which can be resolved from one another. As a consequence, it is possible to begin to identify at which of the two steps various components function. Specific results of the study show that: 1) enhanced transformation associated with overexpression of ComP correlates with increased specific DNA binding in both direct measurement assays and functional assays. Therefore, DNA uptake sequence - specificity in this system is imposed primarily at the level of DNA binding, 2) the pilin subunit protein PilE is essential for functional DNA binding, and 3) the pilus retraction protein PilT is dispensable for functional DNA binding but required for DNA uptake. Another important finding of the study is that by using trans-species complementation, it is possible to show that sequence-specific DNA binding is not attributable to a distinct, intrinsic property of the PilE subunit. Finally, we have demonstrated the existence of nonspecific DNA binding activity associated with the expression of functional Tfp which is unrelated to transformation but which until now has obscured the observation of sequence-specific binding events. This latter activity may have important consequences for host-parasite interactions.

Conclusion: By genetic dissection, it has been possible to gain greater understanding of the early steps of transformation. A model pathway which encompasses all the known factors critical to DNA uptake (including the ComE protein described by Chen and Gotschlich, 2000) will be presented.

SESSION II

Clinical aspects and host defence

Meningococcal infections – from clinical observation to molecular pathology

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Aim: To summarize known relations between intruding *Neisseria meningitidis* and inflammatory responses of the host with different clinical presentations.
Methods: Lipopolysaccharides (LPS) were quantified by Limulus amebocyte lysate (LAL) assay. The real bacterial load (live + dead *N. meningitidis*) was measured by extracting bacterial DNA from plasma, serum and cerebrospinal (CSF) using magnetic beads in a robotized system and real time PCR (Lightcycler technology). Cytokines were determined with ELISA and the net inflammatory capacity of plasma and CSF with a human monocyte target assay. The inflammation induced by meningococcal outer membrane vesicles (nOMV) and purified meningococcal LPS was studied using purified human monocytes or a whole blood assay. The contribution of lipid A of LPS in patient samples was evaluated in assays blocking CD14 and Toll Like Receptor (TLR) 4 with an anti-CD14 mAb and the lipid A antagonist RsDPLA, respectively. The contribution of LPS as a complement activation principle of *N. meningitidis* was studied in vitro by using a whole blood model combined with wild type 44/76 *N. meningitidis*, nOMV, purified *N.m*-LPS and the LPS-depleted mutant lpxA$^-$ 44/76.
Results: The levels of LPS in plasma or CSF are closely associated with development of septic shock and/or meningitis symptoms. Among 135 patients high plasma levels of LPS were associated with high mortality. Few patients with LPS <10 endotoxin units (EU)/mL develop septic shock whereas few patients with LPS >100 EU/mL survive the septic shock. The levels of LPS are closely correlated to levels of *N. meningitidis* DNA in plasma (r=0.86, p=0.0001, n=62) and CSF (r=0.87, p=0.006, n=11). Cytokines including TNF-α, IL-1β, IL-6, IL-10, G-CSF, GM-CSF, M-CSF, MCP-1, MIP-1α, INF-γ, PAI-1 and others are all significantly correlated to the levels of LPS. nOMV are potent activators of monocytes and whole blood. TNF-α secretion induced in monocytes by IL-10 depleted shock plasma was reduced to median 10% by blocking CD14 and 12% by blocking TLR4. The massive complement activation in patients with shock appears to be indirectly related to circulating LPS levels since meningococcal LPS, either purified or bound to the bacterial outer membrane, appears to be a weak complement activator.
Conclusion: The clinical presentations are closely associated with *N. meningitidis*' ability to survive and proliferate within the host's circulation and propensity to penetrate into the subarachnoid space. Rapid bacterial proliferation in the circulation generates large amounts of LPS within 12-24 hours leading to severe shock. The innate immune system in man responds to increasing levels of LPS and possibly other outer membrane molecules in a dose dependent manner. In patients with symptoms of distinct meningitis the bacteremia is low graded and the main bacterial growth is within the subarachnoid space. Experiments employing monocytes as target cells combined with plasma or CSF suggest that lipid A of LPS indeed is the major inflammation inducing principle.

Downregulation of pili and capsule during intimate adhesion of *Neisseria meningitidis* to epithelial cells

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The initial attachment of *Neisseria meningitidis* to target cell surface appears to be largely pilus dependent in capsulated bacteria. Intimate adhesion subsequently occurs to permit effective colonisation where the levels of pili and capsule seem to be decreased. *N. meningitidis* undergoes an adaptive response upon contact with target cells. Several meningococcal genes seem to be induced during this contact. A promoter element CREN (contact regulatory element of *Neisseria*) is responsible for this induction. RT-PCR analysis showed that CREN-harbouring genes were induced upon contact of Nm with target cells. However, the CREN element was absent from some of these genes in other meningococcal strains of different genetic lineages suggesting a role of this element in the output of the bacteria-cell interaction.

The *crgA* (contact-regulated gene A) encodes a LysR-type transcriptional regulator that is under the control of a CREN element and is induced during initial adhesion. *crgA* mutant was shown to be unable to undergo intimate adhesion to target cells. RT-PCR analysis showed that during intimate adhesion, CrgA seems to downregulate genes such as *pilE* and *sia*, involved respectively in pili and capsule biogenesis. Moreover, DNA-binding assays and footprinting analysis indicated that this downregulation was directly mediated by the CrgA protein. RT-PCR analysis showed a constitutive expression of *pilE* and *sia* genes in *crgA* mutant in contrast to the wild-type strain where this expression was greatly reduced. CrgA may play a central regulatory role in meningococcal adhesion, particularly, in switching from initial to intimate adhesion by downregulating bacterial surface structures that hinder this adhesion such as pili and capsule.

Neisserial porin PorB increases expression of TLR2 and TLR4 on B lymphocytes

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Neisserial porins are conserved bacterial outer membrane proteins that are potent immuneadjuvants and have been shown to activate human and murine B lymphocytes. The immunopotentiating activity of porins is due to their ability to stimulate B cells, and potentially other antigen presenting cells, via TLR2, inducing an increase in the expression of the costimulatory molecule B7-2 (CD86). Activation of B cells from LPS hypo-responsive C3H/HeJ mice with *N. meningitidis* porin, PorB, involves NF-κB nuclear translocation, protein tyrosine kinase (PTK) activation and phosphorylation of mitogen activated protein kinases (MAPK) Erk1 and Erk2. The porin induced increase in B7-2 expression is dependent on PTK. However, Erk1 and Erk2 activation are not required for B7-2 upregulation or NF-κB nuclear translocation. Results from RT-PCR show that TLR2 and TLR4 message is increased when B cells from C3H/HeJ mice are stimulated with PorB. This is likely due to the induction of NF-κB nuclear translocation by PorB and may demonstrate a positive feedback loop which can increase the responsiveness of B cells to TLR2 ligands, and more interestingly, also suggests that ligands to one TLR may increase the responsiveness of the B cell (and likely other antigen presenting cells) to other TLR ligands. We have found that induction of NF-κB nuclear translocation by PorB is not dependent on the PorB induction of PTK or MAPK activity, and, therefore, PorB induced increases in TLR2 and TLR4 mRNA, is also, not likely due to PorB induction of PTK or MAPK activity.

Modification of lipid A acylation pattern in *Neisseria meningitidis*: effects on endotoxic and adjuvant properties of LPS, and its interaction with Toll-like receptors TLR2 and 4

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The endotoxic and adjuvant properties of lipopolysaccharide (LPS) are mainly determined by its membrane-anchoring lipid A moiety. By genetic modification of the lipid A biosynthesis pathway in *Neisseria meningitidis*, we have previously isolated various recombinant strains with specific alterations in the lipid A acylation pattern, as well as a strain completely lacking LPS. Mutations in the *lpxA* and *lpxL* genes resulted in longer or shorter fatty acyl chains at the 3 and 3' positions, and penta- or tetra-acylated instead of the normal hexa-acylated lipid A, respectively. Whereas all variations in the fatty acyl pattern resulted in reduced endotoxic activity as measured by TNF-α induction in the human macrophage-derived cell line MM6, the adjuvant activity of the modified meningococcal LPS was, in most cases, not similarly affected. Using CHO cells expressing human Toll-like receptor (TLR) 2 or 4 and an NF-κB-dependent promoter driving expression of a reporter construct, we demonstrated that the recombinant strains are mainly impaired in signaling through TLR4 and not through TLR2. However, immunization of C3H/HeN and C3H/HeJ mice, which contain normal and defective *tlr4* genes, respectively, showed that the adjuvant activity displayed by meningococcal LPS is only partly dependent on TLR4. A missense mutation in the *tlr4* gene of C3H/HeJ mice has rendered them unable to respond to LPS with NF-κB-dependent proinflammatory cytokine induction. Strains producing hexa-acylated lipid A showed reduced immunogenicity of the major outer membrane proteins in these TLR4-defective mice. Outer membrane complexes containing wildtype LPS as internal adjuvant induced significantly lower bactericidal titers and an altered IgG isotype distribution with a higher IgG1 to IgG2a ratio in the TLR4-defective mice, whereas no such difference was found for strains producing penta- or tetra-acylated LPS, or with the adjuvants QuilA and MF59 which are not related to LPS. When murine dendritic cells were activated with LPS preparations from the various mutants, a clear difference between the penta- and tetra-acylated mutants was found, with only the former showing a marker activation pattern similar to wildtype hexa-acyl LPS. Taken together, these results suggest that the ability to stimulate TLR4-mediated proinflammatory cytokine induction is not necessarily linked to LPS adjuvant activity. The implicated existence of additional LPS-signaling pathways may help to explain why endotoxic and adjuvant activity are differentially affected in the recombinant strains producing altered lipid A.

The class A macrophager scavenger receptor is a major pattern-recognition receptor for *Neisseria meningitidis* and determines innate susceptibility to infection

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The initial interactions between humans and *Neisseria meningitidis* (*NM*) and the factors that determine whether colonisation results in asymptomatic carriage or invasive disease are poorly understood. Macrophages (Mϕ) are a first line of defence against invading pathogens and express the class A scavenger receptor (SR-A), one of several cell-surface molecules capable of recognising microbial ligands. SR-A is implicated in the *in vivo* clearance of bacteria and protection against endotoxic shock. Ligand recognition through SR-A is versatile and includes the ability to bind soluble lipopolysaccharide (LPS) and lipoteichoic acid. *NM* has been shown to bind Mϕ directly, but the receptors were not identified until we showed using bone marrow-derived cells from wild type (WT) and SR-A knockout mice (SR-$A^{-/-}$) that almost all *NM* entered Mϕ via SR-A. These findings apply to a variety of pathogenic *NM* strains (serogroups A, B, C and W-135), *N. gonorrhoeae* and to the commensal *Neisseria* strains, *N. lactamica*, *N. sicca* and *N. mucosa*. In *NM*, the presence or absence of pili, capsular polysaccharide and outer membrane proteins (Opa and Opc) did not alter bacterial binding. Surprisingly to us, using various inner and outer core LPS mutants and an LPS-deficient mutant (H44/76*lpxA*), we found no evidence to implicate LPS as a ligand for SR-A. Thus, although *NM* LPS was required for pro-inflammatory cytokine secretion (the LPS-deficient mutantH44/76*lpxA* stimulated only low levels of TNFα, IL-12 and IL-10), LPS did not mediate SR-A dependent ingestion. *In vitro*, interferon-αβ receptor knockout (IFN-αβ$R^{-/-}$) Mf released more TNFα, IL-12, but less IL-10 on exposure to *NM*. *In vivo*, SR-$A^{-/-}$ mice were relatively less susceptible to *NM* infection based on the lower mean bacteraemia and mortality of SR-$A^{-/-}$ compared to WT animals following systemic challenge with virulent *NM* organisms (strain MC58). Following exposure to *NM* organisms *in vitro*, the production of cytokines and nitric oxide (NO) did not depend on SR-A. Using electron microscopy, Mϕ from WT and SR-$A^{-/-}$ animals were equally efficient in killing *NM in vitro* during the first 6 hours of infection. Comparing WT and TLR-4-deficient Mϕ, TLR-4 was required for Mϕ activation, but not for phagocytosis. TLR-4-deficient Mϕ also showed a reduction in cytokine production following exposure to *NM*, although SR-A mediated ingestion of these organisms was normal. We conclude that SR-A is a major receptor for the ingestion of *NM* by Mϕ, but that this function is LPS independent. Furthermore, Mϕ ingestion of *NM* is uncoupled from the LPS and TLR-dependent cytokine responses. Given the reduced susceptibility of SR-A mice to *NM* infection, we speculate that SR-A entry and sequestration within Mϕ may provide a protective niche for *NM* survival.

Unravelling the genetics of the host response to meningococcal disease

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Introduction: In the majority of people, *Neisseria meningitidis* becomes a commensal of the nasopharyngeal epithelium for at least some part in their life. Despite this, meningococcal disease is a rare occurrence, suggesting a complex relationship between colonisation, infection and disease. Susceptibility is likely to be influenced both by the environment (in particular bacterial colonisation) and the host response but, determining the relative contributions of each has proved difficult. We have used the increased risk of disease in siblings (λ_s), to indicate host genetic component and thus characterise susceptibility to both host and environment. A number of genes are likely to contribute to this host response, and variations in them may well form the basis of an individual's susceptibility. We postulated that variation within both the complement regulator, factor H, (fH) and a mediator of intracellular bacterial killing, SLC11A1, (formerly NRAMP1) genes might predict disease.
Subjects: Patients from the paediatric intensive care unit at StMary's Hospital and 2 UK wide studies (including families and controls), donated samples and filled in questionnaires.

Methods and Results:
1. From the questionnaires, 443 cases with 845 siblings were identified and the incidence rates compared to their expected incidence data using notification information from the Public Health Laboratory Service and population information from the Offices for National Statistics. λ_s was calculated to be 30.3 (95% CI 20-44). If siblings contracted disease more than 1 month after onset in the index case, the λ_s was 11.
2. For fH, a polymorphism at position 496 (C to T) was investigated by PCR and restriction enzyme digestion. The proportion of C/C homozygotes was increased in 161 St Mary's patients (59%) compared to 92 controls (33%) ($p = 0.00006$) as was the C allele frequency (0.75 vs 0.57, $p = 0.00001$). An additional 118 nuclear families showed increased transmission of the C allele to patients (61%) compared to the T allele (39%) ($p = 0.04$).
3. SLC11A1 promoter region microsatellite alleles (1 to 4) were categorised by size. The St Mary's patients (215) showed an increased frequency of the 2-2 genotype (15%) compared to 8% in 92 controls ($p = 0.002$). Investigation of disease outcome showed that no St Mary's patients with the 2-2 genotype died ($p = 0.04$). A second UK study ($n = 122$) confirmed this ($p = 0.008$).

Conclusions: This study is the first to calculate λ_s for MD. Using a one-month cut off, host genetic factors were estimated to contribute to at least one third of the risk of disease.

The fH gene C/C genotype is both strongly associated and linked with disease, suggesting that this polymorphism down regulates complement activation during the host response.

The 2 allele of the SLC11A1 promoter region microsatellite is known to drive lower levels of expression, predicting lower levels of intracellular killing and macrophage activation, possibly explaining it's association with disease. Interestingly, the 2-2 genotype also seems to protect from severe disease.

SESSION III

Vaccines (1)

A historical approach to the epidemiology of meningococcal meningitis in Africa

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Meningococcal infection poses as big a threat to the Sahelian and sub-Sahelian countries of West Africa as it did 100 years ago when the first major epidemics of the infection were described in sub-Saharan Africa. The history of meningococcal infection in sub-Saharan Africa will be reviewed to see if this can provide lessons as to how the infection is likely to evolve in the near future. Four topics will be considered.

1. Evidence will be presented that meningococcal infection first reached Nigeria with pilgrims returning from the Holy Places at the beginning of the twentieth century. The critical role that the pilgrimage has played subsequently in the epidemiology of meningococcal infection in sub-Saharan Africa will be discussed. It is likely that pilgrims have played a major role in the recent emergence of W135 meningococcal infections as a major threat to Africa.
2. The evolution of treatment and prevention of meningococcal disease during the past 100 years will be considered and the potential for the emergence of drug resistance will be considered.
3. Information on the serogroup distribution of meningococci isolated in Africa during the past 70 years will be analysed for clues as to what might be expected in the future.
4. Finally, the overall impact of vaccination will be reviewed and the likelihood that vaccination with selective vaccines will give rise to major changes in the serogroup of meningococci responsible for epidemics will be considered.

The Meningitis Vaccine Project (MVP): a new model for public/private sector partnerships for development and introduction of new vaccines for prevention of infectious diseases in the developing world

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Introduction: Epidemics of group A meningococcal disease cause enormous suffering in Sub-Saharan Africa. A vaccine that provides long-term protection and decreases transmission of the organism could eliminate group A epidemics. The private sector is developing tetravalent polysaccharide-protein conjugate vaccines that include groups A, C, W135 and Y, which have the potential for eliminating meningococcal disease in Africa. The products under development are intended for use in premium-price markets, and the price per dose is expected to be too high for introduction and sustaining vaccination in Africa. The Meningitis Vaccine Project (MVP) with a $70 million grant from the Gates Foundation is employing an alternative private-public sector partnership for development and pilot introduction of a meningococcal conjugate vaccine in Africa.
Methods: MVP conducted analyses of costs and timelines, including building manufacturing capacity, process development, and clinical and regulatory activities. The approach assumes contract manufacturing of raw materials (carrier protein and meningococcal polysaccharide) from developing country manufacturers, process development of conjugate technology by a biotechnology company, and technology transfer to a contract manufacturer for large-scale production, filling packaging, release of the vaccine.
Results: 25 million doses per year of a licensed monovalent A conjugate vaccine in lyophilized ten dose vials could be available for the 2007-2008 epidemic season at a price of $US 0.40 per dose. The model allows 1. focus on single product without distraction of competing projects; 2. development of a product intended for use in Africa (rather than adapting an existing product intended for premium priced markets); and 3. a low enough price to make the goal of introduction of vaccination achievable and sustainable. The technical and managerial complexity of this model are far greater than simply relying on an established vaccine manufacturer for development and manufacture. The MVP will be responsible for clinical development and licensing of the vaccine in countries where National Control Authorities may not be fully recognized. Technology transfer from a small biotech company to a newly industrialized vaccine manufacturer will need to be closely monitored to meet the tight deadlines. Finally, additional analyses are examining the feasibility of adding a second capsular group to the vaccine, such as W135.
Conclusions: This partnership could become a model for development of other orphan vaccines or drugs targeted at diseases or strains that are largely limited to the developing world. Newly industrialized vaccine manufacturers will have the technical capacity to produce large quantities of conjugate vaccines targeted for consumption by developing countries at an affordable price. The implications of this project for disease control or therapy of infectious diseases in the developing world are enormous.

MenB vaccine development

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The development and implementation of a menB vaccine is difficult. Our efforts are focussed on a number of approaches.

Firstly, results obtained in Brazil with VA-MENGOC-BC, clearly suggest serotype independent protection in children > 4 years to be feasible.

This cross-protection has been analysed further and our data suggest different minor OMPs to play a role in this cross-protection. Also we have found evidence it is important to avoid PorA dominant immune responses. In infancy immunisation with OMV-WT vaccines lead to PorA specific SBA responses, however a multivalent PorA-OMV approach for pediatric use can be an option. We will show an example of an upsurge of B:2b:P1.2 in Holland – 1966 and Belgium – 1971 that will illustrate the limitations of this approach.

Finally, a generic menB vaccine on the basis of noncapsular antigens is being pursued actively by our team.

A full genomic antigen discovery programme has been carried out as well as careful preclinical evaluations of pre-genomic antigens.

After a pre-selection of 100 candidate antigens a careful analysis into sequence conservation, phase-variation and strain coverage we decided to consider approximately 50 antigens. A quick-and-not-so-dirty expression and purification programme followed by preclinical studies sized the number down to approximately 20.

Currently these 20 antigens are being evaluated either as recombinant subunit or after upregulated meningococcal expression and purification of OMV.

Examples of both approaches including preclinical analysis will be shown.

Genome-derived antigen (GNA) 2132 elicits protective serum antibodies to groups B and C *Neisseria meningitidis* strains

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Introduction: The group B genome sequencing project identified a number of novel genome derived antigens (GNA) as possible vaccine candidates (Pizza et al., Science 2000). One of these, designated GNA2132, is a protein of unknown function that elicited serum bactericidal antibody in mice. Although the amino acid sequence of GNA2132 is variable, the -COOH portion is relatively conserved across *N. meningitidis*. We investigated the ability of mouse anti-GNA2132 antisera to confer passive protection in 5- to 7-day old infant rats challenged with groups B or C *N. meningitidis* strains.
Materials and Methods: Mice were immunized with 3 injections of recombinant GNA2132 (gene from strain 394/98). Bactericidal titers were measured with human complement. Rats were pre-treated with pooled antisera and challenged IP two hours later with ~5000 CFU of group B meningococcal strains 2996 or 394/98 (New Zealand), or group C strains 4335 (O-Acetyl- negative) or 4243 (O-Acetyl-positive). Quantitative blood cultures were obtained at 18 hours.
Results: The bactericidal titer of the pooled anti-GNA2132 antiserum was 1:200 against group B strain MC58 (not tested for passive protection) compared to <1:4 in sera from control mice. The anti-GNA2132 bactericidal titer was 1:5 against strain 394/98 and <1:4 against strains 2996, 4335 and 4243. Despite absence of bactericidal activity when tested with infant rat or human complement, a 1:5 or 1:50 dilution of anti-GNA2132 protected rats challenged with strain 2996 (2/7 rats with bacteremia vs. 6/6 pre-treated with sera from mice immunized with adjuvant alone; respective geometric mean CFU/ml of blood: 4 vs. 7357). A 1:5 dilution of anti-GNA2132 also protected against strain 394/98 (0/8 vs. 8/8 with bacteremia). A 1:10 dilution of anti-GNA2132 serum protected against both group C stains (0/10 with bacteremia vs. 10/10 in rats pre-treated with negative control serum). In contrast, dilutions of 1:6 of pooled sera from two groups of 4-year old children immunized with meningococcal polysaccharide vaccine gave incomplete protection against strain 4335: Pools 1 and 2, 5/5 and 2/5 with bacteremia, respectively; geometric mean CFU/ml, 22,687 and 45. The corresponding results for strain 4243 were: Pools 1 and 2, 4/5 and 0/4 with bacteremia, respectively, geometric mean CFU/ml, 40 and <1.
Conclusions: (i) Mouse antibodies to rGNA2132 protect against meningococcal bacteremia caused by groups B and C strains; (ii) Protection can be observed in the absence of bactericidal activity; (iii) A 1:10 dilution of the mouse antisera is more protective than a 1:6 dilution of sera from 4-year-olds immunized with polysaccharide vaccine, an age group protected by vaccination.

Neisseria lactamica as a vaccine against meningococcal disease

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Introduction: Immunological and epidemiological evidence suggests that carriage of commensal *Neisseria* species is involved in the development of natural immunity against meningococcal disease. *N. lactamica* has many surface structures in common with *N. meningitidis* and may be the most important of these non-pathogenic species. An *N. lactamica*-based vaccine may mimic this natural immunity against meningococcal disease and may pre-empt or enhance protection provided by natural carriage of commensal *Neisseria*. We have investigated the potential of *N. lactamica*-based vaccines to protect against a panel of diverse meningococcal strains in an animal model of meningococcal disease.

Materials and Methods: *N. lactamica* outer membrane vesicles were prepared with deoxycholate extraction (DOMVs) or as natural OMVs (NOMVs). These were used to immunise mice by subcutaneous injection or intranasal instillation. Antibody responses were assessed by ELISA and immunoblots. Antibody surface-labelling of bacteria was assessed by flow cytometry. Protection was determined using a mouse intraperitoneal challenge model of meningococcal disease. In order to identify the antigens that may be responsible for the observed protection, native preparative electrophoresis was used to separate outer membrane proteins. In addition, an *N. lactamica* genomic library expressed in λ phage was screened with sera against *N. lactamica* to identify immunogenic antigens.

Results and Discussion: It has not been possible to colonise the nasopharynx of mice with *N. lactamica*. However, systemic and mucosal responses that cross-react with *N. meningitidis* have been detected following intranasal immunisation with *N. lactamica* NOMVs or live *N. lactamica*. Antisera raised against *N. lactamica* DOMVs and NOMVs were cross-reactive with strains of *N. meningitidis* representing a range of serogroups, serotypes and serosubtypes. *N. lactamica* OMVs have also been shown to protect mice against challenge with meningococcal isolates from the ET-5, ET-37, lineage III and A4 cluster clonal lineages. Two approaches have been taken to identify the *N. lactamica* antigens responsible for this protection. Protein fractions separated by native preparative electrophoresis have been assessed for protective efficacy in the mouse model and a pool of proteins 25–30kDa was identified as protective against meningococcal challenge. Screening an *N. lactamica* genomic library expressed in λ phage against *N. lactamica* sera has identified a number of immunogenic antigens with homology to proteins in the meningococcal genome. The vaccine potential of these antigens is currently being investigated.

We have demonstrated that *N. lactamica*-based vaccines protect against challenge with diverse meningococcal isolates in an animal disease model and have the potential to provide broad protection against meningococcal disease.

SESSION IV

Surface structures (1)

NadA, a novel adhesin and vaccine candidate of *Neisseria meningitidis*

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Introduction: *Neisseria meningitidis* is a human pathogen, which, in spite of antibiotic therapy, is still a major cause of mortality due to sepsis and meningitis. There are no universal vaccines available for serogroup B meningococcus. We have been using a genomic approach (reverse vaccinology) to identify novel vaccine antigens.
Results and Discussion: NadA (Neisseria adhesin A) is a novel surface antigen of *N. meningitidis* that is present in 51 out of 53 strains of hypervirulent lineages ET-37, ET-5 and cluster A4. The gene is absent in the hypervirulent lineage III, in *N. gonorrhoeae* and in the commensal species *N. lactamica* and *N. cinerea*. The GC content, lower than that of the chromosome, suggests acquisition by horizontal gene transfer and subsequent limited evolution to generate three well-conserved alleles. NadA has a predicted molecular structure strikingly similar to a novel class of adhesins (YadA and UspA2), forms high molecular weight oligomers and binds to epithelial cells in vitro supporting the hypothesis that NadA is important for host cell interaction. NadA induces strong bactericidal antibodies and is protective in the infant rat model suggesting that this protein may represent a novel antigen for a vaccine able to control meningococcal disease caused by three hypervirulent lineages.
Conclusions: NadA is a novel vaccine candidate, as it is a surface-exposed molecule, present in almost 50% of the disease-associated strains and in nearly 100% of three hypervirulent lineages, it is able to induce a bactericidal response, not restricted to the homologous strains but to all the strains harboring it. NadA could be taken into account in the light of a vaccine against these three hypervirulent lineages.

Identification of AspA - a novel subtilisin-like serine protease in *Neisseria meningitidis*

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Introduction: In an attempt to identify novel meningococcal virulence determinants, the meningococcal genomic sequence was screened *in silico* for previously unrecognised autotransporter proteins, which play an important role in virulence in many Gram-negative pathogens. This led to the identification of Autotransported serine protease A (AspA: NMA0478; NMB1969), a novel *c.* 112 kDa subtilisin-like serine protease (pyrolysin family). Cell envelope-associated, subtilisin-like serine proteases are important virulence factors and have been proposed as vaccine candidates [1, 2]. AspA exhibits significant homology (30% over 956 amino acids) to SphB1, which is essential for the maturation and secretion of FHA in *Bordetella pertussis* [1]. Here, we describe the molecular and immunological characterisation of AspA.

Methods and Results: *aspA* was cloned and expressed, and mutants were generated in meningococcal strains MC58 and Z4181. *aspA* was fully sequenced in meningococcal strains SD and Z4181 (accession numbers AJ277537 and AJ311654, respectively) and shown to be conserved (>96% identity at the amino acid level) in serogroup A, B and C meningococci. A possible orthologue of *aspA* is present in the available gonococcal sequence database, but the sequence contains numerous stop codons, suggesting that AspA may be a pseudogene in *N. gonorrhoeae*. Antibodies against recombinant AspA (rAspA) were detected in patients' convalescent sera, demonstrating that AspA is expressed *in vivo* and is immunogenic. Rabbit antiserum (RαAspA) was raised against rAspA and used to screen a panel of wild-type meningococcal strains. AspA was expressed in 8/10 hypervirulent lineages examined, but not AspA mutant strains. Strains lacking AspA expression were shown to possess a frame-shift mutation in a 10-bp poly-cytidine tract, suggesting that AspA is phase-variable. Cell fractionation experiments confirmed that the precursor protein (*c.* 112 kDa) was present in the outer membrane, whilst the secreted forms of AspA (*c.* 68 and 72 kDa) were detected in concentrated culture supernatants. Surface-exposure was confirmed by immunogold-staining and electron microscopy. RαAspA exhibited bactericidal activity against the homologous strain (MC58). Site-directed mutagenesis of Serine426 in the putative serine protease site, abolished AspA secretion in *Escherichia coli*, confirming autocleavage.

Conclusion: AspA is a novel, conserved, immunogenic, cross-reactive and surface-exposed meningococcal autotransporter protein, belonging to the pyrolysin family of subtilisin-like serine proteases. AspA shows significant homology to SphB1, which is encoded by a BvgAS-controlled virulence-activated gene and plays an important role in the pathogenesis of *B. pertussis*. Serine426 is responsible for the autocleavage and secretion of AspA. Anti-AspA antibodies exhibit bactericidal activity against meningococcal strain MC58. Further investigation of AspA as a putative meningococcal virulence determinant and vaccine candidate is warranted.

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Surface expression of lipopolysaccharide in *Neisseria meningitidis* requires the function of Omp85, a highly conserved outer membrane protein

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In Gram-negative bacteria, lipopolysaccharide (LPS) biosynthesis takes place at the inner membrane. How the completed lipid molecules are subsequently transported to the outer membrane is still unknown. Omp85 of *Neisseria meningitidis* is representative for a family of outer membrane proteins highly conserved among diverse Gram-negative bacteria. The encoding *omp85* gene belongs to a largely conserved cluster of genes which includes *lpxA* and *lpxD*, encoding acyltransferases required for the first step of lipid A biosynthesis. Using RT-PCR assays, we first demonstrated that the *omp85* gene is part of an operon of eight genes encoding enzymes involved in phospholipid, lipid A and fatty acid biosynthesis. This suggests a common involvement of these proteins in LPS/lipids assembly and transport. *N. meningitidis* is until now the only Gram-negative bacterium in which a completely LPS-deficient but still viable mutant can be obtained. This makes it a uniquely suitable organism for the study of LPS transport. To directly test the potential involvement of Omp85 in LPS transport, we first tried to make a knockout *omp85* mutant but without any success, suggesting that the gene is essential for bacterial survival. To study the function of Omp85 in spite of this limitation, a meningococcal strain was constructed in which Omp85 expression could be switched on or off through a *tac* promoter-controlled *omp85* gene. When cells from this strain are transferred to medium without IPTG, they are first depleted of Omp85 protein and later stop growing, showing that Omp85 is indeed essential for viability. When these cells were examined by transmission electron microscopy, accumulation of electron dense material in the periplasm was observed. By fractionation of inner and outer membranes through isopycnic sucrose gradient centrifugation, we could demonstrate that LPS mostly disappeared from the outer membrane and instead accumulated in the inner membrane, concomitant with the depletion of Omp85. In contrast, Omp85 depletion did not affect localisation of the integral outer membrane proteins PorA and Opa. These results provide compelling evidence for a role of Omp85 in transport of LPS to the cell surface. LPS transport is likely to be a general mechanism involving widely conserved proteins such as Omp85. Our future work will focus on the understanding of the molecular mechanisms by which Omp85 transports LPS. As an *omp85* knockout mutant could also not be isolated in the LPS-deficient strain where LPS biosynthesis is blocked due to an *lpxA* mutation, it is possible that Omp85 has an additional function. We will therefore test its potential involvement in the transport of phospholipids in the outer membrane, similar to the role of MsbA which functions as a general lipid transporter in the *Escherichia coli* inner membrane.

Transport of lipoproteins to the cell surface in *Neisseria meningitidis*

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Introduction: *Neisseria meningitidis* (*N.m*) exposes several lipoproteins at the cell surface. They function, for example, as parts of the receptors for lactoferrin, transferrin and haemoglobin. We are studying the transport pathway to the bacterial cell surface of one of these lipoproteins, the lactoferrin-binding protein LbpB.
In *Escherichia coli,* the Lol system involves in the transport of lipoproteins from the inner membrane through the periplasm to the outer membrane has been described. The system consists of a periplasmic chaperone, LolA, the outer membrane receptor LolB, and an energy-delivering system in the inner membrane consisting of the proteins LolC, LolD, and LolE. For transport via the Lol system, the amino acid residue in position +2 of the lipoprotein is determinative: the presence of an Asp results in inner membrane localization, whereas a Ser or any other residue results in outer membrane localization. However, these outer membrane lipoproteins stay in the inner leaflet of the outer membrane. Thus, in *N. m*, an additional sorting signal and an outer membrane transport machinery must exist for cell surface-exposed lipoproteins.
Results and Discussion: We first decided to investigate whether a Lol system is required for the transport of LbpB to the outer membrane in *N.m.* Inspection of the genome sequences of *N.m* strains revealed homologues of LolA, B, C, and D, but not of LolE. Inactivation by insertion of *lolA* and *lolB* appeared impossible, suggesting that the Lol system is vital to *N.m.* To investigate whether the transport of LbpB to the outer membrane is Lol-dependent, it was expressed and studied in *E. coli.* Expression of LbpB in *E. coli* appeared highly toxic. Immunofluorescence experiments clearly showed no exposition in this organism, and cell fractionations revealed that LbpB was mislocalized to the inner membrane. Moreover and surprisingly, when expressed in sphaeroplasts of *E. coli*, LbpB was not specifically released upon addition of LolA in spite of the presence of an Ile in +2. Replacement of the Ile in +2 by a Ser relieved the high toxicity and resulted in full LolA-dependent outer membrane localization. Our data indicate that sorting of *N. m* surface-exposed lipoproteins occurs at the inner membrane and that transport to the outer membrane involves a Lol-independent transport machinery absent in *E. coli.* Furthermore, sequence analysis of *N.m* lipoproteins suggested a common Lol-avoidance motif specific for cell surface-exposed lipoproteins.

SESSION V

Surface structures (2)

The crystal structure of the OpcA outer membrane protein suggests a model for its adhesion to proteoglycan

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Introduction: OpcA is an integral outer membrane protein from *N. meningitidis* and is functionally similar to the opacity proteins found in *Neisseria*. It mediates binding to epithelial and endothelial cells in nonencapsulated strains and functions independently of pilus-based adhesion. It has also been shown to bind to the same proteoglycan receptors, such as heparin and heparan sulfate, which mediate entry of nonencapsulated meningococci into epithelial cells[1]. The aim of this study was to determine the crystal structure of OpcA and draw conclusions concerning its mechanism of adhesion to proteoglycan.
Materials and Methods: Crystals of OpcA were obtained as described previously[3]. The structure was solved using standard multiple isomorphous replacement methods, in combination with averaging between two different crystal forms.
Results: OpcA adopts a 10-stranded β-barrel structure, in common with other outer membrane proteins whose structures are known[2]. The cross-section of the β-barrel is uniformly flattened along its length, with maximal and minimal diameters of 23 and 15 Å respectively. One end of the barrel consists of much shorter turns and forms the periplasmic end of the molecule, whilst the exterior is made up of five loop regions of varying length which would protrude above the membrane. The loop regions associate to form a continuous surface on the external face: L2 is located in a central position at the top of the barrel and interacts with apolar residues in loops L3, L4 and L5. L1, however, is separate from the other loop regions and does not make any direct interaction with them. This effectively generates a crevice in the external surface of OpcA, approximately 8 Å wide, 22 Å long and 11 Å deep, which is lined by residues from L1, L2 and L5. Examination of the calculated electrostatic potential across the surface showed that the crevice contained a high proportion of basic residues, which is a characteristic of heparin binding sites in other proteins. Given that the crevice is the dominant feature in external surface of OpcA, and also that its dimensions are appropriate to accommodate an oligosaccharide chain, we propose that this is the binding site for epithelial cell proteoglycan ligands.
Conclusions: The crystal structure of OpcA has provided some important clues as to how outer membrane protein structures could be adapted to function as adhesins. The β-barrel framework provides a suitable scaffold for the external loop regions, which can associate to form a wide range of possible binding surfaces. The presence of a defined binding site for proteoglycan ligands also suggests that structural information could be used to design small molecule inhibitors of adhesion.

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The low resolution structure of the neisserial secretin PilQ

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Introduction: The secretins are outer membrane proteins which associate to form complexes consisting of 10-14 identical subunits and are involved in the transport of a variety of proteins across the bacterial outer membrane[1]. PilQ is a member of this secretin family of bacterial membrane proteins and is specifically involved in the secretion of type IV pilus fibres from the bacterial cell surface. The type IV pilus is crucial for natural competence for transformation and twitching motility, but is also a most important organelle in neisserial adherence/colonisation and contains proteins which specifically bind to human epithelial cells[2]. We have shown that meningococcal PilQ is organised as a donut-like complex with 12 identical subunits surrounding a central cavity of sufficient dimensions to accommodate the passage of a nascent type IV pilus fibre[3]. The aim of this study is to determine the 3-D structure of the PilQ complex and draw appropriate conclusions for the function of PilQ in pilus biogenesis.
Materials and Methods: Detergent soluble PilQ complex was purified from native meningoccal or *N. lactamica* membranes and prepared for electron microscopy and image analysis as previously described[2], with minor modifications. The low resolution 3-D structure was determined from samples visualised in negative stain using random conical tilt methodology (www.wadsworth.org/SPIDER).
Results: Here we present the first 3-D data on a secretin complex involved in type IV pilus biogenesis. PilQ forms a ribbed cylindrical complex with a height of 170 Å and width of 165 Å. Viewed from a position above the membrane plane the complex has a circular structure 165 Å wide with 12 areas of density surrounding a cavity of 65 Å width. From the side, the complex is approximately rectangular and is composed of 4 equivalent stacked rings each measuring 165 x 40 Å. Examination of the preliminary 3-D volume reveals that the density through the volume is variable, suggesting that the cavity observed on the membrane face is not continuous through the entire height of the complex.
Conclusions: The overall dimensions of the PilQ complex suggest that a large proportion of the protein is exposed outside the outer membrane, providing potential sites of interaction with other proteins involved in pilus biogenesis. This constitutes further evidence that the secretin component of the type IV pilus secreton does not merely function as a passive sheath but could play an active part in directing pilus assembly. The structure is larger and has significant differences in architecture compared to the PulD-PulS complex from *K. oxytoca*[4], the only other secretin well characterised with regard to 3-D structure. This could be attributed to the different secretion substrates of the two systems.

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Structured diversity, evolution, and implications for protein function: investigation of the *opa* repertoire of *Neisseria meningitidis*

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The high within and among isolate diversity of the Opa proteins of the pathogenic *Neisseria* has been recognised for many years. Here, we describe a systematic survey of the diversity of *opa* loci within and among isolates for more than 300 distinct meningococci, a total in excess of 1100 individual *opa* genes. This survey had three main objectives:

- to define the extent of *opa* gene sequence diversity in *N. meningitidis* populations (thereby to establish '*opa* repertoire' and '*opa* type');
- to determine whether, and the extent to which, this diversity was structured;
- to investigate the evolutionary processes and selective constraints which generated and shaped *opa* diversity; and
- to explore the implications of these processes on the structure and function of Opa proteins

Two well-defined meningococcal isolate collections were studied: the 107 diverse, mainly hyperinvasive, meningococci used for the evaluation of the meningococcal MLST scheme, which had been characterised at a wide number of loci; and a set of 269 carried and disease-associated meningococci collected in the Czech Republic during 1993, all of which had been characterised by MLST and *porA* gene sequencing. The nucleotide sequence data from each *opa* locus (*opaA*, *opaB*, *opaD* and *opaJ*) enabled a number of hypothesis-driven analyses that exploited a range of theoretical approaches. A classification scheme was devised which permitted the accurate description of the distribution of allelic sequences and variable region types among isolates and lineages. Phylogenetic techniques, including split decomposition analysis, allowed the visualization of the phylogeny of the *opa* genes and Opa proteins and permitted the relative rates of inter and intragenic recombination between and among strains to be determined. The structure of the nucleotide sequence diversity of the variable regions was consistent with a model of positive immunological selection. Maximum likelihood analyses permitted nucleotide sites under positive selection to be identified. Homology modelling, using the crystal structure of the related OmpX protein of *Escherichia coli* allowed the transmembrane beta-barrel of the Opa protein to be mapped, while its hypervariable loop structures were investigated using secondary structure prediction software. Together these approaches provide an alternative paradigm for exploring the biological function of these important proteins which takes into account, and indeed exploits, the high levels of variation observed.

Minor pilins involved in type IV pilus biogenesis, retraction, and adherence in *Neisseria gonorrhoeae*

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Introduction: The expression of Type IV pili (Tfp) by *Neisseria gonorrhoeae* is associated with the ability of the bacterium to adhere to the human host cells and to display the properties of twitching motility, DNA binding/uptake during natural transformation and autoagglutination.
Method: To better understand the basis for these relationships, the genomes of pathogenic *Neisseria* were searched for genes encoding molecules related to PilE, the Tfp subunit protein.
Results and Discussion: Five ORFs sharing their highest sequence identity with the N-terminal domain of PilE mapped to one locus. The corresponding proteins, designated PilH-L, were all found to co-purify with pilus fibers. Gene disruption mutants in each of the genes resulted in a dramatic reduction in pilus expression. However their requirement in fiber formation could be suppressed in *pilT* mutants which are defective in fiber retraction. In these mutants, the absence of any on of these molecules precluded the presence of the others in purified fibers. Piliated mutants lacking PilH-L in a *pilT* background failed to adhere to human epithelial cells. From these results it seems that PilH-L act in the same step in the Tfp biogenesis pathway as the fiber associated adhesin molecule PilC. The influence of PilH-L on uptake of DNA during natural transformation will also be discussed.
Conclusion: We propose that PilH-L and PilC function cooperatively as part of a subassembly complex which defines a common step at which fiber growth and retraction take place.

Genetics of O-acetylation of meningococcal capsule polysaccharides

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Introduction: O-acetylation of meningococcal capsule polysaccharides was analyzed by NMR technology (Jennings et al., 1977, Lemercinier and Jones, 1996). Within the serogroups with capsules composed of sialic acid only serogroup B was not acetylated. The frequency of acetylation differed significantly (serogroup C: 88% O-acetylation, serogroup W-135: 8% O-acetylation, serogroup Y: 79% O-acetylation) (Borrow et al., 2000, Longworth et al., 2002). Although, de-O-acetylated serogroup C polysaccharide seemed to be more immunogenic than O-acetylated polysaccharide (Richmond et al., 2001), the biological relevance of O-acetylation is as yet unclear. Furthermore, neither the genes encoding the O-acetyltransferases nor the regulatory mechanisms of O-acetylation are known. In this report we describe the cloning and characterization of putative acetyltransferases of the serogroups C, W-135, and Y.
Material and Methods: The *siaD* downstream regions within the capsule synthesis region of the *cps* locus of different sequence types (STs) of serogroups C, W-135, and Y meningococci were amplified by PCR and sequenced. Knock-out mutants of the O-acetyltransferases were constructed and analyzed by O-acetylation specific monoclonal antibodies kindly provided by Wyeth-Lederle Vaccines (Longworth et al., 2002).
Results and Discussion: In serogroup C meningococci, a 1383-bp orf was found 28-bp downstream of *siaD*. There were no homologies to known genes or proteins, respectively. Nevertheless, knock-out of this gene resulted in a de-O-acetylated capsule. Sequencing of the O-acetyltransferase gene of naturally occuring de-O-acetylated strains revealed two polypyrimidine tracts (poly-T and poly-A, respectively) with repeat unit number variations which resulted in premature stop codons. No correlation between STs and O-acetylation status was found. In both serogroup W-135 and Y meningococci, a 636-bp orf was found 80-bp downstream of *siaD*. The encoded protein showed homologies to known O-acetyltransferases of the CysE-LacA-LpxA-NodL family of various genera. Consequently, a knock-out mutant of this gene expressed the de-O-acetylated phenotype. Sequencing of the *siaD* downstream region of de-O-acetylated wildtype strains showed various deletions and insertions (IS*1301*) in the O-acetyltransferase gene. The gene of the predominant ST of serogroup W-135 meningococci, i.e. ST-22 (de-O-acetylated), harbored the IS*1301* whereas the gene of the predominant ST of serogroup Y meningococci, i.e. ST-23 (O-acetylated), was intact.
Conclusion: Putative capsule O-acetyltransferase genes of serogroup C, and W-135/Y meningococci were sequenced and characterized. Complementations of the W135/Y O-acetyltransferase as well as in vitro assays and transcription analyses are currently being performed.

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SESSION VI

Population genetics and gene regulation

Three contrasting population genetic structures among pathogenic bacteria

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Yersinia pestis, the cause of plague, is a named species only because systematicists thought it was important to avoid confusing medical doctors. *Y. pestis* and *Y. pseudotuberculosis* share identical ssrRNA sequences, a feature that is normally sufficient to assign bacteria to one species. *Y. pestoides* is a further spurious species designation for isolates from Russia with reduced virulence. Multiple housekeeping gene sequences were identical among all isolates of *Y. pestis* and *Y. pestoides*, showing that these bacteria correspond to a recently emerged clone. *Y. pseudotuberculosis* contains a variety of related alleles at these genes and each of the *Y. pestis* alleles, or a close relative, were found among strains of *Y. pseudotuberculosis*. Thus, *Y. pestis* is a clone of *Y. pseudotuberculosis*, calculated to have evolved in the last 1,500-20,000 years. The evolutionary relationship of subgroupings within *Y. pestis* was determined by developing a binary, fine-typing system based on the presence or absence of IS*100* at 12 discrete locations.

Helicobacter pylori is a gram-negative bacterial pathogen that colonizes the stomach, causing gastritis, of over half the human global population. Transmission is often within families and results in decades of carriage in the absence of antibiotic therapy. *H. pylori* is naturally transformable and many individuals are at least transiently colonized by multiple strains, resulting in frequent recombination resembling gene conversion. Genetic variation is more often caused by recombination than by mutation, more so than in any other known bacterium and the local population structure is panmictic. Recombination is so frequent that several percent of the genome differs between bacteria isolated two years apart from single individuals. However, due to geographic isolation between human ethnic groups and due to the largely vertical mode of transmission, distinct populations of *H. pylori* exist in different continents. The analysis of multiple housekeeping gene sequences has demonstrated distinct populations, which evolved separately in Africa, Indo-Europe and East Asia. Human migration between these continents and to the New World has resulted in bacterial spread to novel locations and to recombination between the different populations. In addition, old migrations have resulted in spread of genes from East Asia and Africa to Europe. Due to the extensive sequence diversity within *H. pylori*, these bacteria provide a very sensitive measure for the detection of ancient and modern human migrations.

Neisseria meningitidis recombines less that *H. pylori* and is less deadly than *Y. pestis*. Particular subgroupings, such as subgroup III of serogroup A, are capable of rapid global spread and have been responsible for all the large epidemics in the last decades. Like *Y. pestis*, subgroup III is highly uniform and almost no sequence variation was found within housekeeping genes. Therefore, four variable genes encoding antigens, one somewhat variable housekeeping gene and one IS element were screened within a global collection of subgroup III in order to elucidate its population structure. The data showed that subgroup III consists of a series of so-called genoclouds, of which nine have been recognized from three pandemics since the mid-1960's. Each genocloud consists of a common genotype and its closely

related variants. Genoclouds are transient: they die out in each country within a few years, probably due to herd immunity, and can only survive by epidemic spread. Bottlenecks during spread eliminate variants and purify the genocloud. In addition, many of the variants are apparently less fit than their parental genotype, in particular due to the import of *tbpB* from *N. lactamica*, and are lost due to competition. On occasion, genoclouds are replaced by fitter, related genoclouds but no details are yet available on the molecular basis for increased fitness.

Is the gonococcus a clonal complex of the meningococcus?

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Multilocus sequence typing (MLST) has been used successfully for the characterization of variation among isolates defined as *Neisseria menigitidis* and the data produced have proved to be invaluable for both epidemiological and research purposes. Here we examined nucleotide sequence variation at the same MLST loci for the closely related species, *Neisseria lactamica* and *Neisseria gonorrhoeae*. The similarity of the gene sequences was such that the alleles and sequence types generated were included in the MLST database, so that this database is a *Neisseria* MLST database covering these three related organisms. The close similarity of these sequences further highlighted the previously observed evolutionary relationships among these bacteria, which can be difficult to differentiate by microbiological techniques. To investigate the relationships among them further, from an isolate characterization and evolutionary perspective, we applied a number of phylogenetic and population analyses to the nucleotide sequences obtained from the MLST loci of isolates microbiologically assigned to the three species.

The nucleotide sequences from both *N. meningitidis* and *N. lactamica* were found to be very diverse, whereas the gonococcal isolates examined were virtually identical. When the concatenated sequences of the seven housekeeping gene fragments, obtained from multiple isolates for each of the three species, were analysed two distinct groups were resolved: one corresponding to *N. lactamica* the other corresponding to the meningococcal and the gonococcal isolates. This confirmed the status of *N. lactamica* as a entity distinct from the gonococcus and the meningococcus. The gonococcal isolates, however, formed a distinct cluster of virtually identical organisms within the diversity of the meningococcus. Indeed, on the basis of MLST loci alone they would be classified as a clonal complex within *N. menigitidis*, and further examination of the MLST database revealed that a number of nasopharyngeal isolates originally classified as meningococci were, in fact, misclassified gonococci.

Apart from their genetic homogeneity, a number of features of the gonococcal isolates, especially related to their surface antigens, distinguished them as distinct biologically; however, the data from the multiple housekeeping genes together with data on the diversity of porin genes and the presence of *porA* and *opc* pseudogenes in this organism suggested that it arose once, relatively recently, from an ancestral population which closely resembled the current day meningococcal population. Differences among meningococci and gonococci were largely due to high levels of selection, mainly acting on genes encoding surface components. The very low levels of diversity in gonococcal housekeeping genes suggested that the transition from a meningococcal to a gonococcal ecology represented a tight evolutionary bottleneck, notwithstanding the regular isolation of gonococci from the nasopharynx and meningococci from the urogenital tract.

Mutator clones of *Neisseria meningitidis* in epidemic serogroup A disease

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Introduction: Serogroup A *Neisseria meningitidis* has repeatedly caused wide spread epidemics of meningitis and septicemia throughout the twentieth century. Recently, in a limited collection of strains, epidemic serogroup A isolates were found to have elevated mutation rates due to defects in the postreplicative mismatch repair pathway. To understand the role of these mutators in epidemic spread, we determined the prevalence of hypermutability in a collection of 95 serogroup A *N. meningitidis* disease isolates representing eight of the nine subgroups within the serogroup A lineage. The isolates were obtained from patients from pandemic disease as well as isolated outbreaks between the years 1936 and 1996. The majority of isolates belong to subgroup III pandemics which spread from China through Northern Europe, Asia, Africa, and South America.
Materials and Methods: Overall mutability in *Neisseriae* can be described by determining both missense mutation rates as well as phase variation frequencies of "contingency loci". The rate of phase variation was determined by quantifying the frequency of phase off to on transitions in genes encoding outer-membrane haemoglobin (Hb) receptors, *hmbR* and *hpuAB*. Missense mutation rates were assayed by determining the frequency of spontaneous mutation to rifampicin resistance.
Results: In total, 57% of serogroup A isolates possessed some level of elevated mutability which could be divided into two classes: intermediate and high level. Eleven of twenty high level mutators, which possessed phase variation rates > 100 fold higher than wild type isolates, were defective in mismatch repair. Ten of the thirty-six intermediate mutators possessing > 10 fold increases in phase variation rates could be partially complemented by a wild type *mutL* allele. All high level mutators belonged to pandemic subgroups (I, III, and IV) within serogroup A. Futhermore, in the three pandemic waves of subgroup III, mutators generally appear at the end of individual pandemics and are absent from the origins (China).
Conclusions: Compared with any other bacterial pathogen, a higher prevalence of mutator phenotypes was detected in a collection of serogroup A *N. meningitidis* clinical isolates. This may reflect the advantages *N. meningitidis* isolates derive from the increased phase variation rates which inevitably accompany mutator phenotypes. Higher rates of phase variation will lead to greater diversity within a finite inoculum, thus increasing the population's adaptability upon transmission into new host environments. This "phase variation dependent mutator advantage" may allow fixation of mutator alleles more frequently during epidemic spread, thus explaining the absence of high level mutators from isolated outbreak cases. Mutator phenotypes may prove to be a general phenomenon in pathogens which use "directed mutations" to adapt to the challenges of a new host environment.

The gonococcal Fur regulon: identification of additional genes involved in major catabolic, recombination and secretory pathways

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In several Gram-negative pathogens, the ferric uptake regulator protein (Fur) functions as a general global regulator and controls the expression of not only genes required for iron transport but also genes required for virulence. Although *Neisseria* genes involved in iron transport have been proposed to be regulated by Fur, binding of gonococcal Fur to the promoters of these genes has not been demonstrated. Furthermore the gonococcal Fur regulon is not well defined; the only genes that appear to be regulated by Fur are those involved in iron transport. In this study we have characterized the *in vitro* binding of *Neisseria gonorrhoeae* Fur to several well-defined iron transport genes as well as to additional genes involved in major catabolic, secretory and recombination pathways of gonococci. The gonococcal Fur protein was recombinantly expressed in *Escherichia coli* HBMV119. Fur was isolated from inclusion bodies and was purified by ion-exchange chromatography. The gonococcal Fur was found to bind to the promoter/operator region of a gene encoding the previously identified Fur regulated periplasmic binding protein (FbpA) in a metal-ion dependent fashion, demonstrating that purified Fur is functional. *In silico* analysis of the partially completed gonococcal genome (FA1090) identified Fur boxes in the promoters of several genes including *tonB*, *fur, recN*, *secY*, *sodB*, *hemO*, *hmbR*, *fumC*, and the *opa* family of genes. Using purified gonococcal Fur we demonstrate binding to the operator regions of the *tonB*, *fur*, *recN*, *secY, sodB, hemO, hmbR*, *fumC*, and the *opa* gene family as determined by an electrophoretic mobility shift assay. While gonococcal Fur was demonstrated to bind to the promoter region of all eleven *opa* genes (A through K), we did not detect binding of *E. coli* Fur with 8 of the 11 *opa* members indicating that target DNA sequence specificities exists between these two closely related proteins. Furthermore, we observed differences in the relative strengths of binding of gonococcal Fur for these different genes, which most likely reflects a difference in affinity between gonococcal Fur and its sensitive DNA targets. This is the first report which definitively demonstrates the binding of gonococcal Fur to its own promoter / operator region, as well as to the *opa* family of genes. Our results demonstrate that the gonococcal Fur protein binds to the regulatory regions of a broad array of genes and indicates that the gonococcal Fur regulon is larger than originally proposed.

The role of integrated databases in meningococcal epidemiology and vaccine development

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High-throughput molecular techniques are increasingly exploited to provide portable information on the characterisation of bacterial isolates at high resolution. The data produced are invaluable for epidemiological analysis and vaccine development, but it is essential that the quality of such information is assured and that it is organised and presented in an accessible way. We have developed curated databases that are available via the world wide web (http://neisseria.org/nm/typing/) which integrate quality assured nucleotide sequence data with other information. These databases include DNA sequences from multiple loci encoding housekeeping and antigen genes located around the chromosome and provide a resource for exploring the population biology of the meningococcus and for assessing potential vaccine candidates and virulence determinants within defined isolates collections.

The *Neisseria* MLST site (http://neisseria.org/nm/typing/mlst/) has a distributed structure that stores allelic profiles (defined by Multi Locus Sequence Typing) separately from isolate data. Among other advantages, this allows multiple isolate databases to be set up either locally or remotely which link to the profiles database. The PubMLST database stores allelic profiles and isolate data and accepts and curates all submissions made to it; it aims to contain a minimum of one example of each MLST sequence type described. Other databases which cater for particular data subsets can also be established, for example a database containing information on the 107 isolates used in the original MLST validation has been constructed. This isolate database links to other databases and coordinates a variety of types of information including publications via PubMed and nucleotide sequence data in addition to traditional microbiological, serological, and epidemiological information. As well as making a network connection to the MLST allelic profiles database containing allele designations for seven housekeeping loci, this reference strains database is linked to sequence databases for PorA and FetA variable regions, PorB, NspA, Opa and an additional 13 housekeeping loci providing over 16 kilobases of DNA sequence data for each isolate. Used as a reference set of hyperinvasive meningococci, this database represents the most comprehensively characterised meningococcal isolate collection available to date, which can be expanded further as new vaccine targets and epidemiological markers are identified. This paradigm can be applied to any isolate collection and can incorporate both public and privately held data, readily accessible via the Internet.

SESSION VII

Epidemiology and antibiotic resistance

Multifocal emergence of *Neisseria meningitidis* serogroup W135 within the ET-37 clonal complex.

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The characterization of *N. meningitidis* W135 strains was enhanced since the clonal outbreak of W135 meningococcal disease that occurred in 2000 among pilgrims returning from Saudi Arabia and in their contacts. However, other W135 (ET-37) isolates were detected in France and several African countries (Niger, Burkina Faso, Cameroon, Central African Republic) and Madagascar, but they were slightly different from the Hajj-related clone of 2000, by genotypic analysis. In particular, such strains were found in meningitis cases in Burkina Faso and Niger at the end of the 2001 epidemic (at the time when A and C vaccination had been initiated several weeks ago). Moreover, strains related to those causing the 2000 outbreak have been isolated worldwide in the 1990s, but again were slightly different when several typing methods were combined. Using several gene typing approaches we were able to show that not all of the ET-37 (W135) isolates from France and Africa in 2001 and 2002 are identical to the Hajj 2000-related clone. Indeed, several ET-37 (W135) clones are circulating worldwide. For instance, "local" ET-37 W135 clones are found in 2002 in France and in other countries and particularly in Africa (Burkina Faso). The Hajj 2000-related clone is only a particular clone within the ET-37 complex which, in fact, is composed of a collection of closely related strains. The Hajj 2000 outbreak most likely allowed the expansion (inflation) of one particular clone within the ET-37 complex but other clones are expanding now. The emergence of W135 (ET-37) meningococci raises the hypothesis of a bacterial mechanism of capsule switch as an escape strategy to circumvent host immunity. Most probably, these W135 strains could have been selected by the specific anticapsular immune response against serogroup C in the A and C vaccinated pilgrims, since only capsular antigens distinguish the W135:2a:P1-2,5 from the C:2a:P1-2,5 strains of the same ET-37 clonal complex. Alternatively, the emergence of W135 (ET-37) could be due to the expansion of an "old clone" W135 of this complex, rarely detected before. The global expansion of *N. meningitidis* W135 since 2000 confirms the abilities of the neisserial genome to adapt to host immunity and suggests that other genetic lineages of serogroup W135 could emerge and, therefore, should also be kept under careful surveillance.

Genetic diversity of 822 meningococcal carrier strains from Bavaria

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Introduction: Multilocus sequence typing (MLST) has been used to study the genotypes of carrier strains from the Czech Republic (1). A large number of sequence types and lineages was recovered from this collection of 215 strains. The DNA sequence data can now be used directly for comparisons of strain collections. During the course of the Bavarian meningococcal carriage study performed in the winter 1999/2000, 830 meningococcal isolates were obtained. The collection was compared to the Czech collection on the basis of sequence types and lineages.
Materials and Methods: Retro-pharyngeal swabs were obtained from 8,000 Bavarian subjects in a cross-sectional study (November 1999-March 2000). Kindergarten and school children as well as military recruits were included in the study. MLST of meningococci and lineage assignment by BURST were performed as described (1). Serogroup analysis and determination of the capsular genotype were performed as described (2).
Results and Discussion: 322 sequence types and 50 clonal lineages were recovered in Bavaria, which differed significantly from those found in the Czech Republic. The differences were mostly due to new combinations of pre-existing alleles or polymorphisms. The frequencies of hypervirulent lineages reflected the epidemiology of meningococcal disease in Bavaria: ST-8 complex (former cluster A4, 0.2% of the carrier isolates), ST-11 complex (ET-37 complex, 1%), ST-32 complex (ET-5 complex, 5%), ST-44 complex (lineage 3, 13.5%). The most prevalent serogroup was B (31.8%). However, 40.5% of the isolates harbored the serogroup B specific variant of the *siaD* gene suggesting that 21% of the genotype B isolates were capsule switch-off variants. The overall proportion of *siaD* positive isolates (n=541) which expressed the serogroup B, C, W-135, or Y capsular polysaccharide was 69%. 16.4% of the isolates lacked the genes required for capsule synthesis and transport. Most of these strains belonged to four lineages which do not require polysaccharide capsules for person-to-person transmission (3).
Conclusion: We describe the epidemiology of the largest carrier isolate collection which was characterized by MLST until now. The comparison to the Czech collection, which was established six years before, revealed substantial differences in the bacterial populations. We obtained a representative number for the proportion of unencapsulated *siaD* positive isolates circulating among carriers.

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Identification and characterization of heretofore unknown efflux pumps in *Neisseria gonorrhoeae* and *N. meningitidis*

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Introduction: We have proposed that the efflux pumps possessed by the pathogenic *Neisseria* are of importance in the development of antibiotic resistance and in bacterial survival at sites *in vivo* that contain antimicrobial agents. In this respect, two efflux pumps were previously identified in gonococci: the TX-100 inducible *mtr* (*m*ultiple *t*ransferable *r*esistance) complex composed of MtrC-MtrD-MtrE envelope proteins that exported antibacterial hydrophobic agents (HAs) and the *farAB* (*f*atty *a*cid *r*esistance) complex that exported long chained fatty acids. We also characterized a similar MtrC-MtrD-MtrE efflux pump in meningococci, which was functional but not TX-100 inducible. In this study we questioned whether gonococci and meningococci possess additional efflux pumps that could participate in resistance to antibacterial agents.
Materials and Methods: We used techniques of gene cloning, mutagenesis, DNA sequencing, polymerase chain reaction (PCR), primer extension, RT-PCR, protein analysis and genetic transformation to conduct this work.
Results and Discussion: In our search for other new efflux pumps in *Neisseria* that might be important for antibiotic resistance or survival *in vivo*, we identified and characterized a homologue to the Na^+/drug antiporter NorM from *Vibrio parahaemolyticus*, which, in gonococci and meningococci, exported cationic dyes. The neisserial NorM efflux pump, when overexpressed due to promoter or ribosome binding site mutations, also conferred decreased susceptibility of gonococci to fluoroquinolones. This raises serious concerns about the development or enhancement of fluoroquinolone resistance in the clinical setting. We also characterized a neisserial homologue of the *macAB* ABC transporter of *E. coli*, which has been proposed to export macrolide antibiotics including azithromycin. Transcription studies confirmed that *macAB* is expressed under normal growth conditions *in vitro*. The substrate profile for this efflux pump is now under study using isogenic strains bearing a wild type or inactivated copy of *macAB*.
Conclusion: We propose that the various efflux pump systems in gonococci and meningococci work in synergy to provide the pathogenic *Neisseria* with increased resistance to a large array of antimicrobial compounds. Through their individual or joint effort, the action of the efflux pumps described herein could both promote bacterial survival *in vivo* and impact the efficacy of antibiotic treatment.

Mutation in 23S rRNA conferring macrolide resistance in *Neisseria gonorrhoeae*

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Introduction: Several mechanisms of macrolide resistance have been reported. These included the over-expression of *mtr* efflux pump, the acquisition of methylases (*erm* genes) and efflux genes (*mef*). We have characterized fifty-six azithromycin (Az) resistant (MICs of 2.0 to 4.0 mg/l) *Neisseria gonorrhoeae* strains with cross-resistance to erythromycin (Ery) (MICs of 2.0 to 64.0 mg/l) isolated in Canada between 1997-1999 and their mechanisms of azithromycin resistance were determined. Most of these strains have over-expression of *mtr* pumps and did not have *erm* and *mef* genes described in the literature. In this study, we determined the resistance mechanisms of two clinical isolates.
Materials and Methods: PCR of rRNA methylases (*erm*(A), (B), (C), (F), *mef* genes, and DNA sequencing of the 23S rRNA, the *mtr* efflux pump and the structural genes *rplD* (ribosomal protein L4) and *rplV* (ribosomal protein L22) and sequencing of domain V of 23S rRNA (*rrl*) were used to identify potential mechanisms of macrolide resistance (EryR or AzR) in gonococcal strains. In addition, susceptibility to crystal violet was used to determine membrane permeability. Spontaneous susceptible revertants obtained by subculturing on non-selective media were characterized to confirm that the 23S rRNA mutation conferred macrolide resistance. In addition, spontaneous resistant mutants were obtained by stepwise selection on selective media.
Results and Discussion: The two AzR strains (Ery MIC, 64.0 mg/l; Az MIC, 4.0 mg/l) belonged to A/S class NR/IB-01 with the 2.6 mDa plasmid and crystal violet MICs of 1.0 mg/l. Both strains tested were negative for *erm* and *mef* genes and no mutations were identified in the *rplD* and *rplV* genes or in the *mtr* promotor region or *mtrR* gene. Both strains had a CÆT mutation in the peptidyltransferase loop in domain V of the 23S rRNA alleles (alleles 1 and 2 at nucleotide position 2599 and allele 4 at nucleotide position 2607). To confirm that the mutations in 23S rRNA were associated with macrolide resistance, spontaneous macrolide resistant mutants were generated from susceptible strains. The same CÆT mutation in domain V of the 23S rRNA alleles was identified in all four alleles in the mutant strains. Conversely, the two original clinical strains (Ery MIC, 64.0 mg/l; Az MIC, 4.0 mg/l) were used to derive susceptible mutants. The revertants from resistant to susceptible phenotype showed the reversion of 23S rRNA sequence to wild type.
Conclusion: This is the first observation of mutations in domain V of 23S rRNA alleles associated with macrolide resistance in *N. gonorrhoeae*. These mutations had been reported in other bacteria including *Escherichia coli*, *Streptococcus pneumoniae* and *Helicobacter pylori*.

SESSION VIII

Genome and gene expression

Use of a genome-wide collection of *Neisseria meningitidis* mutants for an in depth analysis of the meningococcal genome

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After the deciphering of the genomic sequence(s) of *Neisseria meningitidis*, the greatest challenge awaiting us is to determine the function of the roughly 2,000 meningococcal genes. The ultimate tool for investigating gene function on a genomic-scale being a comprehensive collection of defined mutants, we constructed such a tool in 8013, a serogroup C meningococcal strain. Meningococcus is one of the most suitable bacterial candidates for such an approach because of its small genome size and tractable genetics. In the first step, high-density random insertional mutagenesis was achieved by *in vitro* transposition of a *mariner*-derived mini-transposon that was engineered for signature-tagged mutagenesis, a high-throughput screening technique allowing the simultaneous analysis of numerous mutants. We thus constructed an ordered library of 4,548 mutants, large enough to contain insertions into most of the non-essential genes. In the second step, we launched a large-scale sequencing program. We sequenced the transposon insertion site in each mutant and are currently completing the genomic sequence of strain 8013 in order to map precisely the transposon insertion sites on its genome. The impact of this library as a tool is best illustrated by the results of a recently completed analysis aiming at identifying the genes involved in meningococcal ability to resist complement-mediated lysis. The identification of more than 80% of the genes known to be involved in the synthesis of the capsule and LOS, as well as several novel candidates, confirmed the quality and the exhaustivity of our library. Preliminary results of another screen hunting for genes important for meningococcal adhesion will also be presented.

Phase variable genes in *Neisseria meningitidis* and genetic factors influencing the phase variation rates

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Interrogation of the complete genome sequence of *Neisseria meningitidis* (Nm) strain MC58 revealed 84 simple sequence repeat tracts indicative of phase variable genes (Snyders *et al.*, 2001, Microbiology).

Twenty one of these putatively phase variable genes were sequenced to identify inter- or intraspecies polymorphisms in the repeat tracts. We included at least one representative of the several different tracts based on the nucleotide composition making up each repeat. From this analysis, we predicted (based on detecting polymorphims and taking into account repeat tract length) that 70% of the genes were likely to be phase variable. Three of these genes were investigated using immunoblotting to identify phenotypic switching using specific antisera to the relevant proteins. Antibodies directed against Cam (NMB2104, presenting $(G)_6$ in the coding region), YadA (NMB1994, $(TAAA)_9$ in the promoter region) and an hypothetical protein (NMB0032, $(A)_{11}$ in the promoter region) of Nm were raised in mice (Pizza *et al.*, 2000, Science). Colonies of strain MC58 showed different levels of reactivity when tested with each of the sera. Preliminary results show that this change in reactivity with anti-YadA antibodies was associated with a change in the number of TAAA repeats, suggesting that the expression of gene NMB1994 is phase variable. Modulation of haemoglobin receptor phase variation rate has been associated with mutator phenotype resulting from mismatch repair deficiencies (Richardson and Stojiljkovic, 2001, Molecular Microbiology, 40: 645-655), and capsule phase variation has been reported to depend on absence of dam activity (Bucci *et al.*, 1999, Molecular Cell, 3: 435-445). Thus, the involvement of trans acting factors in the regulation of the expression of surface structures was studied.

The *dam* gene was investigated in a set of 100 geographically and genetically distinct *N. meningitidis* strains. This revealed that 25% of the strains possessed an active Dam function, of which 44% belonged to hypervirulent lineages. Moreover, disruption of the *dam* gene in various strains of *N. meningitidis* did not lead to a mutator phenotype and to a significant change in the frequency of phase variation of the genes *siaD*, *porA*, *opc* and *lgtG*. On the contrary, a *mutS* derivative of MC58 demonstrated a mutator phenotype and an increase in frequency of *siaD* phase variation (2.2×10^{-4} compared to $10^{-5}/10^{-6}$ in a WT background). Additional capsule negative mutants, which did not result from frameshifts in the *siaD* gene, were also produced in a *mutS* background (2.2×10^{-4}).

Design and construction of a pan-*Neisseria* DNA microarray

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Introduction: DNA microarrays enable genome-wide analyses of bacterial transcription under environmental conditions that are relevant to the infectious process. However, the widespread adoption of this methodology in bacterial systems has been limited by the time and cost involved in the construction of individual microarrays. By pooling resources, a consortium of six laboratories has constructed a microarray containing amplicons from the genes of *Neisseria gonorrhoeae* and *Neisseria meningitidis* strains MC58 and Z2491.

Materials and Methods: We took advantage of the high level of nucleotide sequence identity shared between segments of many genes in both species to design a set of "core" amplicons. These, while derived from the genome of *N. gonorrhoeae* FA1090, can also be used to monitor the expression of the equivalent genes in *N. meningitidis*. Maximally cross-hybridizing segments of common genes were determined and used as templates for primer design. Potential amplicons were then compared against all three genomes, all internal cross-hybridization regions were identified and these regions were then avoided in the design of alternative amplicons. This process was then re-iterated with each genome sequence until adequate specific amplicons were designed for all distinct coding regions.

Results and Discussion: In constructing the microarray, we experienced a failure rate of less than 5% during PCR amplification from five different genomic templates, when the PCR products were checked by agarose gel electrophoresis. A further 5% of the PCR reactions resulted in faint bands, where the concentration of amplified DNA may have been insufficient to result in a quantifiable signal on the microarray. We found that the most efficient means of overcoming these problem cases was to simply resynthesize the same oligonucleotides. The first version of this array contains less than 2700 *Neisseria*-specific amplicons that nonetheless cover more than 99% of the annotated features on each of the three genomes. The overwhelming majority of missing amplicons are from small ORFs where the derived amino acid sequence has no similarity to any entry in the databases. One of the advantages of constructing our own microarray is that it can be easily modified, as new information becomes available. We have added amplicons from a genomic island from *N. gonorrhoeae* strain MS11, and a cassette found in *N. meningitidis* strain NMB. As the annotation of the *N. meningitidis* strain FAM18 genome becomes available, we will add the additional genome-specific amplicons.

Conclusion: This project has demonstrated that a cooperative effort can result in the production of a reagent that is beyond the capacity of any individual laboratory. We intend to make the array available to the wider *Neisseria* community.

DNA microarray and computational-directed identification of novel Fur-dependent genes in *Neisseria meningitidis* group B

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The expression of bacterial iron-transport genes in response to iron in several Gram-negative bacterial pathogens is under the control of the ferric uptake regulator protein (Fur); however, the role of iron and Fur in controlling global gene expression has not been systemically investigated. Here we describe studies of iron global expression patterns together with computational analysis and *in vitro* studies to identify additional targets for the Fur transcriptional regulator in *Neisseria meningitidis*. DNA microarray technology was used to monitor simultaneous expression of the entire gene repertoire of *N. meningitidis* MC58 in response to iron limitation. We demonstrate that iron regulates the expression of at least 257 *N. meningitidis* genes; 166 genes were up-regulated, 86 were down-regulated and 5 were either up- or down-regulated depending upon the time of growth. *In silico* analysis for the identification of Fur box like sequences in the promoter regions of highly iron-repressed and iron-activated cluster of genes, revealed that 40% of the iron-repressed and 55% of the iron-activated genes have the potential to be regulated by Fur. This analysis revealed a broad array of gene families which function in transcription, fatty acid and phospholipid metabolism, protein synthesis, DNA metabolism, energy metabolism and a large group of hypothetical genes. The iron-dependent expression of genes containing highly conserved Fur box sequences in their promoter regions was confirmed by reverse-transcription-PCR analysis. This included representative genes from the iron-activated cluster (*secY*, *sodB* and the hypothetical genes NMB1866 and NMB1436), and the iron-repressed cluster (*fur*, *recN*, *fumC*, *ldh*, *fbpA*, and the hypothetical genes NMB0034, NMB0313, NMB0744, NMB0866). *Neisseria* Fur was demonstrated to bind to the promoter /operator DNA fragments of the representative genes from the iron-repressed and -activated cluster which contained highly conserved Fur boxes. Our studies indicate that the *Neisseria* Fur regulon is larger than originally proposed and expand our understanding of the regulon of this essential regulatory protein. Microarray-based analysis together with computational analysis provides an effective tool for the identification of novel gene targets in bacterial pathogens controlled by regulatory proteins in which the characterization of defined genetic mutants in the regulatory protein are not available. Differential binding of *Neisseria* Fur to various operator sequences is proposed to allow this transcriptional regulatory protein to effectively function in fine-tuning the expression of a broad repertoire of genes under its control.

Genome maintenance in *Neisseria meningitidis*: the role of base excision repair

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Introduction: Genome alterations due to horisontal gene transfer and various recombinational events, as well as environmental DNA damaging agents, will constantly challenge the gene pool of *N. meningitidis*. In addition to being naturally competent for transformation, the meningococcus hosts a genome which is highly recombinogenic and has a relatively high spontaneous mutation rate, representing unusual challenges in terms of genome maintenance. Without DNA repair, every genome undergoes deleterious changes. Mechanisms for rapid genome variability, adaptability and maintenance are a necessity to ensure meningococcal fitness and survival. Indeed, the meningococcus is well equipped for DNA repair[1,2]. The base excision repair pathway is one of the major lines of defence against the deleterious effects of endogenous DNA damage[3]. The enzymes initiating this pathway are called DNA glycosylases, and they recognise and excise different kinds of DNA damage.
Materials and Methods: We have cloned and expressed the meningococcal DNA glycosylases MutY, Nth and Fpg of the base excision repair pathway in *E. coli*. Meningococcal strains and the *E. coli* clones were analysed with regard to DNA glycosylase expression by several biochemical and biological assays. In addition, meningococcal single and multiple mutant strains were constructed, and wildtype and mutant strains were compared with regard to mutagenicity and survival under varying conditions. Bioinformatic searches of the meningococcal genome sequences[1,2] for signature sequences enriched in genome maintenance genes was performed.
Results and Discussion: The DNA glycosylase mutants displayed a decreased survival rate and an elevated spontaneous mutation rate compared to the wild type strain. The importance of these functions for the fitness and survival of *N. meningitidis* is presently being investigated using biochemical and genetic approaches and relevant cellular models. Furthermore, we have identified and characterised other conserved as well as novel DNA binding components. Some of these components are involved in DNA binding on the meningococcal cell surface during transformation while others bind DNA substrates during recombination and repair.
Conclusions: Our findings show that meningococcal DNA glycosylases clearly play a role in genome maintenance, alone and in their interactions with other DNA repair mechanisms[4]. The dynamics of genomic changes and genome maintenance/DNA repair affect the net outcome in terms of DNA sequence variability and conservation.

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SESSION IX

Vaccines (2)

Update on meningococcal C conjugate vaccination programme in England and Wales: coverage, herd immunity, vaccine efficacy, and validation of serological correlates

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Introduction: In November 1999, the UK introduced meningococcal group C conjugate (MCC) vaccines for children under 18 years of age. A total of 12 million children in England and Wales were eligible for immunisation and age groups were prioritised for receipt of the vaccine beginning with the 15 to 17 year olds in November 1999 and ending with 5 to 8 year olds by the end of October 2000.
Methods: Enhanced surveillance was introduced to accurately monitor disease incidence and identify possible cases of vaccine failure. Vaccination history was obtained for all group C meningococcal cases confirmed in targeted age groups. Efficacies estimated for up to 21 months of follow up using the screening method. Evidence of herd immunity was determined by comparing incidence of group C disease in unvaccinated individuals before and after the MCC campaign. To determine the serum bactericidal antibody titre (using baby rabbit complement) (rSBA) that indicates protection, the predicted vaccine efficacy estimates for various rSBA cut-offs were compared with the observed efficacy and its 95% confidence intervals (95% CI) as calculated using the screening method.
Results: By 30th June 2001 overall coverage of approximately 84.6% in children aged 5 years and over and 77.8% in those under 5 years of age had been achieved. Large reductions in group C disease have been observed in all vaccinated age groups. Up to 31/12/2001, 25 confirmed vaccine failures had been identified. Efficacy estimates for 21 months of follow up in England are; for under 1 yr (after 3 doses) 89% (95% CI 60% to 97%), 1 to 2 years 89% (73% to 96%), 11-14 yrs 95% (82% to 99%) and 15 to17 years 94% (83% to 98%). Percentage reduction attack rates in unimmunised individuals are; for 1-4 yrs 50%, 5-8yrs 57%, 9-14 yrs 34% and 15-17 yrs 61%. The rSBA titre correlating with protection was 1:8.
Conclusion: Based on the antibody persistence data from the clinical trials serum bactericidal antibody levels in the youngest age groups are currently declining to near baseline so vaccinees will be reliant on immunological memory for protection. However, efficacy estimates remain high in toddlers. Reductions in group C disease in unvaccinated individuals both in the cohorts targeted for immunisation and those over 20 yrs are consistent with herd immunity. Using the efficacy data we have now been able to determine the appropriate cut-off for the rSBA that is protective.

The effect of meningococcal C conjugate polysaccharide vaccine on the carriage of *Neisseria meningitidis*

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Introduction: The UK was the first country to introduce meningococcal serogroup C conjugate (MCC) vaccines. In an initial introduction phase of one year starting in November 1999, all individuals aged 18 years and under, approximately 15 million people, were offered MCC immunisation. An uptake rate of over 70% was achieved for this population by November 2000, resulting in a rapid decrease in disease caused by serogroup C meningococci in the vaccinated age groups. As routine surveillance would not measure the effects of the vaccine on the carriage of serogroup C meningococci, the United Kingdom Meningococcal Carriage Group was formed to investigate the nature of any such effect over the first three years after the vaccine introduction.
Materials and Methods: This multi-centre study was based around eight sampling centres: Glasgow, London, Nottingham, Oxford, Plymouth, Stockport and North Wales (Bangor) and South Wales (Cardiff). The study design comprised three annual cross-sectional surveys of school students aged 15-19 years of age. This group was chosen as they were one of the first cohorts to be offered vaccination, were accessible through the schools, and were likely to exhibit high rates of meningococcal carriage. Oropharyngeal swabs were collected from 15,206-19,755 (target 16,700) students in each of three successive years, the first sample being collected during vaccine administration in autumn 1999. The swabs were cultured by routine microbiological methods and sent to National Reference Laboratories for phenotypic confirmation of identity and serogrouping. Samples were prepared for genetic characterisation by MLST and molecular analysis of the capsular operon with high throughput techniques.
Results and Discussion: The following results were obtained: 1999, 15,206 students sampled, 2,809 meningococci isolated, carriage rate 18.4%; 2000, 18,273 students sampled, 3,410 meningococci isolated, carriage rate 18.7%; 2001, 19,755 students sampled 3,752 meningococci isolated, carriage rate 19.0%. Comparison of the phenotypes of carried meningococci isolated from 14,064 students aged 15-17 years during vaccination in 1999, with those obtained from 16,583 students of the same age surveyed one year later indicated a 66% reduction in the carriage of serogroup C meningococci ($p=0.004$) pre and post vaccine introduction. Data for 2001 is currently being collated and genetic characterisation of all meningococcal isolates obtained is currently in progress.
Conclusion: These results were consistent with MCC vaccines protecting against carriage of meningococci that express serogroup C polysaccharide capsules with a duration of at least one year. Further analysis of these samples will reveal if this protective effect lasts longer and will indicate whether immunisation has promoted a change in the genetic composition of carried meningococci in the UK.

Immunogenicity and reactogenicity of meningococcal group B OMV vaccine and meningococcal group C conjugate vaccine given in combination.

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Introduction: Most cases of meningococcal disease in Europe are caused by *N. meningitidis* serogroups B and C. To investigate the immunogenicity and reactogenicity of a combination of meningococcal serogroup B outer membrane vesicle (OMV) vaccine and meningococcal C conjugate vaccine, a single blind, randomised, prospective study in healthy adults has been peformed.
Materials and Methods: Healthy students 18-35 years old were randomized in a 1:1:2 ratio to meningococcal group B OMV (MenB) vaccine, meningococcal group C conjugate (MenC) vaccine, or the combination of MenB vaccine and MenC vaccine. The MenB vaccine (MenBvac, Norwegian Institute of Public Health) consists of outer membrane protein vesicles, prepared from a B:15:P1.7,16 meningococcal strain (44/76) and adsorbed to aluminumhydroxide. The MenC vaccine (Menjugate®, Chiron Vaccines) consists of meningococcal group C polysaccharide conjugated to CRM_{197}. In the combination group the lyophilized MenC vaccine was reconstituted with the MenB vaccine immediately before administration. Because three doses of MenB vaccine were given (6 weeks apart), a placebo vaccine containing aluminum hydroxide was given at injection two and three to the MenC vaccine group. Adverse events were monitored after each vaccination. Blood samples were obtained before each immunzation and 6 weeks after the last dose. Serum bactericidal antibodies (SBA) to *N. meningitidis* serogroup B (vaccine strain 44/76) and to *N. meningitidis* serogroup C and ELISA antibodies to C polysaccharide were measured.
Results and Discussion: 73 subjects were enrolled: 19 subjects in the MenB group, 19 subjects in the MenC group and and 35 subjects in the MenBC group. Approximately 60% of the subjects who received MenB or MenBC vaccine and 20% of the subjects who received MenC vaccine, reported local pain of moderate intensity on day 1. In total, 5-20% of the subjects experienced systemic reactions (mild nausea, weakness or feeling unwell on day 1). The adverse events were of short duration. 92% in the MenBC and MenB groups had serogroup B SBA titer ≥4 after three injections. Serogroup B SBA GMT were 28 (95%CI 16-49) in the MenBC group and 16 (95%CI 7-36) in the MenB group. 97% in the MenBC group and 89% in the MenC group had serogroup C SBA titer ≥8 after one injection. Serogroup C SBA GMT were 107 (95%CI 58-196) in the MenBC group and 120 (95%CI 52-273) in the MenC group.
Conclusion: A combined MenB OMV vaccine and MenC conjugate vaccine induced high levels of bactericidal antibodies against both serogroups which indicates that protection against meningococcal serogroup B and C diseases have been induced.

Attacking New Zealand's most urgent infectious disease issue: is a solution in sight?

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New Zealand is in the 11th year of a large and widespread epidemic of meningococcal disease (2001: 17.4/100,000) increasingly dominated by serogroup B meningococci with the subtype (P1.7b,4). To Dec 31, 2001, there have been 4195 cases and 185 deaths. Case numbers notified to public health in 2001 were the highest on record. Infants and children under 5 years (2001: 106/100,000) are most affected.

A "designer" approach to vaccine production to fit the NZ epidemic strain dominating the outbreak has been embarked on with the Norwegian Public Health Institute (NIPH) and Chiron Vaccines. To minimise delays for eventual epidemic control an attenuated clinical development plan has been formulated and peer reviewed internationally. This approach is based on the following assumptions:

- A comprehensively monitored and well characterised outbreak dominated by a single clone
- Demonstration in infants and young children of the ability to mount an homologous immunological response to a strain-specific vaccine
- Serum bactericidal antibody measurements are likely to reflect, as an under estimate, potential vaccine effectiveness.
- Proven efficacy of these vaccines in older children and adults.
- A large body of safety data with the NIPH vaccine and from similar vaccines in both public health and trial situations.
- A comprehensive and active approach in 'real time' to vaccine safety surveillance possible through public hospital database linkage to the national immunisation register. In addition, retrospective database matching, and continued general practice passive surveillance.
- Public Health legislation enabling "provisional consent" to distribute vaccines in response to an epidemic and in proportion to risk.
- Early delivery during the "pilot roll out" of vaccine use in the highest risk areas will be slow and incremental, to monitor for serious adverse events before proceeding with further vaccine use. A Data and Safety Monitoring Board will be in place.

Thus New Zealand's approach through a consortium of the Ministry of Health, Chiron Vaccines and a research team lead from the University of Auckland is to advance to vaccine licensure and epidemic control with vaccine effectiveness evaluation and enhanced national safety surveillance, following immunogenicity trials in adults (phase I/II), school children, toddlers & infants (phase II). Children at highest risk will be prioritised to receive vaccine. The first doses of N.Z strain vaccine were delivered on 30 May '02.

Development of a tailor-made outer membrane vesicle vaccine against the group B meningococcal epidemic in New Zealand

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Introduction: New Zealand (NZ) is now in the 11th year of a large widespread meningococcal disease epidemic caused by serogroup B strains. Case numbers in 2001 were the highest recorded since the start of the epidemic. The NZ outbreak is highly clonal, with an estimated 85% of disease cases caused by B:4:P1.7b,4 lineage III, meningococci. Without a vaccine intervention this outbreak could possibly continue for another five to eight years with potentially 4000 more cases, 200 deaths and perhaps as many as 600 permanently damaged, mostly young persons without underlying diseases.
Materials and Methods: An outer membrane vesicle (OMV) vaccine has been produced at NIPH, based on a "wild-type" meningococcal strain (NZ98/254, B:4:P1.7b,4), using essentially the same technology as for the Norwegian B:15:P1.7,16 vaccine ("MenBvac"), but with growth in a chemically defined medium. The vaccine strain was selected among recent epidemic strains from NZ. The NZ OMV vaccine has been characterised with respect to proteins and lipopolysaccharides (LPS) by SDS-PAGE and by reactions with monoclonal antibodies. The LPS content was determined by a HPLC method and vesicle morphology was studied by electron microscopy. Aluminium hydroxide was used as adjuvant and groups of mice were immunized s.c. The antibody responses in sera were analysed by ELISA and the specificity of the induced antibodies was analysed by immunoblotting. The functional antibody responses were analysed by measuring serum bactericidal activity, towards various MenB strains. Pyrogenicity and toxicity were studied in rabbits and guinea pigs.
Results and Conclusion: Characterisation of the NZ-OMV vaccine showed quite similar protein patterns and LPS concentrations as previously experienced in the development of "MenBvac". The major outer membrane proteins are the PorB and PorA. In addition the NZ-OMV vaccine contains the Opc and some minor membrane proteins and about 6% LPS, relative to protein. Compared to the parent vaccine "MenBvac", the NZ-OMV vaccine consist of more disrupted vesicles and outer membrane fragments. The antibody response in mice against the vaccine strain was significantly raised after immunization with this vaccine. The specificity of the induced antibodies was mainly directed against the PorB, PorA and Class 5 OMPs. Measuring the functional activity in the same sera revealed that serum bactericidal activity had been induced. There was no indication from the toxicological studies that the vaccine would pose a safety risk in humans. The pre-clinical data suggest that the reactogenicity and safety profile of the NZ-OMV will be similar to that of the parent vaccine. The NZ-OMV vaccine is being used in clinical studies in New Zealand, which started in mid-2002.

Structure-based design of subtype-specific peptide vaccines for *Neisseria meningitidis*

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Peptide vaccines can be based on linear epitopes of a pathogen. Outer membrane protein PorA contains two immunogenic, surface-exposed loops, which could be mimicked by a peptide. The conformational mimicry of a peptide-vaccine candidate is of utmost importance for eliciting a functional immune response. Based on the peptide conformation found in crystal structures, we designed small, cyclic peptides for subtypes P1.4 and P1.16, which are capable of eliciting a bactericidal immune response in mice.

The crystal structure[1] of a Fab-fragment of a bactericidal antibody against subtype P1.16 of *N. meningitidis* in complex with its epitope peptide shows that this immunogenic loop of PorA is recognized as a β-turn at residues ^{182}DTNNN^{186}. A peptide based on subtype P1.4 in a similar complex displays a β-turn at residues ^{182}NNKV^{185}. In our designed cyclic peptides the observed β-turns are stabilized by introducing a contra-turn at the other side of the peptide. The conformational stability of the peptides was analyzed *in computro* by molecular dynamics simulations and *in vitro* by surface plasmon resonance binding studies. This showed that of the four designed peptides for subtype P1.16 only one peptide had a reasonably stable β-turn conformation at the correct position. The molecular dynamics simulations did not yield a correct β-turn conformation for the two designed P1.4 peptides, though the observed conformations were reasonably similar.

Immunization experiments in mice using the designed cyclic peptides conjugated to tetanus toxoid as a carrier protein demonstrated that a bactericidal immune response can be elicited by peptides, which contain only six or seven amino acid residues of the cognate protein. The two subtype P1.4 peptide-conjugates elicited high bactericidal titers in some mice, but yielded no response in other mice of the same group. For subtype P1.16 only the peptide with the best conformational properties, as assessed by molecular dynamics and binding studies, is capable of eliciting a high bactericidal immune response in all mice. This response was even higher than the immune response elicited by the cognate protein.

These results indicate that developing peptide vaccines for neisserial subtypes is feasible, if structural information is available. Moreover, this approach could be applicable to many other antigens, especially antigens of β-barrels, which are often found to include ?-turn conformations.

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Cross-reactivity of antibodies against PorA after vaccination with a Men B outer membrane vesicle vaccine

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Introduction: Development of an effective vaccine against serogroup B meningococci is focussed on outer membrane proteins, especially porin A (PorA). Two immunogenic variable regions (VR) on loop1 and 4 of PorA encodes the serosubtype of a meningococcal strain. The RIVM has developed a monovalent outer membrane vesicle (OMV) vaccine against serosubtype P1.7-2,4 (MonoMen) as well as a multivalent vaccine containing 6 different serosubtypes (HexaMen). Antibodies induced by these vaccines are mainly PorA-specific. PorA is a highly variable protein and little is known about the immunodominance of the different VRs and the cross-reactivity of antibodies induced by these vaccines against heterologous strains. This knowledge is important for prediction of the level of protection conferred by vaccines based upon PorA. The cross-reactivity of PorA-specific antibodies induced by MonoMen and/or HexaMen in children was studied.
Materials and Methods: Serum samples of toddlers or school children vaccinated with HexaMen, MonoMen or both were used in serum bactericidal assays (SBA). Besides the isogenic vaccine strains and PorA-identical wild type strains, several patient isolates were used as a target in SBA, varying either single amino acids, a complete VR or having a different combination of VRs compared to vaccine serosubtypes. Expression of PorA, Opa, Opc and LPS phenotype were checked using whole cell enzyme-linked immunosorbent assay (ELISA).
Results and Discussion: Geometric Mean Titers (GMT) in SBA against wild type strains with subtypes P1.5-2,10 and P1.5-1,2-2 after vaccination with HexaMen were generally lower than those against the isogenic strains with the same serosubtype, but the percentage vaccine responders (=> 4-fold increase in SBA after vaccination) was not affected. Interestingly, using various wild type P1.7-2,4 strains GMT as well as the amount of vaccine responders was even higher, indicating that the isogenic P1.7-2,4 strain might have underestimated the immunogenicity of this serosubtype in HexaMen. MonoMen induced P1.4 specific antibodies which are cross-reactive with P1.4 variants like P1.4-1 and P1.4-3. HexaMen induced a broader cross-reactive antibody response against various strains with one VR equal to a vaccine-subtype or a combination of VRs included in HexaMen. Cross-reactivity against heterologous strains ranged from 23 to 92 % depending on the subtype of the tested strain and was mainly directed against VR2.
Conclusions: Using isogenic vaccine strains as a target to measure SBA titers is in general representative for wild type isolates of the same serosubtype, although the immunogenicity of P1.7-2,4 in HexaMen appears to be underestimated.

The monovalent P1.7-2,4 OMV vaccine induces antibodies mainly against VR2 which are cross-reactive with all tested variants of P1.4. HexaMen induces cross-reactive antibodies against vaccine subtypes as well as against VR variant strains and strains containing (combinations of) VRs not present in the vaccine.

A novel approach for eliciting broadly protective antibodies to *Neisseria meningitidis* group B strains

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Introduction: The capsular polysaccharide of *N. meningitidis* group B is an auto-antigen, while noncapsular antigens are highly variable. These present formidable challenges for development of a broadly protective and safe group B vaccine.
Methods: Mice and guinea pigs were sequentially immunized with three doses of microvesicles or outer membrane vesicles prepared from three meningococcal strains that were each antigenically heterologous with respect to the two major porin proteins, PorA and PorB, and capsular groups. Control animals were given three injections of a mixture of the three vesicle preparations, or vesicles prepared from *E. coli.*
Results: Pooled antiserum from guinea pigs (N=8) given sequential immunization was bactericidal (titer ≥1:8) against 9 of 10 group B strains tested that expressed PorA/PorB that were heterologous to those of the three strains used to prepare the vaccines. In contrast, the antiserum from guinea pigs (N=3) immunized with the mixture was bactericidal against only 2 of 10 heterologous strains (P=0.01). Similarly, antiserum from mice given the sequential immunization (N=7) was bactericidal against 7 of 10 heterologous strains compared with 2 of 10 strains for mice (N=7) given a mixture of vesicles (P<0.05). In both mice and guinea pigs, the sequential and mixture immunizations elicited bactericidal antibody against group B strains with PorA/B that were homologous to those of the strains used to prepare the vaccines (titers of ≥1:8 for 10 of 10 strains tested). Antisera from control animals given *E. coli* vesicles were not bactericidal against any strain (titer <1:4). Antisera from animals given sequential vesicle immunization had 5-fold higher ELISA titers (>1:50,000) measured against recombinant Neisserial surface protein A (NspA) as compared to that of animals immunized with the mixture of vesicles. In addition, the bactericidal titer of pooled antiserum from guinea pigs given sequential immunization decreased ~10-fold when tested against a NspA-knockout strain with heterologous PorA/B but was unchanged (i.e., same as for wildtype) when tested with the corresponding PorA-deficient strain. Finally, an anti-NspA mAb isolated from a mouse given sequential immunization was bactericidal against strains previously shown to be resistant to bacteriolysis by polyclonal or anti-NspA mAbs produced against recombinant NspA (Moe et al. Infect Immun. 2001, 69:3762). The new anti-NspA mAb also conferred passive protection against bacteremia in infant rats challenged with group B strain M986, whereas no protection was observed with mouse polyclonal or mAb antibodies prepared to recombinant NspA.
Conclusions: i. Immunization with membrane vesicles prepared from a mixture of three strains elicits predominantly strain-specific bactericidal antibody responses. ii. Sequential immunization with vesicle preparations from heterologous strains offers a novel approach to eliciting broader protective antibody. iii. Antibodies elicited by NspA expressed in Neisserial vesicles may be more broadly reactive than antibodies elicited by rNspA.

POSTER SESSIONS

SESSION I

Genome and gene regulation

Expression of gonococcal Fur regulated genes in response to iron

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An *in silico* analysis of the Neisseria genome indicates that a number of gonococcal genes have the potential to be regulated by the ferric uptake regulatory protein, Fur. These genes contain a Fur consensus binding sequence in their promoter region and electromobility shift assays performed in our laboratory have shown that Fur binds to the promoter regions of a subset of these genes including *rec*N, *sec*Y, *ton*B and *fur*. To study the expression of these genes in response to iron, we constructed transcriptional fusions with the green fluorescent protein (GFP) from *Aequorea victoria*. The promoter region of the *ton*B and *fur* genes which spanned the –10 and –35 regions, and the putative Fur box region were used to construct GFP fusions in *Neisseria gonorrhoeae*. The promoter region of *rmp*, a constitutively expressed gene was utilized as a positive control. These fusions were constructed upstream of the promoterless GFP in pLES 99, a suicide vector for Neisseria, and subsequently transformed into *N. gonorrhoeae* strain F62. Sequencing, PCR and fluorescent microscopy confirmed the proper insertion of the fusions in the gonococcal chromosome. Gonococcal fusions were grown in iron-replete and iron-deplete conditions and growth monitored over a 4 hour period. Differential activity of the promoters was examined by FACS analysis. Gonococcal strains containing the 126 bp promoter region of the *fur* gene fused to GFP demonstrated a 1.7 fold increase in the first hour of growth in iron-deplete conditions as compared to iron-replete conditions and they increased to nearly 4 fold in the fourth hour. Similarly, a 2-fold increase was observed in the gonococcal strain containing the 91 bp *tonB* promoter region fused to GFP during growth in iron-deplete condition as compared to iron-replete conditions. The gonococcal strain containing the *rmp* promoter fused to GFP showed no change in fluorescence during growth in iron-replete versus iron deplete conditions. These results were further confirmed by RT-PCR analysis using internal fragments of the *fur*, *ton*B and *rmp* genes. These results demonstrate that the gonococcal *ton*B and *fur* genes are regulated by iron.

Expression of gonococcal iron regulated genes during natural gonococcal infection

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Iron is limiting in the human host and bacterial pathogens respond to this environment by activating genes required for bacterial virulence expression. Most *in vitro* experiments are designed to determine the response of the gonococcus to an absolute unavailability of exogenous iron. However, *in vivo* if gonococci were able to use iron bound to lactoferrin, transferrin, hemin, or hemoglobin, they might not be exposed to such severe iron limitation. We describe here studies to examine the expression of gonococcal iron and Fur regulated genes during human mucosal gonococcal infection. Specimens were collected from patients with uncomplicated mucosal gonoccocal infection from the Public Health Clinics at Boston Medical Center and the Medical University at South Carolina. Urethral swabs from male patients and cervical swabs from female patients were placed in a TRIZOL reagent and total RNA isolated. The isolated bacterial RNA was then used as a template to amplify by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction), gonococcal genes known to be regulated by iron and Fur (*fbp*A, *tbp*B and *fur*). The constitutively expressed *rmp* gene was used as a positive control. The minimal amount of bacterial RNA required for detection of transcripts by RT-PCR was determined to be 20 ng. RT-PCR analysis indicated that of 30 *Neisseria gonorrhoeae* culture positive samples, 25 (83%) were *rmp* positive. Of these 25 samples, 20 (66.7%) were positive for both the *fbp*A and *tbp*B genes. In addition, we detected a *fur* transcript in 58% of the gonococcal positive cultures. Humoral immune responses to these gonococcal iron-regulated proteins from the gonococcal positive patients was also examined. We observed an increased IgG response to TbpB and FbpA antigens in sera of patients which expressed *tbp*B and *fbp*A transcripts. These results indicate that gonococcal iron and Fur regulated genes are expressed during natural gonococcal infection. Furthermore, these patients mount an antibody response to these proteins.

Bacteriocin secretion by *Neisseria meningitidis* is correlated to repeat-associated phase variation within a gene homologous to the ABC transporter *cvaB* of colicin V

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Screening of Norwegian meningococcal isolates collected in different geographic regions and periods reveal that spontaneous release of meningococcal bacteriocins generally occurs at a frequency of 2-3%. However, among systemic meningococcal isolates collected during the first six months of 1975 in North Norway, *i.e* around the peak of incidence of disease in the Norwegian epidemic, 13.5 % expressed bacteriocin production. Nonproducing serogroup A sulfonamide-resistant (Su ^{R}) isolates from that period were distinctly more sensitive towards bacteriocins than were serogroup B. The serogroup B Su ^{R} strains became predominant among meningococci causing disease in Norway from that time on (1), indicating that bacteriocins have a determinant role in meningococcal epidemiology (*loc. cit.*). The bacteriocins are encoded on the chromosomes and are not induced upon treatment of the cultures with genotoxic agents. Bacteriocin is released during growth and maximum extracellular activity is obtained late in the logaritmic growth phase (2,3). The genome sequencing project for the serogroup B strain MC58 genome has identified three putative islands of horizontally transferred DNA (IHTs) (4). One of these, IHT - A2, contains two disrupted open reading frames with homology to the colicin V ABC transporter *cvaB* and the membrane fusion protein *cvaA*. We have cloned the *cvaB* sequence from the producer strain BT878 and inserted a kanamycin resistance casette. Homologous transformation of the producer with the disrupted gene yields nonproducing, kanamycin-resistant clones. The expression of the *cvaB* homologous gene in MC58 is predicted to be the subject to phase variation (5). In strain MC58 this gene contains an instable G-7 tract, leading to a premature termination of the reading frame. We found the G-7 tract also in the resistant nonproducer 44/76, whereas the producer BT878 instead has G-5 which changes the reading frame yielding an intact ABC transporter gene. We conclude that expression of bacteriocin in the meningococcus is subject to repeat-associated phase variation.

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Yet another *Neisseria meningitidis* genome sequence – serogroup C FAM18

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The genome sequence of a third representative of *Neisseria meningitidis* has now been completed at the Sanger Institute Pathogen Sequencing unit. The sequence is from FAM18, a serogroup C ET-37 complex strain isolated in North Carolina in the 1980s.

The sequence is 2,194,961 bp long, with a G+C content of 51.62%; and the raw sequence data is available from http://www.sanger.ac.uk/Projects/N_meningitidis/seroC.shtml.

We are now in the process of analysing and annotating the genome, and the salient features, along with the latest results from the analysis and comparison with the genomic sequences of MC58 (serogroup B) and Z2491 (serogroup A), will be presented.

Characterization of a nitroreductase-based positive selection system to measure spontaneous mutation frequencies in *Neisseria gonorrhoeae*

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The modified DNA base 5-methylcytosine (5-MC) can spontaneously deaminate to thymine. Although *Neisseria gonorrhoeae* has a high percentage (5%) of this base in its genome, the organism appears to have a low frequency of spontaneous mutation. We are developing a positive selection system that will allow us to study spontaneous mutation due to deamination of 5-MC. Organisms that are sensitive to the antimicrobial agent nitrofurantoin express nitroreductases, which reduce nitrofurantoin. We are characterizing a system based on nitrofurantoin resistance to measure spontaneous mutation frequencies in *N. gonorrhoeae*. To determine if the gonococcus expresses a nitroreductase, cell extracts from an overnight culture of *N. gonorrhoeae* were obtained; nitroreductase activity in the cell extract was found by measuring a loss of absorbance at a wavelength (l) of 365nm in a solution containing cell extract and nitrofurazone, a compound similar to nitrofurantoin. This indicates that the gonococcus expresses a nitroreductase. To identify possible nitroreductase coding regions in the gonococcal genome, a BLAST search of the *N. gonorrhoeae* FA1090 genome was performed using the sequences for the *Escherichia coli* nitroreductase genes, *nfsA* and *nfsB*. A sequence was found with some similarity (25% identity, 42% similarity, E value = 2×10^{-11}) to *nfsB*. A region containing the gonococcal *nfsB* homolog was amplified using the polymerase chain reaction (PCR), and cloned. To determine the spontaneous mutation frequency and minimum inhibitory concentration (MIC) for nitrofurantoin, known quantities of cells of *N. gonorrhoeae* were plated on media containing various concentrations of nitrofurantoin. This assay was performed for various strains of *N. gonorrhoeae*. The data indicate a MIC of ~4 mg/ml and a spontaneous mutation frequency of ~1 in 10^8. The presence of two nitroreductase genes in *E. coli* allows for secondary mutations leading to high-level resistance to nitrofurantoin. To determine if secondary mutations in the gonococcus were possible, primary nitrofurantoin resistant mutants were plated on higher concentrations of nitrofurantoin; no secondary mutations were observed. Using the PCR, the sequence of the gonococcal *nfsB* homolog from various nitrofurantoin-resistant spontaneous mutants was amplified, and the DNA sequences of the amplicons were determined. Both frameshift and point mutations were found. Two nitrofurantoin-resistant mutants and the corresponding wild-type parental strain were analyzed for nitroreductase activity using the biochemical assay. Nitrofurantoin resistant mutants did not express detectable nitroreductase activity. These data indicate that nitrofurantoin resistance can be used to measure spontaneous mutation. In addition, coding sequence of the gonococcal *nfsB* homolog can be modified to direct mutations into 5-MC sites to determine if 5-MC has an effect on spontaneous mutation frequency in the gonococcus.

Role of genes encoded in the gonococcal genetic island in peptidoglycan hydrolysis and peptidoglycan-derived cytotoxin production

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Peptidoglycan (PG) is an important virulence factor in infections caused by *Neisseria gonorrhoeae*. Gonococci have a high rate of PG turnover, and PG fragments generated during growth and division are released into the surrounding milieu. The major fragment released is the 1,6 anhydro-disaccharide tetrapeptide monomer, a 921 Da molecule that has multiple biological effects and is referred to as peptidoglycan-derived cytotoxin (PG-cytotoxin). PG-cytotoxin kills ciliated fallopian tube cells in organ culture, induces IL-1 and IL-6 production and induces arthritis. *N. gonorrhoeae* strains encode up to six putative lytic PG transglycosylases that we have identified using sequence comparisons. These enzymes are predicted to have the enzymatic activity necessary to hydrolyze macromolecular PG and generate PG-cytotoxin. Interestingly, one of the lytic transglycosylases (AtlA) is encoded in a variable section of the Gonococcal Genetic Island (GGI), a region present in most, but not all, strains of *N. gonorrhoeae*. In the limited number of strains we have tested, PG turnover is substantially increased is GGI^+ strains compared to GGI^- strains. We are characterizing the roles of the various gonococcal lytic transglycosylases in PG-cytotoxin production and investigating the possibility that expression of *atlA* or other genes in the GGI results in increased PG-cytotoxin production. We have made mutations in genes encoding three lytic transglycosylases, *atlA* (encoded in the GGI) and *ltgA* and *ltgB* (encoded in the conserved gonococcal chromosome). Mutation of *atlA* or *ltgA* results in lowered PG-cytotoxin production. However, *ltgA* mutants release more multimeric PG fragments compared to wild type while *atlA* mutants do not. *ltgA* and *atlA* mutants are lysis deficient when suspended in buffer. However, *ltgA* mutants exhibit this phenotype at pH 8 whereas *atlA* mutants exhibit this phenotype at pH 6. These results suggest that AtlA and LtgA act in PG-cytotoxin production but may have different substrate specificities and act under different conditions. Further characterization of these and other genes will determine the role of the GGI in PG-cytotoxin production.

Meningococcal genogroup determination using Luminex xMAP flow-analysis technology

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Introduction: Luminex xMAP flow analysis technology allows the simultaneous detection of up to 100 different PCR amplified gene targets in a single tube. This is a major advantage over conventional hybridisation reactions, which require an individual reaction for each gene target analysed. One oligonucleotide primer is labelled with biotin, which is incorporated into the amplicon during the PCR reaction. A fluorescent dye labelled conjugate (streptavidin R-phycoerythrin) is hybridised to the biotin labelled PCR product. The mixture is injected into the Luminex 100 instrument, which aligns the microspheres in single file. Lasers illuminate the colours inside and on the surface of each microsphere with optics capturing the colour signals in real-time. The mean fluorescent intensity (MFI) of the reporter is used to measure the number of molecules attached to the microspheres.
Materials and Methods: An oligonucleotide based direct hybridisation assay, the *Neisseria meningitidis* serogroup specific PCR assays targeting the *siaD* gene were tailored to xMAP technology. Serogroup specific oligonucleotide probes with a C12 linker (B, C, Y, W135) were conjugated to four different microsphere sets with different colour-codes. The probe concentration and hybridisation temperatures for each target were optimised, then as single and multiplex assays the specificity determined and the sensitivity compared using titrated meningococcal DNA. Using DNA extracts of clinical samples, the multiplex assay was compared with gel-based detection and existing 'real-time' TaqMan™ based assays.
Results and Discussion: The same level of sensitivity was achieved for single and multiplex genogrouping assays using titrated meningococcal DNA. The optimal probe concentration was 5 µM for groups B and Y and 10 µM for groups C and W135 and the optimal hybridisation temperature was 43 °C. When the microsphere sets were combined in multiplex, the sensitivity of the Luminex xMAP assay was found to be equivalent to gel-based and ABI7700 TaqMan™ based detection methods. The primer and probe sets were specific for *N. meningtidis* when tested against a range of bacteria and viruses. Multiplexing the bead sets did not affect the sensitivity of the assay. The concentration of probe conjugated to the sphere and the hybridisation signal are crucial to maximising the MFI signal. The sensitivity of the genogrouping assay was equivalent to a threshold cycle (C_T) value of 36 in the TaqMan™ assay, which is nearing the end point of detection.
Conclusion: It is possible to develop sensitive and specific multiplexed genogrouping assays, which have the same level of sensitivity as 'real-time' assays using Luminex xMAP technology.

Sequence and mutational analysis of the gonococcal genetic island

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Approximately 80% of gonococcal strains contain a genetic island, a large region of the chromosome that is not found in other gonococcal strains or in other neisseria species. Genetic islands in other bacteria are often composites of horizontally acquired genetic information from multiple sources and may contain pieces of mobile genetic elements such as plasmids or phages. In bacterial pathogens genetic islands frequently encode pathogenesis functions such as adhesins, toxins, or secretion systems.

To investigate the functions encoded in the gonococcal genetic island (GGI) we have cloned and sequenced this 57 kb region from the chromosome of *N. gonorrhoeae* strain MS11. Consistent with our earlier findings, the complete sequence of the GGI indicates that the GGI was likely acquired by horizontal transfer. It has a low G+C content and contains only 5 copies of the DNA uptake sequence. The GGI is flanked by two copies of a 20 bp sequence identical to the att site of a *Xanthomonas campestris* bacteriophage and highly similar to the *dif* sites of *Escherichia coli* and *Haemophilus influenzae*, sites recognized by the chromosomal separation site-specific recombinases XerCD. This result suggests that the GGI may have inserted into the chromosome by a site-specific recombinase, possibly the bacterium's own XerCD. The most striking characteristic of the GGI sequence is the presence of multiple homologues of type IV secretion genes. The type IV secretion genes are clustered together and make up approximately half of the GGI. The other half of the GGI contains a few genes with similarity to those of conjugative or virulence plasmids of other bacteria and many genes with no significant similarity to published sequences. Type IV secretion systems in other bacteria secrete DNA and/or proteins. We have made mutations in eleven putative type IV secretion system genes and tested the mutants for secretion of DNA into the culture medium. Each of the mutations resulted in loss of DNA secretion into the medium, whereas mutations in genes not homologous to type IV secretion genes or mutations between genes had no effect on DNA secretion. Two of the mutants were tested and found to be deficient in the ability to donate a chromosomal antibiotic resistance marker in coculture transformation. These data suggest that the GGI-encoded type IV secretion system is important for genetic transformation. In addition to determining the characteristics of the secreted DNA, we are searching for other factors secreted by the type IV secretion system and investigating the possibility that the secretion system may play a role in gonococcal pathogenesis.

The use of DNA microarray to identify neisserial genes transcribed using alternative sigma factors

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In addition to the main sigma factor that recognises conventional promoter sequences many bacteria produce alternative sigma factors that recognise distinct promoter sequences. Alterations to the level or activity of an alternative sigma factor provide the opportunity to coordinately regulate the expression of a set of genes. In *Neisseria gonorrhoeae* and *Neisseria meningitidis* two putative alternative sigma factors have been identified. *ecf* is a member of a family of sigma factors that typically respond to extra-cytoplasmic stimuli, while *rpoH* (σ^{32}) is involved in the general stress response. An *ecf* mutant has been constructed in *N. gonorrhoeae* and *N. meningitidis* by insertion of a nonpolar kanamycin cassette. No altered growth phenotype was observed for these mutants. Gene expression in the *ecf* mutant was compared to that of the wild type using the pan-*Neisseria* DNA microarray. A considerable number of genes that had an association with cell surface, lipooligosaccaride biosynthesis and periplasmic or membrane proteins involved in cation binding and transport were down regulated in the *ecf* mutants. These data appear to be consistent with the phenotype observed in other species.

Mutations in *rpoH* appear to be lethal in both *N. gonorrhoeae* and *N.meningitidis*. To overcome this problem we have constructed an expression vector in which a copy of wild type *rpoH* has been placed under the transcriptional control of a P_{tac} promoter and $lacI^q$. This construct is designed to recombine into the *iga* locus. When IPTG is added to the growth medium, overexpression should saturate the usual chaperone mediated suppression of RpoH activity. DNA microarrys will be used to monitor the increase of transcription of genes in the *rpoH* regulon.

Homology constraints in the homeologous pilin system

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Homology drives recombination due to it being an isoenergetic process. Therefore, a conundrum exists with the gonococcal pilin system because the RecA-dependent homologous *pilE/pilS* recombination reaction exchanges homeologous sequences (i.e. *pil* homologues that have noticably diverged in their sequence similarity). Two recent studies have reported that markers placed downstream of the *pilE* locus apparently coconvert with a pilus switch, which would indicate that recombination following a *pilE/pilS* interaction is not constrained to exchanges that simply occur within *pilE* itself. The results presented in this study further extend these observations and demonstrate that coconversion of downstream markers is indeed a frequent occurance that is dependent on RecA protein but is independent of DNA transformation. In contrast, markers placed upstream of *pilE* do not convert. Evidence is also presented that shows that a RecA-dependent recombination tract can be terminated within *pilE* by as little as a four base pair mismatch. In light of these observations, the various pilin models are discussed.

Transcriptome analysis of *Neisseria meningitidis* during different key steps of infection

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During the course of infection, *Neisseria meningitidis* encounters multiple environments within its host, which makes rapid adaptation to environmental changes a crucial factor for neisserial pathogenicity.

Previously employed oligonucleotide-based microarrays (Guckenberger et al., J Bacteriol 2002, 184) encompassing the entire genome of *N. meningitidis* were used as a technology platform to analyse transcriptional changes in meningococci serogroup B during two different stages of infection: the interaction with human nasopharyngeal epithelial cells and brain microvascular endothelial cells. Quantitative Real-Time RT-PCR was used as an independent method to confirm the differential regulation of selected open reading frames (ORFs).

The analysis of *N. meningitidis* interaction with human host cells identified 72 ORFs as differentially transcribed after contact with epithelial cells and 48 ORFs as differentially regulated after contact with endothelial cells. These differentially regulated genes could be clustered in several categories, i. e. genes involved in protein biosynthesis, metabolism, transcription and translation and genes encoding membrane proteins and transporters. Several of the upregulated ORFs are known to contribute to meningococcal virulence. While most well-known virulence genes, which were differentially regulated in bacteria adherent to endothelial cells were also upregulated upon epithelial cell contact, we identified several genes as being induced only after contact to epithelial cells, for example most iron-regulated genes, genes involved in pilus and capsule synthesis and iga. A high portion of the transcriptional deregulated ORFs of both model systems had no known function, as dedicated so far. Interestingly, 5 hypothetical ORFs encode genes with predicted transmembrane regions.

The data obtained with this novel approach may provide insights into pathogenicity mechanisms of *N. meningitidis* and could demonstrate the importance of gene regulation on the transcriptional level during different stages of meningococcal infection. Moreover, the differentially expressed genes identified in this study may serve as a basis for further studies in vaccine-development.

Sequences in the 5' region of the *pilE* gene of *Neisseria gonorrhoeae* are important for efficient antigenic variation

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Neisseria gonorrhoeae is an obligate human pathogen that expresses multiple virulence factors to effectively infect and survive in its host. One of these virulence factors, the pilus, plays a critical role in the establishment of infection. Gonococcal pili are filamentous surface appendages primarily composed of pilin protein monomers. These monomers can undergo antigenic variation leading to antigenically distinct pili. Antigenic variation at *pilE* occurs through a unidirectional, homologous recombination event between a *pilS* silent copy and the *pilE* expression locus. The *pilE* expression locus contains two highly conserved cysteine-containing sequences called *cys1* and *cys2*. It has been shown that proper spacing between *cys1* and *cys2*, as well as the specific sequence of *cys2* are important for efficient pilin antigenic variation. In addition, the *recA*, *recX*, *recO*, *recQ*, *recJ*, and *rdgC* genes have been shown to be involved in this process.

To determine the role of sequences upstream of *pilE* in pilin antigenic variation, either a 3kb IPTG-regulatable cassette or a 0.6kb promoterless *cat* cassette was inserted between the *pilE* promoter and coding sequence. Both insertions significantly disrupted the frequency of antigenic variation to different levels. However, similar insertions introduced ~0.5kb upstream of the *pilE* promoter do not affect the frequency of antigenic variation. Currently, various insertion mutants are being constructed and analyzed to determine the exact nature of this upstream sequence requirement for the variation at *pilE*. Completion of these experiments will determine if specific sequences, specific spacing of sequences, or an intact *pilE* promoter are required for efficient antigenic variation.

Negative regulation of the *farAB*-encoded efflux pump system in *Neisseria gonorrhoeae* due to *cis*- and *trans*-acting factors

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Introduction: Gonococci often infect mucosal sites bathed in antimicrobial fatty acids (FAs). An important factor in gonoccocal resistance to FAs is the presence of an efflux pump encoded by the *farAB* operon. The FarA-FarB proteins were previously shown by us to be similar to the EmrA-EmrB efflux pump proteins of *E. coli*. Expression of the *emrAB* operon is regulated by a transcriptional repressor encoded by *emrR*, which is positioned just upstream of *emrAB*. Our studies failed to detect an *emrR*-like sequence adjacent to *farAB*. Accordingly, we sought to define the mechanism(s) by which *farAB* expression is regulated.
Materials and Methods: We used *farA-lacZ* reporter fusions, promoter deletions, RT-PCR, cloning and expression of *farR*, FarR purification, and gel mobility shift assays to conduct our studies.
Results and Discussion: Using promoter deletion studies, we identified the presence of a *cis*-acting regulatory element. This element is located in the nucleotide sequence –112 to –149 upstream from the translational start of *farA*. Results from the *farA-lacZ* fusion experiments revealed that this sequence negatively controls expression of the *farAB* operon. Furthermore, the results of the promoter deletion and RT-PCR experiments revealed the presence of two sigma-70 type promoters. Interestingly, one of these promoters overlapped the –112 to –149 sequence. Through the analysis of the gonococcal genome sequence database, a novel regulatory protein belonging to MarR family of transcriptional repressors was identified. Insertional inactivation of the gene encoding this MarR-like protein in gonococci resulted in enhanced expression of *farAB*. However, deletion of nucleotides –112 to-149 in this mutant did not affect the level of *farAB* expression, suggesting that the promoter located 30 nucleotides upstream of *farAB* was responsible for directing transcription. These results also indicated that the repressive activity of the –112 to –149 sequence depends on a MarR-like protein. We therefore designated this protein as FarR to signify its role in regulation of *farAB* expression. FarR was purified as a C-terminal histidine-tagged protein by nickel chelate affinity chromatography. Gel mobility shift analysis revealed that FarR directly binds to the –112 to -149 sequence and the nucleotide sequence bearing the promoter region located 30 nucleotides upstream of *farA*.
Conclusion: Taken together, our results strongly suggest that FarR acts as a repressor of the *farAB* operon by directly binding to the –112 to-149 sequence. This activity is of likely importance in modulating *farAB* expression and FA-resistance in gonococci.

The REP2 repeats of the meningococcal chromosome are CREN

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Interaction with host cells is essential in meningococcal pathogenesis especially at the blood brain barrier. This step is likely to involve a common regulatory pathways allowing coordinate regulation of genes necessary for adhesion, invasion and transcytosis through the endothelial cells. In other medically important bacteria, this is often mediated by common regulatory elements or promoter regions. The analysis of the genomic sequence of *Neisseria meningitidis* Z2491 has shown the presence of many repeated sequences. One of these sequences, designated REP 2, is found multiple times in the chromosome, contains a ribosome binding site 5 to 13 bp before an ATG, presents a –24/-12 type promoter and presents the particularity of being in the majority of case immediately upstream of an ORF. Among these are genes implicated in the virulence of the bacteria such as *pilC1* and *crgA*. The copies of REP 2 upstream of *pilC1* and *crgA* have been characterised as a promoter which is induced by contact with human cells and which have been designated CREN (Contact Regulatory Element of *Neisseria*). This characteristic led to the hypothesis that REP 2 may function as an inducible promoter of a variety of genes, and that these repeats could control a regulon necessary for the efficient interaction with host cells. Quantitative PCR in real time confirmed that these elements are indeed Contact Regulatory Elements. Surprisingly subsequent mutagenesis of all the genes located downstream of these CREN demonstrated that onty PilC1 is involved in the adhesion stage.

The role of UP elements and IHF in the transcriptional regulation of the *pilE* gene of *Neisseria gonorrhoeae*

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In addition to conserved –35 and –10 boxes, AT-rich sequences upstream of a promoter may be required for optimal transcription. These AT-rich regions, termed UP (upstream) elements, act as binding sites for the a subunits (RpoA) of RNA polymerase (RNAP). The UP elements consist of distal and proximal subsites, which do not have to be adjacent, and are usually found just upstream of promoter sequences. Footprinting experiments indicate DNA protection by RpoA from approximately –38 to –60 relative to the transcription start point (tsp). It has been shown that the closer an UP element subsite is to the –35 box and to the consensus sequence, the greater the increase in transcription levels. Although genomic sequence data suggests that they are relatively common, few promoters containing UP elements have been investigated to date. It has also been found, *in vitro*, that transcription levels from specific promoters can be boosted in the presence of integration host factor (IHF), a histone-like DNA bending protein. In some instances, this activation does not require additional activator proteins. One mechanism by which this is achieved is via an IHF-induced bend that enables RpoA to interact with an UP element positioned further upstream (-80 to –100).

The *pilE* gene in *Neisseria gonorrhoeae* (Ng) encodes the subunit of the type 4 pili. Upstream of *pilE*, two potential UP elements and a confirmed IHF binding site (IHFBS) (Hill *et al.*, 1998) have been identified. In contrast to previously characterised systems, we have shown in footprinting experiments that gonococcal RpoA protects an upstream region (approximately –40 to –100) which spans both of the UP elements. We propose these entities form part of what we have termed a 'shift-UP' promoter. We propose that IHF may allow RNAP to shift from using a relatively weak UP element (immediately upstream of the promoter) to a stronger UP element, upstream of the IHFBS. If the 'shift-UP' promoter proposal for increased levels of *pilE* transcription is correct, then phasing between the IHF binding site and promoter will be important, as IHF would have a direct role in promoting interaction between RpoA and the UP elements. Experiments to confirm this are underway. As IHF levels seem to directly determine the level of transcription of *pilE*, we are also investigating the transcriptional regulation of the *ihfA* and *ihfB* genes.

Iron and Fur mediated transcriptional tegulation of gonococcal Fur

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Regulation of iron-regulated genes in several gram-negative pathogens has been postulated to occur via a ferric uptake regulator (Fur) mechanism. Fur functions as a global regulator and controls the expression of not only genes required for iron transport but also genes required for virulence. In this study we have defined the operator region of *Neisseria gonorrhoeae fur* by DNase I foot printing using purified gonococcal Fur and have determined the levels of *fur* transcript and protein from gonococcal cultures grown under iron-deplete and iron-replete conditions. The *N. gonorrhoeae fur* promoter region contains overlapping Fur boxes, which exhibits a 68% and 57% identity respectively to the *E. coli* (Ec) and gonococcal Fur (Gc) consensus binding sequence. Electrophoretic mobility shift assays confirmed the ability of both the Fur homologs (Ec-Fur and Gc-Fur) to bind to the gonococcal *fur* operator. DNase I protection experiments using a 356-bp restriction fragment encompassing the promoter region and the putative iron boxes of the *fur* promoter identified an apparent protected zone at 230 nM Fur and a clear zone of protection (46 bp) at 460 nM Fur. As calculated from the DNA size markers, the protected region covered the –10 promoter motif, a –35 region, and the *in silico* identified Fur box which overlaps the –10 region. The extent of the *fur* operator sequence (46 bp) as defined by our foot printing analysis, would suggest the binding of two repressor dimers.

RT-PCR experiments to examine the levels of the *fur* transcript in gonococcal cultures grown under iron-deplete and –replete conditions indicated that *fur* mRNA was detected at low levels in RNA extracted from gonococci grown under iron-replete conditions but at much higher levels in samples obtained from cultures grown under iron-deplete conditions. To correlate transcription of *fur* with expression, the presence of Fur protein was determined by Western blot analysis using gonococcal Fur specific antiserum from samples removed at 1-h intervals from gonococcal cultures grown under iron-replete and –deplete conditions over a 5 h growth period. Our results indicate that cultures grown under iron-restricted conditions produced Fur throughout the entire growth cycle. With same concentration of total protein however, Fur expression was moderately repressed in cell lysates from cultures grown under iron-sufficient conditions. Our *in vitro* results together with the growth studies suggest that in *N. gonorrhoeae,* the *fur* promoter is under auto regulation by Fur and iron.

Localization and differential expression of the neisserial LOS specific α-2, 3-sialyltransferase

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Pathogenic Neisseria sialylate their lipooligosaccharide (LOS) with an a-2,3-sialyltransferase (Lst) that can protect them from complement mediated killing. Lst is a 43 kDa membrane protein that is constitutively expressed. However, the membrane localization of Lst has not been determined. Although *lst* is constitutively expressed, *Ng* strains generally express more sialyltransferase activity than *Nm* strains. Here, we extend the characterization of Lst by determining its subcellular location and present a mechanism of transcriptional control responsible for differential *lst* expression. Western analysis of subcellular fractions of *Ng* F62 and *Nm* MC58¢3 using Lst-specific antiserum localized the 43 kDa Lst exclusively to outer membrane preparations. Outer membrane fractions of *Ng* were also enriched for sialyltransferase activity, substantiating our Western results. Furthermore, anti-Lst antibodies bound to whole *Ng* and *Nm*, but not *lst* knockouts, demonstrating that Lst is an outer membrane surface exposed protein. To our knowledge this is the first Gram negative glycosyltransferase localized to the outer membrane.

Regarding *lst* expression, *lst::lacZ* fusions (485 bp of *lst*, from –463 to +22) from *Ng* exhibit more b-galactosidase activity than 5¢ *lst:: lacZ* fusions of *Nm* (546 bp) when integrated into their respective chromosomes [155 (*Ng*) vs 30 (*Nm*) MU (n=3)] or when expressed as plasmids in *E. coli* [3500 vs 1600 MU (n=3)] suggesting transcriptional or post-transcriptional control. Deletion of 30 bp and 13 bp sequences that exist as tandem repeats in the *Ng* 5¢ *lst* region but as single copies in the *Nm* 5¢ *lst* region did not account for differential expression. In addition, the presence or absence of a 105 bp CREE (Correia Repeat-Enclosed Element) located 110 bp upstream of the *lst* ATG of *Nm*, which is absent in *Ng*, does not significantly affect *lst* expression. Sequential deletions of the *lst* upstream region suggest the *lst* promoter is within -109 and -81 upstream of ATG in *Ng*. The putative promoter of *lst* in *Nm* and *Ng* differs by a single nucleotide at the –35 site. Primer extension studies suggest that *lst* transcription is initiated at different points in *Ng* and *Nm*. Further molecular analyses are being conducted to confirm these findings and the mechanism for differential *lst* expression.

Biochemical properties of *Neisseria gonorrhoeae* LgtE and its role in the modulation of LOS expression.

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The *lgt* gene cluster encodes most of the proteins needed for the biosynthesis of the lacto-*N*-neotetraose LOS component. While biochemical and genetic data have defined the role of LgtA-D in the overall biosynthetic process, the role of LgtE in this process has only been defined genetically. Furthermore, the role of LgtE in extending the gonococcal $-chain is unclear. We used a series of biochemical and genetic tools to define the role of LgtE in gonococcal LOS expression. A fragment of chromosomal DNA encoding the *lgtE* gene of *Neisseria gonorrhoeae* strain F62 was amplified by the polymerase chain reaction and cloned into the expression vector pET15b. Functional LgtE was purified and its biochemical properties determined. The purified enzyme was maximally active in buffer containing manganese; minimal activity was obtained in buffer containing other divalent cations. LgtE was only able to mediate the addition of Uridine diphosphate-galactose into Neisserial Lipooligosaccharides. We used a variety of genetically defined and chemically verified LOS structures to determine substrate receptor specificity. LgtE was able to mediate the addition of galactose into a variety of LOS structures, indicating the this enzyme possesses broad receptor substrate specificity. Furthermore, it was able to add multiple galactose residues onto LOS. Biochemical analysis indicated that LgtE was able to modify both the « and $ chains of Neisserial LOS. We introduced transcriptional terminators into a variety of positions within the *lgt* gene cluster. This type of genetic analysis indicated that multiple promoters were found within this region. RACE analysis allowed us to define several transcriptional start sites within this region. Through the use of *xylE* transcriptional fusions, we were able to determine that the overall transcription of this region is quite low, and that LgtE is expressed in limiting quantities; subtle modulations of LgtE expression resulted in alterations in the LOS SDS-PAGE profile. Given that the level of expression of LgtE is low, and that subtle modulations in its expression can have a dramatic impact in the overall SDS-PAGE profile provide an explanation of how changes in growth rate can effect changes in LOS expression.

Differential cross-complementation patterns of *Escherichia coli* and *Neisseria gonorrhoeae* RecA proteins

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Homologous recombination is an essential function in all organisms, important for maintaining genomic integrity and repairing damage to DNA. The RecA protein and its homologues are central to these recombination processes. In *Escherichia coli*, the RecA protein is required for genetic transfer (via transduction and conjugation) and repair of damaged DNA. In *Neisseria gonorrhoeae* (Gc), the RecA protein is necessary for efficient DNA transformation, pilus antigenic variation, and DNA repair. Many bacteria contain a gene, *recX*, which has been hypothesized to be important for RecA activity. We have shown that the Gc RecX potentiates, but not is not essential for, all RecA-mediated processes in Gc. Recently we have also shown that the *E. coli* RecX inhibits RecA activities both *in vitro* and *in vitro*, and have suggested that *E. coli* RecX functions during the SOS response.

If the different cellular processes catalyzed by RecA require specific interactions with other proteins or involve unique biochemical properties of the RecA proteins themselves, then expression of RecA from a different species may not result in full complementation. By expressing the *recA* genes from Gc and *E. coli* under control of *lac* regulatory sequences in *E. coli*, we have shown that the Gc RecA fully complements an *E. coli recA* mutant for homologous recombination, but only partially complements for survival to DNA damage. By expressing similar constructs in Gc, we have shown that the *E. coli* RecA complements for pilus antigenic variation, partially complements for DNA transformation, but does not complement for survival to DNA damage, suggesting that species-specific interactions are important for DNA repair, but not for homologous recombination. Co-expression of the *E. coli recA* and *recX* genes in Gc suggested that in this heterologous system *E. coli* RecX modulates RecA-mediated processes. Based on our findings implicating both the Gc and *E. coli* RecX proteins as important for RecA function, we have constructed site-directed mutants in conserved amino acid residues of both the *E. coli* and Gc RecX proteins. We are currently assessing whether these residues are important for protein activity in these two bacteria.

Analysis of the DNA content of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae is an obligate human pathogen expressing type IV pili. Pili function to enhance adherence between the bacterium and host cells and tissues, and are required for transformation competence. Pilin, the product of the *pilE* gene, is the main component of the gonococcal pilus. Recombination between *pilE* and silent loci containing copies of partial pilin coding sequences occurs at a high frequency, and results in antigenic variation of pilin and altered pilus expression. Based upon our model of p ilin recombination, this RecA-dependent recombination event requires at least two gene copies, although how two gene copies are produced within a haploid cell is unknown. Multiple copies of *pilE* may arise from having more than one chromosome per cell, and during DNA replication two copies of *pilE* are transiently present. Identifying the number of genomes per gonococcal cell will clarify our model, and test whether DNA replication is directly linked to the process of pilin antigenic variation. We are using FACS analysis and quantitative PCR to determine the number of chromosomes present in each gonococcal cell grown under conditions promoting fast and slow growth. These results will aid in our understanding of the biology of this important human pathogen and also provide insights into the mechanisms underlying gonococcal pilin recombination.

OxyR acts as a repressor of catalase expression in *Neisseria gonorrhoeae*

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Hydrogen peroxide is a potent bacteriocidal agent. It can react with Fe(II) to generate the hydroxyl radical ($OH^{\cdot}$) a particularly potent oxidising agent that causes damage to DNA, lipids and proteins. Thus, the removal of hydrogen peroxide is a critical step in the bacterial defense against oxidative killing. It has been reported that *Neisseria gonorrhoeae* possesses a very high level of catalase activity, but the regulation of catalase expression has not been investigated extensively. In *Escherichia coli* and *Salmonella typhimurium* it has been demonstrated that OxyR is a positive regulator of hydrogen peroxide-inducible genes, including catalase. The *oxyR* gene from *N. gonorrhoeae* was cloned and used to complement an *E. coli oxyR* mutant, confirming its identity and function. The gene was inactivated by inserting a kanamycin resistance cassette and used to make a knockout allele on the chromosome of *N. gonorrhoeae* strain 1291. In contrast to *E. coli*, the *oxyR*::kan mutant expressed nine-fold more catalase activity and was more resistant to hydrogen peroxide killing than the wild-type. These data are consistent with OxyR in *N. gonorrhoeae* acting as a repressor of catalase expression. Furthermore, unlike *E. coli*, the putative promoter region of the catalase gene in *N. gonorrhoeae* strains 1291 and FA1090 revealed no obvious OxyR-binding site. The reason why *N gonorrhoeae* has evolved a system that responds to hydrogen peroxide via OxyR, but does not de-repress to maximal expression, effectively capping catalase activity, remains to be resolved.

Virulence factor expression in *Neisseria gonorrhoeae:* regulation by manganese and the Mn(II) binding protein MntC

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The mechanism by which the majority of *N. gonorrhoeae* strains overcomes the effects of superoxide is unclear. Only 20% of strains surveyed possess any superoxide dismutase activity (SOD), all of the reported activity is SodB. The high level of catalase found in *N. gonorrhoeae* cannot substitute for the role of SOD. We have previously shown that the accumulation of manganese (Mn) in *N. gonorrhoeae* protected gonococci from oxidative stress and was independent of SodB. We identified and characterised MntC, the periplasmic binding protein of the MntABC transporter. This is a high affinity Mn transporter in *N. gonorrhoeae*. This MntABC transporter belongs to a group, cluster IX, of a recently characterized ABC permeases. These are identified by the nature of the solute that is bound by their extracytoplasmic binding protein component. In this study, we further characterised the regulatory role of Mn and the phenotype of the *mntC* mutant in oxidative stress and gene regulation. The addition of Mn^{2+} to the growth media of wild-type cells dampened catalase induction by hydrogen peroxide and repressed pilin expression two-fold, as shown by catalase activity measurement and Western blot, respectively. MntC may also have a regulatory role in both catalase and pilin production as pilin expression was abolished and catalase activity reduced in a *mntC* mutant. A series of *in vitro* killing assays demonstrated that the *mntC* mutant was more sensitive to oxidative killing than the wild-type cells. These data indicated MntC is important in oxidative stress defense. In addition, two dimensional gel analysis demonstrated a pleitropic effect of the *mntC* mutant on gene regulation, suggesting MntC may be part of a global regulatory system.

Meningococcal DNA binding proteins involved in transformation

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Introduction: Natural transformation is a major source of neisserial genomic variability. DNA binding and uptake in transformation in the pathogenic *Neisseria* is dependent on the presence on the 10 base pair long sequence 5'- GCCGTCTGAA-3', termed the DNA uptake sequence (DUS). Our aim was to identify and characterise DNA binding components located in the meningococcal outer membrane.
Materials and Methods: Based on genome-wide searches for outer membrane proteins, we have investigated a number of neisserial outer membrane proteins and other proteins for the ability to bind DNA with and without the neisserial DNA uptake sequence (DUS). Protein-DNA binding was monitored by gel retardation assays. Recombinant proteins and proteins purified from the meningococcal outer membranes were analysed. The proteins investigated were mixed with radioactively labelled DNA substrates with and without DUS. The DNA-protein interactions were characterised under various conditions with regard to salt, temperature and relative amounts of protein and DNA, as well as with homologous and various heterologous competitive DNA substrates. Meningococcal mutant strains were constructed, and wildtype and mutant strains will be compared with regard to functional activities.
Results and Discussion: We have identified two meningococcal candidate outer membrane proteins that bind double stranded (ds) and single stranded (ss) DNA in a DUS-specific manner. It turned out that under the conditions used, ss DNA binding was stronger than the binding of ds DNA to these components. The function of these proteins are being assessed by analysis of wildtype and mutant strains in functional assays. Degradation of.one strand of the transformingDNA has until now been thought to take place in the periplasm.The fact that DUS-specific ssDNA binding was stronger than dsDNA binding to the two protein candidates might indicate that during transformation either one strand of the transforming DNA is degraded on the outer leaf of the outer membrane, or that the DNA binding epitopes are located on the periplasmic surface of the outer membrane.
Conclusions: We have identified two novel outer membrane ligands that bind neisserial DNA in a DUS-specific manner. These components are likely to be involved in DNA binding on the meningococcal cell surface during the transformation process.

Isolation and identification of genomic islands in *Neisseria meningitidis*

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Introduction: Recent comparisons of sequenced genomes of related bacteria strike the impact of lateral gene transfer on the evolution of bacterial genomes. The chromosomes of several pathogenic bacteria were shown to harbour genomic islands (GI) which may be acquired from unknown sources. These GI often contain clusters of genes contributing to the fitness and virulence of the bacteria. Therefore, identification of GI in pathogenic bacteria may allow the discovery of genes encoding novel virulence factors. At present, identification of GI based on nucleotide composition, e.g. GC content, codon usage and dinucleotide frequencies, occurs *in silico* and requires substantial sequence information of the strain of interest.
Hypothesis: Genomic islands can be recognised by anomalous clustering of dinucleotides. Hence, hexanucleotide frequency in GI might be different from that of the rest of the genome. Many restriction endonuclease recognition sites are palindromic hexanucleotides, which can be either overrepresented or underrepresented in a genome. A genomically underrepresented hexanucleotide sequence may be relatively overrepresented in GI. Such local overrepresentations would yield small, clonable fragments upon digestion. We hypothesise that local overrepresentations of restriction sites in GI clusters may be used to isolate novel virulence factors.
Approach and Results: A preselection of restriction enzymes underrepresented in the genome of *Neisseria meningitidis* MC58 was made by analysing the *in silico* digestion profiles using TIGR software. Next, fragments up to 5 kb were identified for each of the underrepresented restriction enzymes. A number of these enzymes yielded fragments solely originating from GI. This technique was validated *in vitro* for MC58. Chromosomal DNA was digested with the selected endonucleases, the resulting restriction fragments were amplified via adaptor-linked PCR, cloned and identified by sequencing. This procedure yielded predominantly the fragments from the GI as calculated *in silico*. Experiments to identify GI in the hypervirulent *N. meningitidis* lineage III are in progress.
Conclusions: A fast and simple method was developed to isolate sequences belonging to genomic islands. Besides the isolation of sequences belonging to known GI, this technique also allows the isolation of non-clustered anomalous DNA in *Neisseria meningitidis* MC58.

IgA1 protease repression by iron and its significance in *Neisseria* pathogenesis

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IgA1 protease plays a multitude of roles in the virulence of pathogenic *Neisseriae*. The transcriptional regulation of the *iga* gene, however, remains unknown. It has long been assumed to be constitutively expressed. Recent results linking protease activity with severity of disease, suggest that variations in the level of the protease may, itself, participate in virulence of these pathogens. We identified a potential Fur (Ferric uptake regulator)-binding region in the promoter of the *iga* gene in both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. We show that *iga* transcription is repressed under iron-rich conditions and increased under iron-poor conditions. Protease activity itself, however, is not affected by iron. Studies with GFP promoter fusion constructs demonstrate that mutations in the putative Fur-binding sequence abolish iron repression of *iga*. Finally, we address a role this regulation may play in *Neisseria* pathogenesis. Previous work from our group showed that IgA1 protease is important in the trafficking of gonococci across an epithelial monolayer; an *iga*- mutant is compromised in its ability to transcytose. In this work, we show that with the addition of human transferrin, wild-type gonococci behave identically to their *iga*- isogenic counterparts in epithelial transcytosis. Taken together, our results clearly demonstrate the iron regulation of IgA1 protease and suggest the importance of this regulation in *Neisseria* pathogenesis.

Genome sequencing of meningococci isolated from healthy carriers

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Introduction: *Neisseria meningitidis* is a commensal of the human nasopharynx. Approximately 10% of the isolates from healthy carriers may be considered as potentially pathogenic genotypes. Disease develops after colonization by a pathogenic strain in hosts showing risk factors and genetic predispositions which are not yet completely understood. Because of the medical importance of meningococcal disease, two complete genome sequences of pathogenic isolates have been published until now, i.e. one serogroup A meningococcal genome (strain Z2491; Sanger Centre) and one serogroup B genome (strain MC58; TIGR). Furthermore, complete sequencing of a pathogenic serogroup C strain and of *N. gonorrhoeae* is in progress at the Sanger Centre and at the University of Oklahoma, respectively. The projects provided invaluable insight into meningococcal diversity and mechanisms of genome variation. However, genome sequencing of pathogenic isolates did not explain why certain genotypes are pathogenic and others not although pathogenicity factors such as capsule and LPS structure are shared. We suggest that pathogenicity is determined by a complex interplay of different pathogenicity factors.
Materials and Methods: The genomic approach to test the hypothesis is sequencing the genomes of apathogenic variants. We recently set up a large carrier strain collection comprising 830 isolates which were characterized by MLST (Claus, Maiden, Frosch, Vogel, unpublished). Two apathogenic genotypes were selected for genome sequencing: strain 710 (ST-136) has been assigned to the ST-44 complex or lineage 3, the ST comprised 1.5% of the isolates of the Bavarian carriage strain collection, in the MLST database only one invasive ST-136 isolate has been deposited until now; strain 14 (ST-53) is one of few meningococcal complexes regularly lacking the capsule synthesis and transport operons (Claus et al., Microbiology 2002. 148: 1813-1819).
Results and Perspective: Until now, a 10.3-fold sequencing coverage was achieved for strain 14 (153 contigs, maximum length 146,317 bp); and a 5.4-fold coverage for isolate 710 (419 contigs; maximum length 65,116 bp). We intend to present the complete sequence of strain 14. The partial genome sequence of isolate 710 will be analyzed in conjunction with comparative microarray hybridizations. We expect to obtain an overview of genomic differences between pathogenic and apathogenic meningococcal isolates.

SESSION II

Physiology and metabolism

Iron acquisition from human lactoferrin by *Neisseria gonorrhoeae*: an evolutionary dilemma

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Neisseria gonorrhoeae have evolved elaborate mechanisms for iron (Fe) acquisition from heme, hemoglobin (Hb), human transferrin (Tf) and human lactoferrin (Lf). All gonococci express a Tf receptor, but only 50% express a Lf receptor. It is uncertain what role the Lf receptor plays in gonococcal infection. We have used the male human challenge model to address the contribution of the Tf and Lf receptors as virulence factors for urethral infection.

FA1090 (Tf+Lf-) is a naturally occurring Lf receptor mutant that expresses the Tf receptor. The estimated ID_{50} of FA1090 in male volunteers is about 1x 10^5 colony forming units (CFU). A Tf- mutant of FA1090 is markedly reduced in infectivity. We constructed a derivative of FA1090 that does not express the Tf receptor but does express the FA19 Lf receptor (Tf-Lf+); this strain infected four of eight male volunteers at inocula of ~10^5 and ~10^6 CFU. Thus, the Lf receptor alone was sufficient to permit urethral colonization and infection. We then constructed a Tf+Lf+ derivative of FA1090 and asked if there is a selective advantage to expression of both the Tf and Lf receptors. Twelve subjects were inoculated with 2 x 10^5 CFU of a 50/50 mixture of wildtype FA1090 (Tf+Lf-) and FA7163 (Tf+Lf+). Five subjects (42%) became infected, which was close to the expected result for an ID_{50}. Initial reisolated colonies from some subjects contained both organisms, but by the final day of experimental infection, 100% of colonies from all infected subject were Tf+Lf+ ($p<0.001$, Paired t-test). Thus, expression of both receptors was advantageous in the male urethral challenge model.

Tf and Lf ELISAs were performed on pre and post trial semen and urine samples and daily urine samples. Seminal Lf concentrations ranged from 0.16 uM to 21 uM and Tf concentrations from 0.18 uM to 1.7 uM. In urine, Lf concentrations ranged from 0.07 nM to 5.4 nM and Tf concentrations ranged from 0.7 nM to 28.3 nM. The gonococcal Lf and Tf receptors are saturated at 1uM of ligand, but urine and semen levels are often less than 1uM, which apparently explains why expressing both the Tf and Lf receptor during infection of the male urethra provides a selective advantage.

Although there is a selective advantage to Tf+Lf+ strains during male urethral infection, only 50% of clinical isolates express the Lf receptor. Thus, there must be a selective disadvantage to expression of the Lf receptor under other conditions, possibly in the female genital tract.

Identification and functional characterization of an SRP-dependent protein of *Neisseria gonorrhoeae*

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The prokaryotic signal recognition particle (SRP) is a complex of two proteins, FtsY (PilA in *Neisseria)* and Ffh, and a 4.5S RNA that is necessary for the targeting of a subset of proteins whose final destination is the cytoplasmic membrane. Each of the SRP components are essential for bacterial cell viability, indicating that this targeting system is critical for the localization of proteins involved in essential cell processes. A genetic screen designed to identify SRP-dependent proteins in *Escherichia coli* was modified and used to screen a *Neisseria gonorrhoeae* plasmid library for SRP-dependent proteins. Sequence analysis of 6 plasmids identified in this screen showed that each encodes one or more putative cytoplasmic membrane proteins. One of these plasmids, pSLO7, has four open reading frames (ORFs), three of which encode proteins with no similarity to known proteins in GenBank other than sequences from the closely related *N. meningitidis*. Transposon mutagenesis showed that one of these, SLO7ORF3, encodes an SRP-dependent protein and that this gene is essential in *N. gonorrhoeae*. While appearing unique to *Neisseria* at the primary sequence level, more detailed analyses revealed that SLO7ORF3 shared some features with the cell division gene *zipA* of *E. coli*. These features included similar chromosomal locations of the genes (with respect to linked genes) as well as similar protein sequence motifs. ZipA is an essential cell division protein that appears to be anchored to the cytoplasmic membrane and directly interacts with FtsZ, the major component of the septal ring. ZipA is thought to tether FtsZ to the membrane and is required for recruitment of additional division proteins to the Z ring at the division septum. Like the other major cell division proteins, ZipA is essential in *E. coli*, although it is the least conserved of this group of proteins. Predicted proteins with significant homology to ZipA have not been identified in genome sequences of gram-positive bacteria, archaebacteria, and several gram-negative bacteria, leading to the conclusion that it has either divergently evolved or other proteins serve its function in the cell. In this study, we present evidence showing that SLO7ORF3 can complement an *E. coli* conditional *zipA* mutant, and therefore encodes a functional ZipA homolog in *N. gonorrhoeae*. This is the first identification of a ZipA homolog in a non-rod-shaped organism. Additionally, the identification of ZipA as SRP-dependent makes it the fourth cell division protein (the others are FtsE, FtsX, and FtsQ) shown to be dependent on the SRP for localization, which may explain the essential nature of the SRP genes in bacteria.

A role for both RNA and DNA in protein targeting by the signal recognition particle in *Neisseria gonorrhoeae*

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The prokaryotic signal recognition particle (SRP) is a complex of two proteins, FtsY (PilA in *Neisseria*) and Ffh, and a 4.5S RNA that targets a subset of proteins to the cytoplasmic membrane (CM) co-translationally. In the current model for prokaryotic SRP function, Ffh binds to 4.5S RNA, the Ffh-4.5S RNA complex binds to the signal sequence of nascent peptides and then docks with FtsY at the membrane. GTP is hydrolyzed by FtsY and Ffh synergistically and the nascent peptide is transferred to the translocon, and subsequently inserts into the CM as it is being translated. In this work, we have isolated each of the SRP components from *Neisseria gonorrhoeae* and characterized them in various combinations. We present evidence that the in vitro properties of the gonococcal SRP differ from previously described systems, and suggest a modification of the model for SRP-dependent protein targeting based on these results. In the absence of 4.5S RNA, a modest (~2-fold) stimulation of total GTPase activity was observed when both PilA and Ffh were present. The inclusion of 4.5S RNA had a small effect on the combined activity of the two proteins, although the stimulation was likely due to a stimulation of PilA, rather than Ffh GTP hydrolysis. The addition of 4.5S RNA to PilA alone resulted in a ~5-fold stimulation of GTP hydrolysis, whereas the addition of 4.5S RNA to Ffh alone (in the absence of PilA) had no effect on activity. The effect of 4.5S RNA on PilA GTP hydrolysis was sequence-specific. The addition of similar molar equivalents of 3 different small RNAs; tRNA, a highly-structured mRNA, or a guide RNA (involved in RNA editing), had no effect on PilA GTP hydrolysis. These results represent a significant difference from other systems in which the interactions between FtsY and 4.5S RNA are reported to be dependent on Ffh. A direct interaction between the docking protein, PilA, and 4.5S RNA was shown by gel retardation analyses in which PilA and Ffh, both alone and together, bound to 4.5S RNA, forming complexes of different electrophoretic mobilities. Thus, although the 4.5S RNA does not affect Ffh GTP hydrolysis, it is bound by this protein. An additional novel finding was that P_{pilE} DNA, previously shown by us to bind PilA in vitro (Arvidson and So, 1995, J. Bacteriol. 177:2497) also stimulates PilA GTP hydrolysis, but has no effect on Ffh enzymatic activity. This stimulation was more dramatic (~12-fold) than the stimulation by 4.5S RNA, and was also sequence-specific. This is the first demonstration of an involvement for DNA in protein targeting, and has led us to propose an alternate model for prokaryotic SRP-dependent protein targeting in which RNA and DNA play a role.

PilQ point mutation results in hemoglobin utilization in the absence of HpuA/B

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Neisseria gonorrhoeae ordinarily requires both HpuA and HpuB to use hemoglobin (Hb) as a source of iron for growth. Deletion of *hpuA* resulted in reduced Hb binding and failure of growth on Hb. We identified rare Hb utilizing (Hb^+) colonies from an *hpuA* deletion mutant of FA1090, which fell into two classes. One required intact TonB and a point mutation in HpuB to grow on Hb (J. Bacteriol.184: 420-426), while the other required neither TonB nor HpuB. The Hb^+, HpuB and TonB-independent mutants all had increased sensitivity to antimicrobial hydrophobic agents, and, in this sense, phenotypically mimicked *mtr* mutants. Insertional inactivation of efflux pump related genes, *mtr*CDE, of the Hb^- parent resulted in the expected sensitivity to antibiotics and detergent, but did not lead to the Hb^+ phenotype. This suggested that a novel gene product be involved. Screening of pooled plasmids containing chromosomal DNA from the Hb^+, HpuB independent mutant revealed a point mutation in *pilQ* was responsible for the growth on Hb. PilQ is the subunit of the macromolecular complex formerly designated OMP-MC and is required for pilus biogenesis. The same PilQ point mutation was later identified from an independently isolated mutant of the same phenotype. Characteristics of these two PilQ mutants were further studied. Insertional inactivation of *pilQ* resulted in Pil^-, Hb^- phenotype confirming that expression of mutant PilQ was essential to Hb utilization. Human serum albumin inhibited Hb dependent growth, suggesting that extracellular heme released from Hb was used for growth. Consistent with their increased permeability, the PilQ mutants were highly sensitive to the concentration of heme in growth media and only grew on a narrow range of free heme concentrations. The results suggest TonB-independent heme and antibiotic passage through the mutant form of the assembled PilQ complex and therefore, the PilQ complex may be acting as a gated pore. The effect of this PilQ mutation on expressed pili and transformation competence is under investigation. We are also seeking the identity of other mutations, which are not in PilQ but that result in a similar phenotype.

Assembly and retraction of neisserial type IV pili are controlled by pilin translocation across the cytoplasmic membrane

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Introduction: Many Gram-negative bacteria express architecturally and functionally diverse pili on their surface. Pathogenic *Neisseriae* express type IV pili (tfp), which are involved in adhesion to human cells, DNA transformation and twitching motility. Tfp biogenesis is a complex and dynamic process that involves pilus fibre protrusion on the bacterial surface as well as fibre retraction. The components of the tfp machinery have been described, and recent findings show that tfp formation is a two-steps process. The first step leads to the presence of pilus fibres within the periplasm, which are, in a second step, translocated across the outer membrane (OM) through a multimeric secretin. The aim of this work was to get insight into the mechanisms responsible for fibre formation that precede pili extrusion through the OM.
Results and Discussion: By studying the periplasmic pilin content of the wild type meningococcal strain Nm2C4.3 and of a series of isogenic tfp defective mutants, we first show that tfp assembly is intimately linked with the translocation of mature pilin across the cytoplasmic membrane, and that detection of periplasmic pilin appears as a marker of fibre assembly. The OM PilC proteins have a positive effect on piliation and were shown to increase the pool of periplasmic pilin, probably by facilitating the translocation of mature pilin across the cytoplasmic membrane. On the other hand, the PilT-mediated pilus retraction is characterised by the reduction of the periplasmic pilin pool. Furthermore, on the basis that meningococcal adhesion represents a unique physiological model for studying tfp retraction, time-course monitoring of *pilC* and *pilT* transcription confirmed that the modification of the piliation status during bacteria-host cell interaction is linked to a modification of the balance between PilT and PilC. Finally, pilin gold-labelling on ultrathin sections of resin-embedded infected cell monolayers showed that pilus retraction correlates with the redistribution of pilin subunits within the bacterial cell, leading to the concentration of gold-labelled pilin along the bacterial cytoplasmic membrane.

Taken together, our results suggest that the biogenesis of tfp is under the control of both PilC and PilT proteins, which both mediate opposite actions on pilin translocation across the cytoplasmic membrane.

Cross-complementation of C-terminal domains of MinC from *Neisseria gonorrhoeae* and *Escherichia coli* and identification of essential residues for protein functionality

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Introduction: MinC acts as a cell division inhibitor by preventing septation in *Neisseria gonorrhoeae* (Ng) and *Escherichia coli* (Ec). Structural studies of MinC revealed that its N- and C-termini are connected by a flexible linker. Our comparison of MinC from different prokaryotes indicated that only the C-terminus is highly similar within species and contains five conserved amino acids: R136/R133, G138/G135, G157/G154, G164/G161 and G174/G171 of *N. gonorrhoeae* and *E. coli,* respectively. The C-terminus is essential for the self-interaction of $MinC_{Ec}$ and for $MinC_{Ec}$-$MinD_{Ec}$ interaction. Due to the evolutionary conservation between MinC C-terminal domains, we hypothesized that orthologous MinC domains could complement one another and that the conserved amino acids were essential for MinC functionality.
Materials and Methods: Chimeric proteins were constructed by cloning combined N- and C- termini of $MinC_{Ng}$ and $MinC_{Ec}$. Each of the conserved residues was replaced with radically different amino acids by site-directed mutagenesis of MinC from both, Ng and Ec. Function of the mutated and chimeric proteins was tested using microscopic, flow cytometric and yeast two-hybrid methods. Loss or conservation of protein functionality, due to possible structural changes in the C-termini from the various MinC proteins, was assessed using protein modeling.
Results and Discussion: Overexpression of wild type $MinC_{Ec}$ or $MinC_{Ng}$ in *E. coli* induced cell filamentation demonstrating activity of the two proteins as cell division inhibitors. Overexpression of the chimeras in *E. coli* induced filamentation indicating cross-functionality of these orthologs despite their combined domains. When the site-directed MinC mutants were overexpressed in *E. coli,* filamentation was not observed indicating that the conserved residues are essential for proper MinC function. Yeast two-hybrid assays demonstrated that all $MinC_{Ec}$ mutants lost their ability to interact with either $MinC_{Ec}$ or $MinD_{Ec}$ indicating that the conserved residues may be necessary for protein function through interaction with these proteins. Upon modeling, the mutant MinC proteins did not show any configuration changes. Although $MinC_{Ng}$ does not interact with any of the other Min proteins, chimeric MinC proteins interacted with MinD. Three-dimensional analyses of the chimeras revealed structural changes in the C-termini that may be responsible for the observed changes in protein interactions.
Conclusions: We demonstrated that the MinC domains from different bacteria complement one another. Furthermore, the conserved residues of the C-terminus of MinC are essential for its proper function as a cell division inhibitor. These findings are being exploited to develop novel antimicrobial targets.

Conservation of dynamic localization among MinD and MinE orthologs: oscillation of *Neisseria gonorrhoeae* proteins in *Escherichia coli*

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Introduction: The MinC, MinD and MinE proteins are involved in the correct placement of division septa in many Gram negative bacteria including *Neisseria gonorrhoeae* (Ng). Our recent studies have shown that heterologous expression of $MinC_{Ng}$ and $MinD_{Ng}$ in wild-type *Escherichia coli* (Ec) induces filamentation and *E. coli minC* and *minD* mutants were complemented by their orthologous gonococcal genes. These observations indicated similarities of MinC and MinD function across bacterial species of different morphology. Since Min_{Ec} function is associated with the pole-to-pole oscillation of these proteins within the cells, we hypothesize that *N. gonorrhoeae* Min proteins may also oscillate.

Materials and Methods: The heterologous dynamic behavior of gonococcal Min proteins in *E. coli* was investigated using various genetic and microscopic methods. Green fluorescent protein (GFP) fusions to wild type and various mutants of MinD and MinE, from both *N. gonorrhoae* and *E. coli,* were constructed. Time-lapse images of GFP localization were recorded and viewed in succession as movies. Protein-protein interactions in various combinations were studied using yeast two-hybrid assays.

Results and Discussion: Gonococcal Min proteins exhibit dynamic behaviour in *E. coli* and also act in concert with *E. coli* Min proteins. GFP-$MinD_{Ng}$ moved from pole-to-pole in rod-shaped *E. coli* cells, in 30-45 sec periods, when $MinE_{Ng}$ was expressed *in cis*. The oscillation time of GFP-$MinD_{Ng}$ was reduced when wild-type $MinE_{Ng}$ was replaced with $MinE_{Ng}$ carrying a R30D mutation. By contrast, the oscillation period of GFP-$MinD_{Ng}$ is increased almost 2-fold when it is activated by *E. coli* MinE. Several mutations in the N-terminal domain of $MinD_{Ng}$, including a K16Q mutation and 4- and 19-amino acid truncations, resulted in loss of oscillation; this was probably due to the lack of self-interaction between the $MinD_{Ng}$ mutants or between them and $MinE_{Ng}$. Gonococcal MinE-GFP oscillated in *E. coli* cells when $MinD_{Ec}$ was expressed *in cis* and exhibited movements similar to the oscillation of $MinE_{Ec}$-GFP. We used round *E. coli* to determine patterns of intracellular movement in cells of differing morphologies. The GFP-$MinD_{Ng}$ fusion also oscillated in round *E. coli* cells.

Conclusions: This is the first study to show conservation of the dynamic localization among Min proteins from bacterial species of different morphology. Thus, Min orthologs are cross-functional.

A comparative study of oxidative stress defence mechanisms in *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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Mechanisms for coping with oxidative stress are crucial for intracellular survival of Neisseria. Reactive oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) are encountered by Neisseria during aerobic respiration and interactions with phagocytic cells. Bacterial defences used to counter these reactive oxygen species include catalase, peroxidase (remove H_2O_2), and superoxide dismutase (SOD) (remove $O_2^{\cdot-}$), comprising cytoplasmic Sod A and Sod B, and periplasmic Sod C. Reports have recently highlighted the importance of manganese uptake, via MntC (the periplamsic binding protein of an ABC type transport system), in the protection from oxidative stress in *Neisseria gonorrheae*. In this report we present a comparison of the mechanisms employed by *Neisseria gonorrheae* and *Neisseria meningitidis* to survive oxidative stress. These organisms have a different complement of genes involved in oxidative defence, most likely a result of their localisation in different ecological niches. *N. meningitidis* contains MntC, SodC and SodB, whereas *N. gonorrheae* lacks SodC and has an inactive or weakly active SodB. In *N. gonorrheae* it is believed that intracellular oxidation of Mn(II) to Mn(III) substitutes for SodB or SodC activity in removal of superoxide. SodC, however, is a key component in *N. meningitidis* defence. The presence of *mnt*C, *sod*C and *sod*B was examined in a range of strains and insertion inactivation mutants were made in these genes in *N. meningitidis* strain MC58 ¢3. Resistance to killing by oxidative stress was investigated in the presence and absence of manganese using a paraquat killing assay (generates intracellular $O_2^{\cdot-}$) and a xanthine / xanthine oxidase killing assay (generates extracellular $O_2^{\cdot-}$). These assays revealed that MntC plays a key role in *N. meningitidis* in defence against oxidative stress.

Glutamine regulates hydrophobic antimicrobial agent resistance through an Mtr-dependent mechanism

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Introduction: We have previously shown that mutations in the *mtrF* gene result in phenotypic suppression of high-level hydrophobic agent (HA) resistance provided by the MtrCDE efflux system of *Neisseria gonorrhoeae*. MtrF was shown by us to define a protein family; however, it shows homology to only one characterized protein, AbgT from *E. coli*, and MtrF was unable to complement an *abgT* mutation. Since this indicated that MtrF was likely not functionally identical to AbgT, we sought to define the function of MtrF and its role in gonococcal HA resistance mediated by the *mtr* system.
Materials and Methods: We used RT-PCR and 2-dimensional gel electrophoresis to examine gene and protein expression levels, agar-dilution MICs to determine HA susceptibility, and glutamine uptake assays to investigate glutamine transport.
Results and Discussion: Inactivation of *mtrF* results in an unusual colony phenotype, which we have termed the "clear colony" phenotype. We discovered that growth of wild-type gonococcal strains on media lacking the GC agar supplement I, which contains glucose, cocarboxylase, and glutamine, resulted in the same colony phenotype; omitting only glutamine (i.e., plus glucose and cocarboxylase) gave the same result. Glutamine supplementation reversed this clear colony phenotype, while supplementation with various other amino acids did not, indicating specificity for glutamine. The presence or absence of glutamine supplementation also resulted in several other changes. The absence of glutamine resulted in a decrease in the expression of *mtrC* and *mtrF*. Conversely, several proteins were found to be upregulated in the absence of glutamine, one of which was identical to a meningococcal protein (GNA1946) that is a proposed vaccine candidate. The high-level HA resistance of strain FA140 was eliminated in the absence of glutamine supplementation. The changes in protein profile, colony phenotype, and HA resistance were reminiscent of that seen in *mtrF* mutants, leading to the hypothesis that MtrF might function as an inwardly-directed transporter of glutamine. MtrF mutants were shown to have decreased glutamine uptake while maintaining similar levels of leucine uptake, indicating that *mtrF* mutants have specifically lower glutamine acquisition.
Conclusion: Our results demonstrate that glutamine supplementation is required for high-level HA resistance, and that glutamine supplementation mediates changes in gonococcal transcription, indicating a potential signaling pathway. In addition, the results suggest that MtrF might be a transporter of glutamine and thus have an integral role in this process.

Motifs in gonococcal MinD essential for structure and function

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Introduction: MinD is a bacterial cell division site determinant that recruits the division inhibitor MinC to regions where septation is undesirable. We have shown that *minD* disruption in *Neisseria gonorrhoeae* (Ng) results in cells with grossly aberrant morphology, reduced viability, and improper division patterns. Furthermore, we showed that MinD from different genera self-associates; thus, dimerization may be important for MinD function across species. Although the crystal structure of bacterial MinD is unavailable, Archaeal MinD structures have been solved and they most closely resemble nitrogenase iron proteins, which are dimeric. MinD is highly conserved among bacteria and contains an ATP-binding site. Studies have shown MinD is an ATPase, and it has been suggested that this activity requires dimerization. The present study was conducted to identify potential motifs in gonococcal MinD ($MinD_{Ng}$) that may be important for self-association and/or protein function.
Design: Analyses of the $MinD_{Ng}$ sequence and of Archaeal MinD structures identified several regions that may be involved in protein dimerization. Mutations to disrupt a conserved ATP-binding site ($MinD_{Ng\text{-}ATP}$ at K16Q), a predicted coiled-coil motif ($MinD_{Ng\text{-}coil}$), and a polar loop region ($MinD_{Ng\text{-}loop}$) were constructed. Their effects on $MinD_{Ng}$ function and interaction were assessed by morphological and biochemical studies.
Results and Discussion: The three $MinD_{Ng}$ mutant proteins were unable to inhibit cell division when expressed in *E. coli*, showing a loss of function in this indicator background. Yeast two-hybrid studies showed that each mutant had significantly decreased or lost self-interaction; however, gel-filtration of purified $MinD_{Ng\text{-}loop}$ mutant indicated that the protein was still self-associated. Thus, the MinD dimer interface may be more extensive than the predicted loop region. Fusions of $MinD_{Ng\text{-}ATP}$ and $MinD_{Ng\text{-}loop}$ mutants to green-fluorescent protein failed to oscillate in an *E. coli* background, in contrast to the characteristic oscillation of wild-type $MinD_{Ng}$. The $minD_{Ng\text{-}ATP}$ gene was homologously recombined into the gonococcal genome, resulting in aberrantly shaped cells, indicating the importance of the K16 residue for protein function. Furthermore, $MinD_{Ng\text{-}ATP}$ and $MinD_{Ng\text{-}loop}$ mutant proteins have decreased ATPase activity when compared to wild-type control.
Conclusions: Various mutations in $MinD_{Ng}$ result in non-functional proteins that fail to influence cell division in either gonococcal or *E. coli* backgrounds. We show that dimerization alone is insufficient for $MinD_{Ng}$ function. This is the first study to determine the ATPase activity of MinD mutants, and our results demonstrate a correlation between non-functional protein and reduced ATPase. The conservation of self-interaction and function across species may make MinD a suitable target for novel antimicrobials.

Identification of the *Neisseria meningitidis* lactate permease and its role in pathogenesis

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Within the host, pathogenic bacteria are exposed to combinations of nutrients that can affect their success during initial lodgement or invasive disease. For example, at mucosal surfaces and in the bloodstream, pathogenic *Neisseria* grow in the presence of a mixture of carbon sources including glucose and lactate. We have previously shown that the addition of lactate to media containing glucose stimulates the metabolism of *Neisseria gonorrhoeae* and increases its serum resistance through sialylation of LPS. To investigate the impact of lactate on meningococcal pathogenesis, we identified and inactivated a gene, NMB0543, encoding a putative lactate permease in the serogroup B *N. meningitidis* strain, MC58. The mutant strain grows at the same rate as the wild-type bacterium on glucose as the sole carbon source, but, in contrast to the wild-type, is unable to grow with lactate as the only carbon source. Furthermore, the mutant strain is defective for the uptake of C^{14}-labelled lactate, providing evidence for the function of the NMB0543 gene product as a permease. Comparison of the mutant and wild-type strains in the infant rat model of meningococcal sepsis demonstrates that the mutant is attenuated during systemic infection. The results indicate that the utilisation of lactate by *N. meningitidis* is important during pathogenesis, and may be relevant to other pathogens that encounter mixed carbon sources *in vivo*.

SESSION III

Surface antigens

Neisseria meningitidis App: a new adhesin with autocatalytic serine-protease activity

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Introduction: The recently described Adhesion and Penetration Protein (App) of *Neisseria meningitidis* is a protein belonging to the autotransporter family that is cleaved and secreted in *N. meningitidis*. App has high homology with the Haemophilus adhesion and penetration protein (Hap) of *Haemophilus influenzae*. The biological role of App is not known, however, its homology to the Hap protein suggests that App could be involved in the interactions of meningococcus to host cells.
Results: We used *Escherichia coli* expression of *app* to study the functional properties of the protein. After export to the *E. coli* surface, the protein is cleaved and the amino terminal 100 kDa fragment released in the culture supernatant. The cleavage has shown to be mediated by an endogenous serine-protease activity, which is abolished by site directed mutagenesis of the catalytic serine residue. The kinetics of App expression revealed that at mid-log phase there is a high level of App surface expression, whereas secreted App is minimal. In contrast, in stationary phase surface expression of App is minimal, whereas there is a high level of secreted App in the culture supernatant. The close homology to Hap of *H. influenzae* suggests that App may mediate meningococcal-host cell interactions. *E. coli* expressing *app* on the surface adheres to Chang epithelial cells, thus proving that App is indeed able to mediate bacterial adhesion to host cells. To verify the role of App in meningococci an isogenic App mutant was generated. Adhesion assays using Chang epithelial cell line revealed that the meningococcus isogenic *app* mutant is less adhesive than the wild type strain.
Conclusions: These results indicate that App has autocatalytic serine protease activity and is involved in *Neisseria*-host cells interactions.

The meningococcal TspA protein is vital for normal type IV pilus biogenesis and function

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Introduction: TspA is a potent T-cell and B-cell stimulating protein of *Neisseria meningitidis* and is conserved among meningococcal isolates. It is a high molecular weight protein that migrates at twice the predicted size. In silico analysis shows that it has a highly positively charged N-terminal half and a highly negatively charged C-terminal half, separated by a hydrophobic, predicted membrane-spanning region. In this study, the molecular features, functional role and subcellular localisation of TspA were investigated.
Results and Discussion: TspA was cloned, expressed, purified and used to raise rabbit polyclonal antiserum. Western immunoblots confirmed the constitutive expression of the protein in all meningococcal strains examined but not in *N. gonorrhoea* or *N. lactamica*. To study the biological properties and functional role of the protein, an isogenic deletion mutant and two truncated TspA mutants (lacking part of the N- or C-terminal halves) were produced in strain MC58. TspA mutagenesis did not affect the growth of the meningococcus in broth cultures; however, it markedly impaired its growth on solid media or co-culture with human cells. TspA mutagenesis reduced the bacterial adhesion and invasion capacity and its ability to exhibit twitching motility, a property that is attributed to the type IV pilus of the organism. These findings imply that TspA must interact with the meningococcal pilus. Indeed, on examination under the electron microscope, cells of the null mutant were grossly abnormal with deformed pili and disorganised microcolonies. They had unusual projections at the cell surface presumably composed of disorganised pilus subunits. Electron microscopy of these cells confirmed that the projections did not affect the structure and integrity of the outer membrane or the rest of the cell envelope. Electron microscopy of the truncated TspA mutants confirmed that the C-terminal half of TspA is necessary for normal pilus biogenesis. The mutant lacking part of the N-terminus was almost identical to the wild type, whereas the C-terminal mutant resembled the null mutant. Immunoblots of fractionated cells and immunogold transmission electron microscopy confirmed that the protein is transported to the outer membrane and is surface exposed on the wild type organism. The null mutant and the truncated mutant lacking the C-terminus were not surface labelled, indicating that the functional C-terminal half of the molecule is also surface exposed.
Conclusion: TspA is conserved, constitutively expressed and surface-exposed on the meningococcus. It is required for type IV pilus biogenesis and function. We propose that TspA, which is a potent T-cell and B-cell immunogenic, may have a vital role in the pathogenesis of meningococcal disease.

Functional and structural analysis of NadA, a novel adhesin of *Neisseria meningitidis*

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Introduction: In a previous study, using a genomic-based approach, we identified novel proteins considered as vaccine candidates against *Neisseria meningitidis* sero-group B, some of which share homologies to known bacterial virulence-associated factors (1). One of these proteins, NMB1994, named NadA (Neisseria adhesin A,) is homologue to YadA of enteropathogenic *Yersinia,* a non-pilus associated adhesin, and to UspA2 of *Moraxella catarrhalis,* involved in serum resistance. While sequence homology is limited to the carboxyl terminal region (membrane anchor domain), an overall similarity in predicted secondary structure is shared among the three proteins. We have shown that, like YadA and UspA2 also NadA forms very stable and difficult to dissociate high molecular weight oligomers anchored to the outer membrane of meningococcus (2).

Here we studied the structure-function relationship of NadA using *Escherichia coli* as a model system of surface-expression.

Materials and Methods: *NadA* full-length gene and different mutants were cloned and expressed in *E. coli* BL21(DE3) under the control of T7 promoter. Expression was achieved activating the promoter with IPTG or under uninduced conditions. Localisation and surface-exposure of the protein(s) were assayed by cell-fractionation experiments (SDS-PAGE and Western blot) and FACS analysis. Adherence of *E. coli* strains and binding of recombinant NadA to eukaryotic cells was assayed by FACS analysis and immunofluorescence microscopy

Results and Discussion: We show that in *E. coli*, NadA is exported to the outer membrane and assembled in stable oligomeric protein as in *Neisseria*, suggesting that NadA is localised to the bacterial surface without the help of specific meningococcal factors. Moreover, we show that *E. coli* expressing *nadA* on the surface adhere to Chang epithelial cells but not to Huvec endothelial cells, providing evidence that NadA is able to mediate bacterial adhesion to host epithelial cells. Adhesive properties of NadA were confirmed by *in vitro* binding experiments using the recombinant protein (rNadA) to different cell lines. We found that rNadA binds differentially to the diverse epithelial cells tested, suggesting a differential expression of the eukaryotic receptor molecule(s).

Conclusion: Our results suggest a role of NadA in the interaction of meningococcus with human epithelial cells. The mechanism that mediates the export and assembly of NadA, as well as its role in the virulence of meningococcus is under investigation.

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Characterization of *Neisseria gonorrhoeae* pilin glycans

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Introduction: *Neisseria gonorrhoeae* (gonococcus, GC) causes not only local infection or uncomplicated gonorrhea (UG) but also pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI). The pilin glycoprotein is the main building block of the GC pili. Pilin of GC strain MS11 (variant C30) carries an O-glycan, Galα(1–3)GlcNAc, on its Ser 63 residue. Recently, we reported characterization of the *pgtA* gene, which codes for the galactosyl transferase that catalyzes synthesis of the GC pilin glycan Galα(1–3)GlcNAc bond. In DGI isolates, *pgtA* carries a poly-G tract and undergoes phase-variation (Pv). However, in UG strains, it usually lacks poly-G and expresses constitutively. Pv of *pgtA* is likely needed for UG to DGI conversion.
Materials and Methods: In the current study, pilin glycans from more GC strains, particularly from those having *pgtA* Pv, are being characterized in details by various analytical methods. These techniques include high performance anion exchange chromatography (HPAE), mass spectrometry (MS) and NMR. Additionally, NMR and TLC analyses of GC pilin glycans of the existing *pgtA* mutants and their isogenic wild type strains are also being used for detecting the alternative glycoforms that likely arise in the *pgtA* knockouts due to a lack of the PgtA activity. Moreover, the existing *pgtA* mutants are being moved into a regulatable *recA* background for studying the effect of pilin antigenic variation on the glycosylation of this molecule. Finally, radioactive transferase assays were performed in order to directly demonstrate the glycosyl transferase activity of several pilin glycosylation genes.
Results and Discussion: The pilin glycans of the GC strains (like FA1090 and 15253) possessing Pv of *pgtA* likely differ markedly from that of the previously analyzed strains (like MS11 and F62), which lack this Pv. A few novel glycoforms of the GC having *pgtA* Pv may correspond specifically to the off-phase of *pgtA*. The past studies of GC pilin glycosylation, which only used the GC strains that carry the constitutive *pgtA*, could not have found such glycoforms whose syntheses require an absence of the PgtA activity.
Conclusion: Pilin glycoforms of the GC strains possessing *pgtA* Pv are likely to be significantly different from that of the GC lacking this Pv. The differences in the glycoforms of the two groups of GC may partly be responsible for their distinct pathogenic manifestations.

Identification of a novel vaccine candidate for group B *Neisseria meningitidis*

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Introduction: Group B *Neisseria meningitidis* strains are a major cause of meningococcal disease throughout the world. No vaccine currently exists to prevent group B disease. Studies in humans as well as animals indicate that the serosubtyping antigen, PorA, elicits bactericidal antibodies. The immune response to Por A is generally serosubtype specific, therefore 6-9 PorAs will be needed to cover 70-80% of group B strains. Our earlier work with LOS depleted outer membrane proteins identified strain 8529 as producing an outer membrane protein(s) other than PorA which were capable of eliciting bactericidal antibodies to strains expressing heterologous serosubtypes. Here we describe an outer membrane lipoprotein, P2086, which can reduce the number of proteins required in a meningococcal vaccine.
Results: Subcellular fractionation, differential detergent extraction, isoelectric focusing, and ion exchange chromatography were used in conjunction with immunization and bactericidal assays against multiple strains to identify small groups of proteins of interest. Direct sequencing of the main components indicated that the N-termini were blocked. Internal protein sequences were obtained by direct sequencing of polypeptides derived from chemical and proteolytic digests. The genomic sequence of a group A meningococcal strain was downloaded from the Sanger Center and analyzed by our Bioinformatics group using existing and proprietary algorithms to create a searchable database. The peptide sequence data indicated that ORF2086 was of interest. Primers based on this orf were used to PCR the P2086 gene from strain 8529. Analysis of the gene sequence, the fact that the N-terminus was blocked, and its subcellular location indicated that P2086 is a lipidated outer membrane protein(LP2086). rLP2086-8529 and variants from other meningococcal strains were recombinantly expressed as lipoproteins in *E.coli* using the *H.influenzae* P4 signal sequence. These recombinant proteins were isolated from *E.coli* membranes by differential detergent extraction, purified using ion exchange chromatography, and used to immunize mice. Mouse anti-LP2086 sera were able to facilitate bactericidal activity against several different serosubtype strains of *N.meningitidis*. Further analysis of the P2086 genes from many *N. meningitidis* strains showed that these sequences fell into two groups designated Subfamily A and Subfamily B (see Farley J et. al. poster). The antisera raised against the Subfamily B proteins were bactericidal against nine strains expressing Subfamily B proteins, and one strain expressing a Subfamily A protein. Subfamily A antisera were bactericidal against Subfamily A strains. A mixture of one rPorA and one rLP2086 elicited complementary antibodies extending vaccine coverage beyond that induced by either protein alone.
Conclusions: rLP2086 antigens are capable of eliciting bactericidal antibodies against meningococcal strains expressing heterologous PorAs and heterologous P2086 proteins. The P2086 family of antigens may be a useful vaccine either alone or in combination with other neisserial antigens.

Structure-function analysis of neisserial surface protein A

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Neisserial surface protein A (NspA) is a highly conserved outer membrane protein (OMP) present in all meningococcal strains tested to date. Anti-NspA monoclonal antibodies were shown to be bactericidal and to mediate protection against serogroup B strains in a mouse model. Therefore, NspA is considered a potential vaccine candidate (1). However, the accessibility of NspA on the surface of the meningococcus varies among strains, hampering its use as a broad protective vaccine (2). Nevertheless, it remains one of the most broadly protective neisserial antigens found so far. In order to understand its success as a vaccine candidate, we set out to determine the structure and function of this protein.

Recombinant mature NspA was overexpressed as inclusion bodies in *E. coli*. Solubilized, denatured protein was refolded using the detergent SB-12 and subsequently purified on a mono S ion-exchange column. NspA crystallized using the detergent $C_{10}E_5$ in the space group R32. The crystal structure was determined to a resolution of 2.6 Å using the single wavelength anomalous scattering (SAS) method. Each monomer of NspA consists of an eight-stranded antiparallel β-barrel with short turns at the periplasmic side and long loops at the extracellular region. The only other eight-stranded β-barrel structures solved to date are the *E. coli* OMPs OmpX and OmpA. Comparison of these structures to NspA revealed that NspA resembles OmpA with respect to shape and shear number although NspA shows only about 15% homology to OmpA. NspA shows high homology to the Neisserial Opa protein family of adhesins, especially in the transmembrane regions. Therefore, the NspA structure may aid in modelling the structure of Opa proteins. Furthermore, the structure of the extracellular loops may be used for vaccine design (3).

NspA knock-out mutants in *Neisseria gonorrhoeae* and *Neisseria meningitidis* were constructed as well as an *E. coli* strain expressing meningococcal NspA in its outer membrane. The phenotype of these strains is currently under investigation in order to gain insight in the function of NspA. Growth characteristics, potential adhesin function and role in outer membrane stability of NspA are being studied.

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Characterization of neisserial outer membrane phospholipase A

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Outer membrane phospholipase A (OMPLA) is one of the few enzymes present in the outer membrane of Gram-negative bacteria. For the human pathogens *Yersinia pseudotuberculosis* and *Helicobacter pylori,* OMPLA was shown to be required for colonization of mice, indicating that the enzyme may be important in the pathogenesis of human disease as well (Karlyshev *et al.*, 2001, Infect Immun 69: 7810; Dorrell *et al.*, 1999, Gastroentrology 117: 1098). The presence of the *pldA* gene, encoding the OMPLA protein, was appreciated from the neisserial genome sequences (Brok *et al.*, 1998, Res Microbiol 149: 703). To address a possible role of OMPLA in neisserial disease, we investigated the presence and function of this enzyme in the pathogenic *Neisseriae.*

Immunoblotting of cell extracts showed that OMPLA was expressed by six out of six investigated gonococcal and by 27 out of 32 investigated meningococcal strains from five different serogroups. Sequencing of the *pldA* gene of the five meningococcal strains that lacked the protein revealed that four of them had an identical premature stop codon in the 5' part of the gene. The other strain contained a different premature stop codon. The commensals *N. cinerea, N. flavescens,* and *N. subflava* did not express the protein, while two out of four *N. lactamica* strains were positive for OMPLA expression. To facilitate functional analysis of neisserial OMPLA, the gene was cloned and mutants were constructed in *N. gonorrhoeae* (MS11) and *N. meningitidis* (H44/76) by insertion of a kanamycin-resistance cassette in the *pldA* gene. Phenotype analysis indicated that the mutants exhibited unaltered exponential growth rates compared to the parent strains. However, the optical density of the mutants remained stable for longer times after prolonged growth. Lower levels of DNA were found in the spent medium of the mutants, indicating reduced autolysis. The most prominent demonstration of a change in autolysis was seen when the bacteria were kept as a pellet: after several hours, it was impossible to resuspend the aggregated wild-type bacteria, while the mutant cells could be resuspended into a smooth suspension for at least 24 hours. Further analysis of the mutants showed that the LPS profile was unchanged and that Opa-mediated adherence to epithelial cells was not affected in the mutant.

In conclusion, our data indicate that OMPLA is present in most but not all pathogenic neisserial strains and that it has a role in autolysis. This phenomenon is well known for the *Neisseriae* and may aid in the survival and fitness of a population of bacteria.

Analysis of PorA variable region 3 in meningococci. Implications for vaccine policy?

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Introduction: An effective vaccine against *Neisseria meningitidis* serogroup B is urgently needed. Outer membrane protein vaccines are being developed which may provide protection against common circulating serotypes and serosubtypes in some countries. However, limited data is available in Europe from genosubtyping meningococci because most laboratories use monoclonal antibodies directed against a limited number of specific types and subtypes.
Materials and Methods: We undertook a retrospective analysis of the three main variable regions, VR1, VR2, as well as VR3, of the *por*A gene from *N. meningitidis* strains isolated from a number of countries, mainly Scotland and Sweden.
Results: Analysis of the *por*A gene showed that, amongst 226 strains studied, there were a total of over 50 different strains. Five new VR3 variants are described (39, 40, 40-1, 40-2 and 41).
Conclusion: Our data indicates the importance of analysing the VR3 region of PorA in addition to VR's 1 and 2 and also highlights, in general terms, the need for genosubtyping meningococci. Such analyses have major implications for the design of new meningococcal vaccines.

NadA genetic diversity and carriage in *Neisseria meningitidis*

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Introduction: NadA is a promising vaccine candidate against meningococcus, sharing significant homologies with an adhesin and a vaccine candidate of *Yersinia pestis* and *Moraxella catarrhalis*, respectively. The *nadA* gene is present in approximately 50% of clinical isolates, and over-represented in the hypervirulent clusters (ET-5, A4 and ET-37) of *Neisseria meningitidis*. NadA is able to raise a bactericidal immune response against all the strains harbouring it. The *nadA* gene is present in clinical isolates in three well-defined and conserved alleles, named I, II and III: here we describe the presence and properties of *nadA* in a panel of meningococcal strains isolated from healthy carriers in six countries and report the discovery of a new, carrier-associated form of NadA.
Results and Discussion: We detected by PCR and dot/blot hybridization the presence of the *nadA* gene in 23 out of 120 strains isolated from healthy individuals. The overall percentage of the gene presence (19%) is much lower than in clinical isolates (50%). The *NadA* gene found in 18 out of 23 positive carrier strains were identical to allele I and II, suggesting that although isolated from carriers, these strains are ET-5 and ET-37 strains undistinguishable from those isolated from patients. Five of the 23 positive strains contained a variant sequence that we have never found in strains isolated from clinical cases. This variant was harboured by strains isolated in the same country, at three different surveys, spanning eight years. We named it NadA carrier (*NadAc*) allele. The protein encoded by the carrier allele, NadAc, has a smaller size (323 aa) as compared to other NadAs (ranging from 362 to 405). It is predicted by computer analysis to share a limited homology to the other NadA alleles, but in its C-term part (the NadA predicted membrane anchor motif), where it is 100% conserved. It is expressed in diverse amounts depending on the strain. It is exported to the outer membrane, where it is still able to form oligomers. It evokes a bactericidal antibody response against homologous strains, which does not cross-react to the strains harbouring NadAs encoded by other alleles.
Conclusion: NadA was present in carrier strains belonging to the hypervirulent lineages (15%) or, in a few isolates (4%), as a protein which had never been found in disease cases. This new form appears to be strictly carrier-associated, and shows different features when compared to the ones described in clinical isolates.

Functional activity of antibodies against recombinant OpaJ protein from *Neisseria meningitidis*: blocking of Opa-CEACAM interaction

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Introduction: The Opacity proteins belong to the major outer membrane proteins of the pathogenic *Neisseria* and are involved in adhesion and invasion. We studied the functional activity of antibodies raised against OpaJ protein from strain H44/76. Recombinant OpaJ protein was obtained from *E.coli* in two different ways: cytoplasmic expression in the form of inclusion bodies followed by purification and refolding, and cell surface expression followed by isolation of outer membrane complexes (OMCs).
Materials and Methods: BALB/c mice were used for subcutaneous immunization with 5 ?g purified protein with or without QuilA adjuvant, or with OMCs on days 0, 14 and 28, and on day 42 sera were obtained. ELISA was used to determine the presence of total and isotype specific IgG antibodies and the reaction with chimeric Opa proteins. Functional activity of antibodies was measured in bactericidal and opsonophagocytosis assays. To test for blocking activity, a highly defined *in vitro* infection assay was used with Opa-expressing *E.coli* and HELA cells expressing either CEACAM 1 or 5.
Results and Discussion: Immunization with purified protein and QuilA induced high levels of Opa-specific antibodies, while the *E.coli* OMC preparations induced generally lower levels of antibodies. Purified denatured OpaJ induced a two-fold higher level of total IgG compared to H44/76 OMCs expressing OpaJ. Two chimeric Opa proteins were generated, and used to demonstrate that the HV2 variable region is immunodominant. Strikingly, denatured OpaJ with QuilA induced high levels of IgG2a in addition to IgG1, while refolded OpaJ with QuilA induced IgG1 exclusively. This appeared not to be associated with the ability of these sera to induce complement-mediated killing, which was very low in all cases. The sera were tested for their ability to block the binding between OpaJ and OpaB proteins and their receptors, the CEACAM1 and 5 proteins. In addition to blocking of the OpaJ-CEACAM1 interaction, cross-reactive blocking of the heterologous OpaB protein with both CEACAM 1 and 5 was found for all sera with significant IgG titres. Sera raised against purified OpaJ and against OpaJ-containing OMCs could also be used for blocking of the non-opsonic interaction of Opa-expressing meningococci with human polymorphonuclear leukocytes.
Conclusion: Purified recombinant OpaJ protein with QuilA is highly immunogenic in mice, while OpaJ presented in *E.coli* OMCs is less immunogenic. Although hardly any bactericidal and opsonophagocytic antibodies were found, OpaJ could induce antibodies with blocking activity, both for homologous and heterologous Opa-CEACAM interactions. This implies the presence of conserved surface-exposed epitopes on Opa proteins with distinct HV1 and 2 regions. If similar cross-reactive blocking antibodies can also be induced with purified Opa protein at mucosal surfaces, this would constitute a novel protective mechanism for meningococcal vaccines.

A novel approach for purifying post-transcriptionally modified proteins: homologous expression and purification of His-tagged pilin from *Neisseria meningitidis*

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Glycosylation of proteins in prokaryotes was until recently regarded as uncommon and thought to be limited to special cases such as S-layer proteins and some archeal outer membrane proteins. However, there are now an increasing number of reports in prokaryotes of not only protein glycosylation, but also of the importance of glycoproteins in pathogenicity. The most extensively studied glycoproteins of bacterial pathogens are the pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Pili of pathogenic *Neisseria* are typical of a family of adhesins, type IV fimbriae, found in a wide range of Gram-negative pathogens. These long polymeric proteins protrude from the bacterial surface and have a crucial role in both colonisation of the host and adhesion to host cells. Although there are other accessory proteins, pili are composed primarily of thousands of subunits, called pilin. Pili of the pathogenic *Neisseria* are also post-translationally modified. Four different types of post-translational modifications have been described in detail. A phosphodiester-linked glycero substituent has been reported at serine 93 of the pilin molecule, a covalently linked phosphorylcholine has been reported in *N. meningitidis* and *N. gonorrhoeae*, a phosphate group has been identified at serine 68 in *N. gonorrhoeae*, and finally, pili of both *N. meningitidis* and *N. gonorrhoeae* are glycosylated. In *N. meningitidis* strain C311, a detailed structural study revealed that pilin is glycosylated at serine 63 with an unusual trisaccharide molecule, Gal (β1-4) Gal (α1-3) 2,4-diacetamido-2,4,6-trideoxyhexose. In order to conduct structural and immunological studies of pilin, it is imperative to have large quantities of pure protein. Current published pilin purification methods were found to be time consuming, non-reproducible, included interfering proteins and had low efficiency. Therefore, a novel, rapid and simple method for the purification of pilin was developed. A 6-Histidine tag was inserted at the C-terminus of the pilin structural gene, *pilE* of C311, on the chromosome via homologous recombination. This has allowed purification of pure pilin from C311 under non-denaturing conditions using immobilised metal affinity chromatography (IMAC). Analysis of the purified pilin by Western blotting with antisera specific for the trisaccharide molecule and phosphorylcholine showed that both post-translationally modifications were present on the his-tagged pilin. This purified pilin may be used for structural studies and as a "native" substrate to study the transferases involved in its glycosylation. The simplicity of this new method makes the study of many mutations in several strains feasible. In addition, this approach may be generally applicable to the study of post-translationally modified proteins or other proteins that are difficult to express and purify in conventional *E. coli* based systems.

A study into the sequence variability, distribution, structure and mechanism of HmbR, a haemoglobin receptor of *Neisseria meningitidis*

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Introduction: HmbR is a TonB-dependent, iron-regulated, outer membrane protein that is responsible for the transport of haem into the periplasm of meningococci. To date, only a limited number of sequences of the *hmbR* gene from *N. meningitidis* have been compared[1]. Other outer membrane proteins from *N. meningitidis* are characterised by a high degree of sequence variability within regions of the protein that are surface-exposed. In order tTo ascertain whether this was the case for *hmbR*, the gene was sequenced from a diverse collection of 30 strains derived from the MLST dataset.

Results and Conclusions: There was a limited level of nucleotide sequence variability in the *hmbR* gene, with sequence substitutions confined to three separate regions. A structural model for HmbR was constructed, based on the crystal structure of the related iron-transporter FepA from *E. coli*. The model consisted of a 22-stranded β-barrel with 11 extracellular loop regions and an N-terminal globular domain. The regions of sequence variability were concentrated within extracellular loops 2, 4 and 8, which are probably surface-exposed. The distribution of the *hmbR* gene within the MLST set and 10 different commensals was also investigated using a hybridisation probe. Of the MLST strains studied, 80 % contained the *hmbR* gene, including all serogroup C strains strains. Of the commensals tested, only *N. polysaccharea* appeared to contain the gene. Sequencing revealed that in this case a premature stop codon occurs after 54 amino acids, indicating a non-functional product. A premature stop codon in the *N. gonorrhoeae hmbR* gene, after 338 residues, has been reported previously[1]. *N. meningitidis* HmbR was expressed from a T7-based promoter vector in *E. coli* without a signal sequence and the protein appears to be produced into inclusion bodies. Refolding and purification studies are currently underway.

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Characterization, cloning and expression of different subfamilies of the ORF 2086 gene from *Neisseria meningitidis*

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Background: The quest for a serogroup B *Neisseria meningitidis* vaccine has explored the use of several surface exposed components of the meningococcus as antigenic targets. In the process of testing soluble outer membrane components from various meningococcal strains for their immunogenic properties, some strains yielded soluble fractions that showed cross-functional activity against heterologous serogroup B strains in a bactericidal assay (BCA). In particular, soluble outer membrane components from strain 8529 showed the highest level of cross-functional activity. N-terminal and internal amino acid sequence was obtained for proteins in the BCA positive fractions, and these sequences were compared to the Sanger Centre serogroup A meningococcal genomic sequence. Bioinfomatics analysis identified an ORF predicted to encode a lipidated surface protein of approximately 28 Kd in molecular weight. (see Bernfield, *et al*. poster.)
Results: The putative vaccine candidate, lipidated protein 2086 (LP2086), generates the same immunogenic cross-reactivity that was previously displayed in the outer membrane protein extracts of meningococcal strain 8529. This protein has an unknown function and its sequence appears to be unique to the *Neisseria* genus. A large number of heterologous *Neisseria* strains have been screened for the presence of a "2086 gene homolog" by PCR amplification (N>100) and DNA sequencing (N>50). There appears to be at least two distinct subfamilies of the P2086 protein, which share approximately 60 to 75% homology at the nucleic acid level. Amino acid sequence variations within the families define the phylogenetic clusters. Representative genes from each subfamily were recombinantly expressed with and without a heterologous lipoprotein signal sequence. The lipidated version of the protein generates significantly greater antibody titers which have greater killing capacity in the BCA. In general, the expression level of recombinant P2086 in *E. coli* represents between 2 - 5% of total cellular protein. In an attempt to maximize the yield of recombinant P2086 and produce enough material for animal studies, a variety approaches were tested.
Conclusions: A unique surface exposed protein (P2086) is capable of generating antibodies that recognize and are bactericidal against heterologous meningococcal strains. A homolog of the P2086 protein has been identified in all the pathogenic *Neisseria* strains tested to date. Two distinct subfamilies of the P2086 protein have been identified, with the subfamilies sharing 60-75% homology. The lipidated 2086 protein is more immunogenic and generates stronger functional antibody activity.

The twitching motility protein PilT is an oligomeric ATPase

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Introduction: Surface motility in gonococci and other bacteria works by retraction of surface-attached type IV pili. This retraction requires the PilT protein, a member of a large family of putative NTPases from type II and IV secretion systems. Our eventual goal is to characterize Neisserial PilT by x-ray crystallography. In this study we have purified and biochemically characterized a hyperthermophilic PilT.
Methods: PilT from the hyperthermophilic eubacterium *Aquifex aeolicus* has been used as an experimentally tractable model for gonococcal PilT. PilT was cloned from genomic *A. aeolicus* DNA, overexpressed in *E. coli* as a hexahistidine fusion protein, and purified by Ni++ chelate chromatography and a heat shock step. In order to assess the enzymatic activity of this protein, we adapted a well-known real-time assay for mesophilic enzymes, separating high temperature ATPase activity from moderate temperature end-point read out of [ADP] using standard coupling enzymes. The oligomeric state of PilT was probed by sedimentation equilibrium, size exclusion chromatography, and dynamic light scattering.
Results and Discussion: The thermostable protein remained soluble at 40°C, facilitating its purification from the *E. coli* background. PilT is an ATPase with a specific activity of 15.7 nmoles ATP hydrolyzed/min/mg protein *in vitro*. This activity was abolished when a conserved lysine in the nucleotide binding motif was altered. Presence of the hexahistidine tag at the C- or N-terminus led to similar behavior during purification and similar specific activity. Both PilT and Lys149Gln variant PilT were identified as an ~5-6 subunit oligomer at concentrations above ~1.1 mg/ml. At lower concentrations, the protein is apparently monomeric. Circular dichroism measurements also yielded virtually indistinguishable results for mutant and wild type protein, and indicated an alpha-helical content of ~30%.

An implication of the demonstrated ATPase activity is that PilT could act directly as a retraction motor. The modest specific activity observed would likely need to be stimulated *in vivo* by another interacting protein or membrane component to achieve the observed retraction rates. Alternatively, ATP hydrolysis may not be required for each pilin monomer retracted but could instead set PilT in a retraction-ready open conformation. We have crystallized this protein and are pursuing structural studies in order to further address mechanistic questions.

Evidence that the PilV protein of *Neisseria gonorrhoeae* interacts with the Tfp biogenesis machinery and influences post-translational modification of PilE, the pilin subunit

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The expression of Type IV pili (Tfp) by *Neisseria gonorrhoeae* is crucial for the bacteria's ability to adhere to epithelial cells and colonise its human host. PilV, a prepilin-like molecule identified by data mining of the gonococcal genome sequence, is dispensable for Tfp expression but involved in Tfp mediated adherence. The role of PilV in adherence is unkown although it appears to influence the levels of the adhesin PilC which co-purifies with Tfp. In addition, PilV co-purifies with Tfp in a PilC dependent fashion. As opposed to many of the other Tfp involved genes in *N. gonorrhoeae, pilV* did not appear to be linked to other genes influencing Tfp biology. To examine this in more detail, transposon insertion mutations in the surrounding genes were constructed but were found to have no discernible effects on Tfp associated phenotypes.

PilV has a consensus PilD prepilin peptidase cleavage site at its N-terminus and its migration in SDS-PAGE is decreased in a *pilD* background. To verify that the altered migration is due to lack of processing, a PilV missense mutant was created in which the Gly_{-1} residue (essential to PilD proteolysis in Tfp subunits) was substituted by a Ser. This altered PilV migrated at the same retarded position as PilV in the *pilD* background. Surprisingly, the PilV missense mutant did not express Tfp as assessed by purification and EM techniques. Therefore, this defective form of PilV impacts on the biogenesis pathway. Remarkably, the Tfp expression defect in this background could be suppressed by either loss-of-function mutations in *pilT*, encoding a pilus retraction ATPase, or overexpression of *pilE*, encoding the pilus subunit protein. These findings indicate that the missense PilV molecule disrupts the biogenesis pathway by altering the dynamics of fiber growth and retraction and demonstrates for the first time that the levels of the Tfp subunit (PilE) can influence the equilibrium balancing polymerization / depolymerization. Biochemical characterization of these piliated suppressor mutants reveal that the mechanism by which Tfp expression is restored is different in the two backgrounds. Results of human epithelial cell adherence assays support this interpretation since in one case, adherence is restored while in the other it is not.

In the course of investigation, we noted that PilE in wildtype (wt) background actually migrates as a doublet in SDS-PAGE, and that in *pilV* null mutants only the slower migrating form is present. The influence of PilV on PilE migration was confirmed by the observation that overexpression of PilV resulted in the presence of only the faster migrating form. Mass spectrometry analysis of purified PilE from these backgrounds confirm that PilV is associated with an altered pattern of a post-translational modification of PilE.

Together, these findings raise the possibility that the altered adherence phenotype of *pilV* mutants may be a consequence of altered post-translational modification of PilE.

Towards an improved neisserial surface protein A (NspA)-based vaccine

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Introduction: Recombinant NspA vaccine is currently in clinical trials. Although NspA is highly conserved across *N. meningitidis*, both native and rNspA are poorly immunogenic in experimental animals. rNspA also has been difficult to formulate into an effective vaccine for humans. Our previous studies showed that despite *nspa* gene conservation there are large differences in surface-accessible NspA among *N. meningitidis* strains (Moe et al, Infect Immun 1999 and 2001). Further, both the anti-NspA antibody concentration and epitope specificity are important in conferring protection. With the long-term goal of developing a more effective NspA-based vaccine, we produced a panel of bactericidal anti-NspA mAbs for use in epitope mapping. Initial attempts to identify epitope specificity using overlapping peptides proved unsuccessful.
Methods: We are now using a genetic approach to identify naturally-occurring polymorphisms in different Nm and non-*meningitidis* strains [i.e., *lactamica* (Nl) and *gonorrhoeae* (Ng)] that express NspA and have heterogeneity in mAb binding. The respective NspA genes have been cloned into a multicopy plasmid for sequencing, expression of recombinant protein in *E. coli*, and for site-directed mutagenesis.
Results: We observed heterogeneity in binding of two bactericidal mAbs, AL12 (prepared to rNspA) and 14C7 (prepared to native NspA), amongst a panel of Nm, Nl and Ng strains. For example, both AL12 and 14C7 bind strongly to Nm strain 8047. Neither mAb recognizes NspA in Nm strain MCH88 by whole cell ELISA or dot blot assays although MCH88 produces NspA as detected by Western blot developed with polyclonal anti-rNspA antisera. A comparison of the respective amino acid sequences inferred from the *nspa* genes of Nm strains 8047 and MCH88 reveals two amino acid differences in putative loop 2 (YK-*A*PS vs. YK*QV*PS, respectively), and two in putative loop 3 (VD*LG*GS vs. VD*FN*GS, respectively). Nl strain A5906 has the same loop 2 sequence as that of Nm MCH88 but still binds with mAb AL12, a result suggesting that the loop 3 polymorphism accounts for lack of mAb binding with MCH88. To test this hypothesis, we mutated the gene encoding loop 3 of Nm 8047 to match that of Nm MCH88 and expressed the native and mutated genes in *E. coli*. Although both proteins were expressed by *E. coli* , the anti-NspA mAbs no longer recognized the mutant protein. The converse mutation whereby the Nm MCH88 loop 3 is changed to Nm 8047 loop 3 sequence is being made.
Conclusions: (i). Loop 3, which is highly conserved across *N. meningitidis*, is an important target of anti-NspA bactericidal antibodies. (ii). The lack of binding to overlapping peptides is consistent with the epitopes being discontinuous and/or being conformationally-dependent. (iii). Vaccine formulations that optimally present loop 3 epitopes may enhance protective antibodies.

The addition of O-6 linked PEA to the lipooligosaccharide inner core is regulated by PhoP in *Neisseria meningitidis* strain NMB

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Introduction: Meningococcal lipooligosaccharide has been classified into 12 immunotypes (L1-L12) based upon monoclonal antibody recognition. Examination of the chemically defined structures reveals that the inner core, $Glc_1GlcNAc_1Hep_2Kdo_2$-lipid A has three variable substitutions made to Heptose II (HepII). These are 1) the addition of O-6 or O-7 linked phosphoethanolamine (PEtn) as seen in immunotypes L2/4/6, 2) the addition of O-3 linked PEtn by Lpt-3 as seen in L1/3/7/8/9, and 3) the addition of 3-linked glucose by LgtG as seen in L2 and L5. The roles of these substitutions are currently not well understood, although the addition of O-3 linked PEtn to HepII has been recently shown to improve resistance to bactericidal killing and opsonophagocytosis. We are currently investigating the genetic basis for the addition of O-6 linked PEA to the LOS inner core of the immunotype L2 strain NMB.
Materials and Methods: We examined the LOS structure of the *phoP* mutant of strain NMB (NMBΔ*phoP*) which is four fold more sensitive to the antimicrobial peptide polymyxin B than the parent strain. Sensitivity to polymyxin B has been shown to correlate with the PEtn content on the inner core and lipid A of LPS in *Salmonella* and *Pasteurella*. Glycosyl composition analysis of the LOSs of NMBΔ*phoP* was accomplished by GC-MS analysis of trimethylsilyl (TMS) methylglycosides. The oligosaccharides (OSs), liberated from the LOS by mild acid treatment, were incubated with aqueous HF to remove all phosphate moieties. The resulting OS-HF samples were then analyzed for glycosyl composition as above and for glycosyl linkages by GC-MS analysis of partially methylated alditol acetates (PMAAs).
Results and Discussion: MALDI-TOF MS analysis of OS-HF samples from the NMBΔ*phoP* mutant LOSs in the positive mode gave two molecular ions at *m/z* 1681 $[M+Na-H2O]^+$ and 1699 $[M+Na]^+$; which were consistent with the reported major L2 oligosaccharide structure observed in NMB LOS. However, comparison of the NMBΔ*phoP* LOS with NMB LOS showed a significant difference in the level of 2,3-linked Hep II. In the NMBΔ*phoP* LOS there was virtually no difference between the LOS and the OS-HF sample; ie. levels of 2,3- linked Hep II were 10% and 12% respectively. However, in NMB LOS, only trace levels of 2,3-linked Hep II were observed, which increased to 10% upon HF-treatment. These results indicate that the Hep II residue in the LOSs from NMBΔ*phoP* mutant is not phosphorylated (at the O-6 or O-7 position) with a PEtn group, while in the wild type NMB LOS the Hep II is completely substituted with PEtn.
Conclusion: The loss of O-6/7 linked PEtn from the L2 immunotype LOS in the *phoP* mutant of strain NMB indicates that the transferase required for this step is transcriptionally regulated by *phoP*. Thus, PhoP appears to control the heterogeneity of the LOS inner core structure and may infuence immunogenicity.

A system for efficient cloning and expression of meningococcal proteins

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Introduction: Many meningococcal proteins, mainly outer membrane proteins, are toxic for *E. coli* and are therefore very difficult to clone and express. Cloning of intact toxic proteins is only possible when systems with very tight regulation of expression are used. Proteins are less toxic for *E. coli* when cloned without their corresponding signal sequence, but then the protein is often localized in inclusion bodies, raising the problem of subsequent denaturation and correct in vitro folding of the protein.
Materials and Methods: In an attempt to find a system for routine cloning and expression of a large variety of meningococcal proteins, we tested three different vector/host systems: the pET system (Novagen), the pQE system (Qiagen) and a heat-inducible vector (pMA5-8P_L). As a model protein known to be very difficult to clone and express in *E. coli*, we used three different fragments of the IgA1 protease of *Neisseria meningitidis*: 1) the 196 kDa precursor, 2) the precursor lacking the 27 aa long N-terminal leader peptide and 3) the mature protease (105 kDa) lacking both leader peptide and the C-terminal helper domain required for secretion. The desired gene fragments were amplified by PCR, ligated into the vector and electro-porated into the appropriate *E. coli* strains. Protein expression of whole cell lysates was analysed by SDS-PAGE and western blotting before and after induction.
Results and Discussion: The best results were obtained using the pQE system, with cloning efficiencies of up to 100% and very strong expression of mature IgA1 protease and weak secretion of mature protease into the medium resulting from precursor processing. To confirm the suitability of this system, we tested it with four further toxic proteins: the adhesin OpcA, the porins PorA and PorB and the pilus secretin PilQ from *N. meningitidis*. We were able to clone the genes for these proteins both with and without leader peptide. Protein expression was intermediate to strong, and processing of the precursor proteins was also shown.

Using the pQE system, we have cloned 200 meningococcal open reading frames (ORFs). 80% of the proteins ranging in size from 8 kDa to 200 kDa were expressed by *E. coli* and can be purified via their N-terminal His-Tag. These proteins comprise known virulence factors and outer membrane proteins, as well as putative membrane proteins and hypothetical proteins of unknown function. Although the proteins were cloned with their corresponding signal sequences, strong protein expression is seen in most cases, a goal that is very difficult to achieve with other vector systems. Due to the tight control of expression in the pQE system, meningococcal proteins that were thought to be toxic for *E. coli* were efficiently cloned and expressed after induction.

Redirecting the immune response towards conserved domains of the *Neisseria meningitidis* FrpB protein

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FrpB is the most abundant iron-limitation-inducible outer-membrane protein of *Neisseria meningitidis*. The high expression levels together with considerable sequence conservation between strains and its ability to induce bactericidal antibodies make it an interesting vaccine candidate for the prevention of meningococcal disease. However, the protein does contain some variability, which is mostly confined to two large extracellular loops, L3 and L5, respectively (van der Ley et al. 1996).

Immunization of mice with outer-membrane complexes of iron-stressed cells induced FrpB-specific bactericidal antibodies (Pettersson et al. 1990). However, these antibodies were all directed against the variable loop L5, which is apparently immunodominant. Consistently, these antibodies were poorly cross-reactive between strains. In an attempt to direct the immune response to the more conserved parts of the protein, a deletion of 28 amino acid residues was created in L5. Immunization with outer-membrane complexes of the L5 mutant yielded three monoclonal antibodies, which were bactericidal against strains expressing the mutant protein. However, Western-blot analysis of six different strains did not show cross-reactivity of these antibodies. Consistently, epitope mapping showed that these antibodies were directed against the other variable loop, L3. Furthermore, no bactericidal activity was observed against cells expressing the wild-type protein, indicating shielding of other parts of the protein by L5. Indeed, in ELISAs with antisera directed against synthetic oligopeptides corresponding to various cell surface-exposed loops, improved accessibility of FrpB domains was observed after removal of L5.

By removing L3 in addition to L5, we hope to be able to construct an FrpB protein that consists of only very-well conserved domains of FrpB, which could potentially induce broad-spectrum bactericidal antibodies. As immunization studies with outer-membrane complexes require a lot of screening, large quantities of pure protein would facilitate our studies. Therefore, FrpB and FrpB loop-deletion mutants were produced in inclusion bodies in *Escherichia coli* and subsequently refolded *in vitro*. Refolding was successful and immune responses against these proteins will be analysed.

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Production and characterization of monoclonal antibodies specific for PorA loop 5 of *Neisseria meningitidis*

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Introduction: Meningococcal outer membrane vesicle vaccines maily induce antibodies against the outer membrane protein PorA. Bactericidal antibodies are predominantly directed to loop 4 and to a lesser degree to loop 1 of this protein. Next to the variable regions (VR) on loop 1 and loop 4 of PorA, a third VR is situated on the top of loop 5. This region is more conserved than VR1 and VR2 and a monoclonal antibody (Mab) named MN19D6.13 (a-P1.6), specific for loop 5, was bactericidal. This Mab was elicited by immunising Balb/C mice with outer membrane complexes of strain 6940 (serosubtype P1.18,25,6). In this study we raised antibodies to loop 5 by immunising mice with conjugated cyclic peptides and analysed their bactericidal activity.
Materials and Methods: Three cyclic peptides with the sequences LFLIGSGSDQA (cVR3-1), LFLLGSGSDQA (cVR3-2) and LFLLGRIGDDD (cVR3-3) were chosen because they cover the far majority of all loop 5 sequences of meningococci. Peptides were prepared by "head to tail" solid-phase cyclisation and conjugated to tetanus toxoid. Balb/C mice were immunised with 50 µg of conjugate and Quil A as adjuvant on days 0, 14, 28 and boostered at day 52. Three days after the booster spleen cells were used for fusion with SP/0 myeloma cells. Specificity of Mabs was tested in whole cell ELISA on several reference strains and in inhibition ELISA using overlapping linear peptides as inhibitor: HANVGRDAFNLFLLG (I), RDAFNLFLLGRIGEG (II), LFLLGRIGEGDEAKG (III) and RIGEGDEAKGTDPLK (IV). Functional activity of antibodies was measured in serum bactericidal assay (SBA).
Results and Discussion: Immunisation with peptide cVR3-1 yielded Mab-1 which recognised strains with LFLIGSGSDQA but also strains with LFLIGSATSDQA on loop 5. Mab-2 was elicited with cVR3-2 and recognised strains with GSGSDQA but not GSGSDEA on loop 5. Peptide cVR3-3 yielded two Mabs: Mab-3 bound only to strains with the same sequence as the immunogen and Mab-4 bound to strains with LFLLGRIGDDD, LFLLGRIGEDD or LFLLGRIGEGD. The a-P1.6 Mab reacted with LFLLGRIGEDD or LFLLGRIGEGD containing strains. An inhibition ELISA showed that the binding of the a-P1.6 Mab on OMCs of strain 6940 was blocked by peptides I and II while Mab-4 was inhibited by peptide II and III. These results pointed to distinct specificity for the a-P1.6 Mab and Mab-4. None of the four new Mabs showed bactericidal activity.
Conclusions: Mabs elicited by immunising with cyclic peptides are useful reagents for detection of a broad range of PorA proteins in intact bacteria and also in denatured bacterial fractions. Apparantly only some epitopes on loop 5 are capable of inducing bactericidal antibodies.

Peptides binding the LOS specific monoclonal 9-2-L379 expressed as fusions to the hepatitis B core protein

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Introduction: Lipooligosaccharides (LOS) are important antigens on the surface of *Neisseria meningitidis* and antibodies to LOS are bactericidal. However, LOS is highly toxic in humans and it is a T-cell independent antigen. Therefore considerable interest exist in developing peptide-mimotopes of LOS epitopes, thereby being able to convert them to T-cell dependent antigens which can be used in a vaccine.
Materials and Methods: The monoclonal antibody (mAb) 9-2- L3,7,9 (mouse IgG2a) reactive against Neisseria meningococcal LOS immunotype L3,7,9 was kindly obtained from Dr. W.D. Zollinger (WRAIR), and used to select antibody binding peptides from two different phage display libraries with cyclic 6-mers and 9-mers, respectively. A system was developed for the direct cloning of phage selected peptides as fusions to the immunodominant loop of the core protein of hepatit B virus (HBcAg). HBcAg- peptide fusions were expressed in *E. coli* and shown to form HBcAg particles.
Results and Discussion: Several peptides which were able to inhibit LOS binding of mAb 9-2 L3,7,9 in a dose dependent manner, were selected by panning procedures. We investigated whether the peptides could represent LOS mimotopes and give rise to an antiLOS specific immune response. In an ELISA assay mAb 9-2-L3,7,9 reacted specifically with core particles displaying the selected peptides. Immunization of Balb/C mice with either these particles or with the peptides conjugated to KLH revealed a significant anti-peptide specific immune response, but no detectable anti-LOS activity. These results suggest that the peptides are functionally presented on the HBc particle, although they do not act as LOS mimotopes.
Conclusion: The HBcAg system reveals an easy way to screen a large number of phage display derived peptides as fusions to the HBcAg. The selected peptides do not act as LOS mimitopes neither when conjugated to KLH protein nor as HBcAg-fusion protein.

Novel putative surface antigens from *N. meningitidis* strain MC58 which are consistently recognised by antibodies from patients convalescing from meningococcal disease

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Development of a vaccine against serogroup B meningococcal disease has been hampered by a lack of bacterial antigens capable of eliciting a strong bactericidal antibody response against a broad range of meningococcal isolates. In order to discover new antigens which are antigenically conserved in the target bacterial population and can elicit bactericidal antibodies, the genome sequence of the strain MC58 has been studied for genes encoding novel proteins predicted to be exported out of the bacterial cytoplasm. As reported previously[1], 350 such proteins have been purified from *E. coli* in a recombinant form and used in immunisation studies in animals.

We report here the results of screening a subset of these novel recombinant antigens using human sera in order to select those which are consistently recognised by antibodies from young patients convalescing from meningococcal disease. Subjects aged 4 years and under were chosen in order to select antigens which are immunogenic in the age group most susceptible to disease.

Antibody responses to more than 180 recombinant antigens were examined in convalescent sera using a quantitative dot blotting procedure. 19 of the proteins stood out in eliciting significant antibody responses in more than two thirds of 31 sera from patients infected with a broad range of serogroup B and C strains. 12 of the 19 proteins were particularly strongly recognised by the majority of the sera (at least 28 out of 31).

Further dot blotting, comparing convalescent sera to sera from uninfected children in the same age group, revealed that many of the 19 proteins that were consistently recognised by convalescent sera were as strongly recognised by sera from uninfected children. This was unexpected. We speculate that this reaction may be due to these antigens being conserved between *N. meningitidis* and commensal *Neisseria* species such as *N. lactamica*, but this will need to be investigated further. Four proteins appeared to be more strongly recognised by convalescent sera than by control sera, suggesting the possibility that these proteins may have elicited antibodies during disease. The presence of the gene encoding one of the four proteins, NadA, was determined in all of the meningococcal strains responsible for disease in our patients. (It was present in 12 out of 31.) Comparison of meningococcal *NadA* genotype with dot blotting results revealed that sera containing the highest levels of anti-NadA antibody did indeed come from the patients infected with strains carrying *NadA*. Western blotting confirmed that anti-NadA antibodies bound to the target protein. NadA is a candidate vaccine antigen potentially protective against disease caused by approximately 50% of currently prevalent serogroup B strains[2].

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Recombinant NspA incorporated into liposomal vesicles induces functional antibodies

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Background: The NspA protein was shown to be antigenically highly conserved and present in the outer membrane of *Neisseria meningitidis* where it is accessible to specific antibodies. It was observed that after purification, in vitro folding of the recombinant NspA (rNspA) was required to induce bactericidal antibodies.
Objective: Evaluate the efficiency of liposomal vesicles to generate correctly folded rNspA and analyze the immune response induced by these NspA-liposomal vesicles.
Methods: Highly purified rNspA was unfolded in vitro and then reconstituted into different liposomal formulations. Mice and rabbits were immunized with these rNspA-liposomal formulations or with rNspA adsorbed onto Alhydrogel adjuvant. Sera were analyzed by ELISA using meningococcal outer membrane preparations as coating antigen, cytofluorometric and in vitro bactericidal assays.
Results: NspA-specific monoclonal antibodies directed against conformational epitopes recognized the rNspA that was incorporated into liposomal vesicles. Immunization of mice and rabbits with these NspA-liposomal vesicles induced rise in antibodies (Abs) directed against the native NspA protein comparatively to the Abs response determined for sera obtained from mice immunized with purified NspA adsorbed to Alhydrogel. Cytofluorometric assay also indicated that the NspA-specific Abs present in these sera efficiently recognized their specific epitopes at the surface of serologically distinct meningococcal strains. In vitro bactericidal assay confirmed that immunization with rNspA incorporated into liposomal vesicles can induce the production of functional antibodies capable of killing meningococcal cells.
Conclusion: Reconstitution of the rNspA into its native conformation was efficiently achieved after incorporation into liposomal vesicles.

Identification and molecular characterization of a novel ADP-ribosyltransferase protein from *Neisseria meningitidis*

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Introduction: Mono ADP-ribosylation is a posttranslational modification that profoundly affects eukaryotic pathways modulating or inhibiting protein functions.

In Prokaryotes proteins of this family are classified as toxins and are often responsible for the pathology caused by the related microorganisms. A large number of Gram-positive and Gram-negative bacteria have shown to produce ADP-ribosylating toxins and although they display the same catalytic activity, their primary structures are poorly conserved. Nevertheless, the active site organization is conserved and the similarity is restricted to short regions, which delineate the structure of the catalytic cleft. By computational analysis, we have identified a gene coding for a putative ADP-ribosylating toxin in Meningococcus B. Here we describe the properties of this novel enzyme, the sequence similarity with other ADP-ribosyltransferases, the conservation among different Neisseria species and the *in vitro* activity.

Materials and Methods: A degenerated amino acid pattern, based on the consensus derived from known prokaryotic ADP-ribosyltransferases was used (FindPatterns) to screen the MC58 genomic sequence for novel putative members of this group of bacterial toxins. One of the hits, NM-ADPRT, could be nicely aligned to LT and CT ADP-ribosylating toxins, and displayed a good sequence conservation in the regions of the catalytic site (Pileup). To verify the presence of *nm-adprt* in other selected Neisseria isolates, PCR experiments were designed using external primers and the product was directly sequenced. The *nm-adprt* gene product was expressed in *Escheichia coli* as C-terminal Histidine-tag fusion protein and purified to homogenicity. Recombinant NM-ADPRT was used in assays containing [*adenine*-U-^{14}C]NAD to monitor the synthesis of ADP-ribosylagmatine and the release of nicotinamide from NAD, respectively.

Results and Discussion: The *nm-adprt* gene is characterized by a surprisingly low GC content of 35% with respect to the average GC composition of 51,5% calculated for *Neisseria meningitidis*. The gene is present in all strains belonging to the ET-5 and Lineage 3 hypervirulent complexes analyzed, whereas is absent in the ET-37 and in all the serogroup A strains analyzed. When the gene is present, the sequence is 100% conserved. These observations suggest that the gene might have been acquired by a mechanism of horizontal transfer. The recombinant protein has been purified and used to verify the predicted enzymatic activities. The results show that NM-ADPRT possesses both NAD-glycohydrolase and ADP-ribosyltransferase activities. Using different substrates we have shown that, as in the case of CT and LT toxins, the amino acid acceptor for the ADP-ribose is the arginine.

Conclusion: We have identified a novel ADP-ribosylating enzyme produced by ET5 and lineage 3 hypervirulent clusters of *N. meningitidis*. This protein may represent a strain-specific virulence determinant of Meningococcus. *In vitro* experiments are underway to evaluate the role of this protein in host cell damage and in the virulence of *Neisseria*.

Immunogenicity of individual epitopes of gonococcal transferrin binding protein A (TbpA)

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TbpA is a TonB dependent, integral outer membrane protein, which comprises one component of the gonococcal transferrin receptor complex. This complex is the first point of contact between the pathogen and the host iron binding protein transferrin. TbpA is proposed to form a gated transporter, through which Tf-released iron passes and gains entry to the periplasm. TbpA is highly conserved among the pathogenic *Neisseria*, and it is absolutely required for Tf-iron acquisition, while its counterpart, TbpB is lipid modified, more variable, and thought to increase the efficiency of iron uptake from Tf by binding the ferrated form of transferrin preferentially. TbpA's critical role in Tf-iron uptake and its conservation make it an attractive vaccine candidate. More specifically, we propose that individual epitopes of TbpA may be sufficient to elicit an effective, more targeted immune response. To determine if individual epitopes of TbpA are immunogenic, we immunized rabbits with recombinant fusion proteins in the form of inclusion body preparations, and assessed the generated antisera for reactivity to denatured TbpA by Western Blot analysis and to native TbpA utilizing a whole cell dot blot format. All but one of the anti-epitope sera recognized full length denatured TbpA in Western Blots. Antisera elicited against constructs L5, L4 + 5, and L2 appear to recognize native TbpA on the surface of whole GC, consistent with our current topology map that suggests these regions are surface exposed. We also evaluated the cross reactivity of the epitope fusion antisera among TbpAs expressed by 13 different gonococcal strains in Western analysis. The epitope specific sera were found to be more cross-reactive to a gonococcal panel than the previously developed anti-TbpA peptide sera, albeit with variable intensities, indicating that epitope conservation exists within these longer regions of TbpA. Additionally, we investigated whether polyclonal sera, produced against full length TbpA recognize specific linear TbpA epitopes preferentially via Western Blot analysis. A polyclonal serum raised against denatured gonococcal TbpA recognizes the plug region predominately, whereas sera raised against native gonococcal or meningococcal rTbpA principally recognize putative L2. We are in the process of functional characterization of the loop-fusion sera with regard to complement-mediated bactericidal killing and blocking of Tf binding, to assess whether specific TbpA epitopes may be sufficient to elicit a protective immune response.

The P1.16 epitope on the PorA protein of *Neisseria meningitidis* is a better target for antibodies to induce bactericidal activity than the P1.7 epitope, but the two epitopes are equally potent for antibodies to induce phagocytosis

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Introduction: The PorA porin protein on the outer membrane of *Neisseria meningitidis* is an important vaccine antigen. The protein is one of the main targets for bactericidal antibodies induced by the Norwegian outer membrane vesicle (OMV) Group B meningococcal vaccine. The PorA protein express two immunodominant epitopes located to variable region 1 (VR1) and variable region 2 (VR2) on the extracellular loops I and IV, respectively. It has been a matter of debate how important these two regions are in inducing protective immunity.
Materials and Methods: We constructed chimeric antibodies of human IgG1, IgG3 and IgM isotype against a VR1 epitope (P1.7) and a VR2 epitope (P1.16) by isolating the V_L- and V_H -genes of three mouse mAbs and subcloning them into expression vectors followed by transfection to NS0 cells. Chimeric anti-P1.16 and anti-P1.7 mAbs produced by these cells, were tested for bactericidal activity (SBA) and opsonophagocytosis (OP) against group B meningococci by a colony forming unit (CFU) reduction assay and by flowcytometry, respectively.
Results and Discussion: On molar basis the P1.16 antibodies were 10 times more efficient in SBA than the corresponding P1.7 antibodies. On the other hand, in an opsonophagocytic assay they were equally efficient. A possible explanation is the more distant localization of the P1.7 epitope relative to the bacterial membrane, since VR1 has a longer loop than VR2. Thus, P1.7 antibodies might be expected to be less efficient in depositing membrane attack complexes (MAC) on the bacterial membrane than P1.16 antibodies, and are therefore less bactericidal. OP on the other hand, is induced by Fc-receptors (FcγR) and complement receptors (CR) on effector cells. Interaction with these receptors is less dependent on the distance from the bacterial membrane to the target epitope. On the contrary, the further out the bacterial epitopes are, the better interaction with phagocytic effector cells? This observation is important in relation to development of vaccines and antibody preparations for clinical use.
Conclusion: The P1.16 epitope is a better target for SBA activity of antibodies than the P1.7 epitope, while both these PorA epitopes are equally potent targets for OP activity of antibodies.

Antigen discovery in *Neisseria meningitidis* using human antibody phage display and meningococcal genomic DNA phage display

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Introduction: A vaccine to prevent disease caused by *Neisseria meningitidis* serogroup B is an acknowledged health priority. Much work has focused on the search for a subcapsular antigen or group of antigens widely recognised by the human immune system, a search so far frustrated by the extreme variability of exposed protein structures in the meningococcus. The recent publication of the genome sequence of *N. meningitidis* has allowed a number of molecular approaches to be applied. We have chosen to explore the meningococcal surface using human antibody libraries displayed on bacteriophage. Subsequent identification of the antigens recognised by our selected antibody clones has been achieved by panning a second phage library displaying peptides generated from random segments of meningococcal DNA against the purified antibodies.
Materials and Methods: Adult humans have naturally-acquired bactericidal antibody against *N. meningitidis*. A diverse (>1.3x 10^{10} recombinants) library of human single chain Fv (scFv) antibody fragments, expressed on the surface of M13 bacteriophage through cloning of antibody V-region genes in a phagemid vector, was panned against outer membrane vesicles (OMVs) produced from *N. meningitidis* strain 44/76 (B:15:P1.7,16). Three scFv clones were identified after three rounds of panning which showed strong binding to the OMVs by ELISA. Soluble scFvs from two of these clones were expressed in E.coli TG1 and purified. These scFvs were then used to pan a phage library expressing random fragments of meningococcal DNA from strain MC58 (also B:15:P1.7,16). The library was prepared by ligation of sheared meningococcal chromosomal DNA (200-500 base pairs in length) into the phagemid vector pG8SAET allowing random fusions with Protein VIII of filamentous phage. The peptide products of these fragments are then expressed on the surface of the M13 bacteriophage.
Results and Discussion: After two rounds of panning a dominant meningococcal clone was identified which showed binding to both scFv clones. Sequencing revealed that it contained virtually the complete open reading frame of a meningococcal stress response protein, one of the cold-shock family.
Conclusion: The use of two different phage display techniques in concert will allow us to identify meningococcal antigens with vaccine potential. ScFv libraries prepared from lymphocytes harvested from patients convalescent after meningococcal disease are being constructed which should be enriched for meningococcus-reactive antibody clones, which may identify prospective protective vaccine antigens.

Production of "immune" antibody phage display libraries from the peripheral B-cells of patients convalescent following infection with *Neisseria meningitidis* serogroup B. A tool for the study of antibody responses and antigen discovery

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Introduction: *Neisseria meningitidis* serogroup B vaccine research has largely focused on the search for an antigen or group of antigens that is both well conserved among meningococcal strains and immunogenic in humans. Patients convalescent after meningococcal disease have been shown to possess immunity against multiple meningococcal strains and are the study of their immune responses may prove in discovering such antigens. Antibody phage display libraries, consist of antibody variable (V) domains expressed on the surface of M13 bacteriophage (producing phage antibodies) and can be used to select specific antibodies. Such libraries may be based on B-cells from either a non-immune subject (naïve libraries) or from a subject immunised through exposure to antigens of interest (immune libraries). Immune libraries are enriched with antibodies which have undergone affinity maturation by the immune system and are useful both as sources of high affinity antibodies and as a means of analysing immune responses.

This project aims to utilise immune antibody phage display libraries to discover conserved epitopes on the surface of *N. meningitidis* serogroup B which are immunogenic in humans.

Materials and Methods: 100ml blood samples were collected from 10 patients 4-8 weeks convalescent after meningococcal disease. Peripheral blood mononuclear cells and serum from each patient were separated and stored. The infecting strain for each of these patients was also archived. Serum samples were analysed for cross-reactive immunogenicity using the serum bactericidal assay against multiple clinical serogroup B strains. The mononuclear cells for those patients with good cross-reactive responses were taken forward for library production.

The heavy chain variable (VH) genes were amplified using PCR and VH family specific primers containing restriction sites for cloning. The amplified VH genes were then cloned into the phagemid vector pHen-VL-rep that contains a naïve repertoire (10^4) of human VL genes. Following transformation into *Escherichia coli* the libraries were rescued using VCS-M13 helper phage to produce phage-displayed libraries of single chain Fv fragments (scFvs).

Results: Two antibody libraries, each containing $>10^7$ clones were produced. PCR fingerprinting and V gene sequencing showed that both libraries contained a diverse repertoire of VH genes. The libraries were panned against *N. meningitidis* outer membrane vesicles, recombinant Neisserial antigens and whole *N. meningitidis* cells. To date, 10 antibody clones that bind intact *N. meningitidis* cells have been isolated.

Discussion: Two immune libraries have been produced from the peripheral B-cells of patients convalescent after serogroup B meningococcal disease. From these libraries antibodies with specificity for surface antigens of *N. meningitidis* have been isolated. Experiments to identify the antigens recognized by these antibodies are underway.

A paucity of local and systemic antibodies to recombinant *Neisseria gonorrhoeae* transferrin binding proteins A and B

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The transferrin binding proteins, TbpA and TbpB, have generated considerable interest as possible vaccine antigens to protect against infection by both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Vaccine studies with *N. meningitidis* Tbps have demonstrated production of bactericidal antibodies, and protection from systemic infection in immunized mice. TbpA is an especially attractive antigen because of its antigenic stability, and its ability to produce antibodies that are cross-reactive against *N. meningitidis* TbpA. The importance of these antigens in virulence has also been demostrated in a human male experimental infection model. A TbpA-/TbpB- derivative of a gonococcal strain that naturally lacks the lactoferrin receptor (FA1090) was constructed and inoculated into human male volunteers. Individuals inoculated with this mutant strain demonstrated no signs or symptoms of urethritis, indicating that expression of these proteins is important in enabling *N. gonorrhoeae* to gain a foothold in the host. By contrast, little is known about the immunogenicity of these proteins during a natural gonococcal infection. To address this issue, we performed enzyme-linked immunosorbant assays (ELISA) to quantitate the immune response generated during a natural infection, and to better characterize any antibody isotypes elicited. To this end, we tested a panel of male and female serum and mucosal secretions from individuals infected with *N. gonorrhoeae*. Individuals with no history of gonorrhea were used as negative controls. Recombinant TbpA and TbpB were used as antigens for our ELISAs. Tbp antigens were over-expressed in *E. coli* and purified by affinity chromatography under non-denaturing conditions. Subsequent to purification, the recombinant Tbp proteins were analyzed in dot blot assays to assess their ability to bind HRP-conjugated human transferrin. Serum and mucosal secretions were then tested for antibodies specific to these rTbps using ELISA. Anti-Tbp antibody concentrations were calculated from a standard curve generated with anti-human capture antibody bound to the plate with a known concentration of human immunoglobulin serving as antigen. Total serum and secretion antibodies were calculated using the same capture anti-human immunoglobulins. Although Tbp-specific antibodies were detected in sera of both male and female patients, concentrations were relatively low, and did not significantly differ from those detected in negative control samples. Mucosal responses were even lower than those seen in sera, and generally below the limits of detection of our assay. Overall, this study demonstrated a paucity of systemic and local antibody responses to rTbps as a result of natural infection. These results are consistent with those of a previous study, which showed limited antibody responses to whole, formaldehyde-fixed gonococci. Taken together, these studies could have significant implications for the development of a vaccine, as induction and sustained production of anti-Tbp antibodies could be important in generating immunity against infection.

Peptide-peptide interactions between transferrin and transferrin binding protein B

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Pathogenic bacteria of the Neisseriaceae are able to acquire iron from their mammalian hosts via a receptor-mediated interaction with the iron binding protein transferrin. Transferrin (Tf) is a bi-lobed monomeric glycoprotein found in the serum that binds iron in order to sequester it from invading organisms. The two lobes are virtually identical in overall structure, each consisting of two domains connected by a hinge region with an interdomain cleft that serves as the site for metal and anion binding. The bacterial Tf receptor consists of two iron repressible outer membrane proteins, transferrin binding protein A and B (TbpA, TbpB). TbpA is an integral transmembrane protein that may act as a gated pore for iron passage. TbpB is a peripheral outer membrane lipoprotein anchored to the outer membrane via a N-terminal linked fatty acid.

TbpB is a bi-lobed protein with Tf-binding regions present in both the N and C-terminal halves of the protein. To investigate the lobe-lobe interactions, recombinant forms of the individual hTf lobes and of the individual lobes of TbpB from *N. meningitidis* were prepared. Their interactions were assessed by a combination of solid-phase binding assays, affinity isolation assays and a novel frontal affinity chromatography method coupled to mass spectrometry. A lobe-lobe preference was demonstrated that predicts a preferred orientation of Tf in the receptor-ligand complex.

To further delineate the Tf-TbpB interaction, a solid-phase overlapping synthetic peptide library of hTf was probed with labeled TbpB from two representative strains, M982 and B16B6. The results demonstrated that these two strains identified an identical set of peptides on hTf. When labeled hTf was used to probe libraries representing the two TbpBs, a series of hTf-binding peptides were identified but clearly not an equivalent set of identical peptides from the two different TbpBs. To confirm the validity of the hTf binding regions on TbpB and compare the binding regions between TbpBs from the two species, a series of truncations of the TbpB N-lobe or TbpB C-lobe were used to probe the hTf binding peptides from the hTf C-lobe and N-lobe, respectively. This analysis also enabled us to identify several of the specific peptide-peptide interactions between hTf and TbpB. The results demonstrated that the equivalent binding regions from the two TbpBs that recognized identical peptides from hTf did not contain identical binding peptides. This indicates that the conserved structural features of these binding determinants must be provided by regions consisting of differing amino acid sequence. Structural studies with receptor-ligand complex will be required to substantiate this hypothesis.

Identification of genes necessary for pilin variation in iron-replete or iron-limited *Neisseria gonorrhoeae*

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Introduction: *Neisseria gonorrhoeae* antigenically and/or phase vary a number of surface exposed molecules. Antigenic and phase variation are responsible for immune evasion during an infection, and contribute to reasons why antibodies produced during an infection do not protect an individual from a subsequent infection. One molecule that antigenically varies is the pilin protein, the principle subunit of the type IV pilus. Pilin variation results from high frequency, RecA-dependent gene conversion events mediated by the RecF-like pathway members *recO, recQ,* and *recJ*. Our lab recently found that iron-limited growth of gonococci results in increased pilin variation as well as DNA transformation and DNA repair.
Results: We have conducted a genetic screen to identify the regulators and/or effectors of the iron-dependent recombination response. In addition, this screen enabled us to identify other genes required for efficient pilin variation. *In vitro* transposition was used to generate random insertions in gonococcal genomic DNA, which were then transformed into the gonococcal chromosome. Transformants were selected and screened concomitantly for mutants that exhibited decreased colony variation on iron-limiting, antibiotic-containing media. Transformants that had reduced levels of colony variation were reexamined in both iron-replete and iron-limiting conditions to identify mutants that lack the iron-dependent recombination response. Of roughly 11,000 transformants screened, 70 show decreased levels of colony variation. Of these 70, 20 have an iron-dependent phenotype in that they show decreased colony variation only when grown on iron-limiting media. The remaining 50 mutants exhibit a decrease in colony variation on both iron-replete and iron-limiting media and thus have an iron-independent phenotype. Identification of the sites of transposon insertion has revealed disruptions in both genes linked to, and genes not linked to recombination processes. Additional phenotypic analyses of mutants were performed to assess effects on DNA repair and DNA transformation. These analyses revealed three types of mutants: mutants that had a defect only in pilin variation, mutants with a defect in both pilin variation and DNA repair, and mutants with a defect in both pilin variation and DNA transformation.

Structure-function relationship of meningococcal pili

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Pili of *Neisseria meningitidis* are required for efficient attachment of capsulate bacteria to target tissues and hence are regarded as important virulence factors. Meningococcal pili undergo structural variations not only in their primary amino acid sequences but also in post-translational modifications. We are examining the contribution of these variations in distinct facets of pilus-associated functions.

Pilins of strain C311: In previous studies we reported capsulate strain C311 variants #3 and #16, which exhibited markedly different levels of adhesion to epithelial cells. The pili of both variants were glycosylated at serine 63, contained phosphodiester-linked glycerol at serine 93[1], and a phosphorylcholine (ChoP) moiety[2]. The variants were also similar in their expression of several known adhesins. However, analysis of their primary PilE sequence showed some minor amino acid substitutions at three positions (designated A, B and C). In molecular models of #3 and #16 pili that we have developed, these regions from neighbouring pilin monomers cluster together creating ordered and repeating patterns along the length of the pilus. To evaluate if such amino acid differences could affect the adhesion properties of the C311 pilin variants, site-directed mutants of C311 pilins have been constructed, containing hybrid *pilE* sequences consisting of a combination of the three regions from variants #16 and #3. Using these mutants we have been able to identify that substitutions in regions B and C of variant #3 pili, affect not only the adhesion phenotype but also the migration of pilins on SDS-PAGE. In addition, substitutions in the region B alone, comprising amino acid 127-132 (distinct from a previously reported single amino acid substitution[3]), alter the adhesion phenotype. Additional mutant strains have been constructed with conservative and non-conservative substitutions at this site to identify how these affect pilus adhesion and pilin mobility on SDS-PAGE.

ChoP modifications of pili: In addition to pilin sequence variation, we have recently studied the phosphorylcholine modifications of pili. Unexpectedly, we found that ChoP moiety was present on pili of pathogenic *Neisseriae* but not of commensal spp. In the latter case, ChoP was found on their lipopolysaccharides. In addition, distinct genetic elements are involved in ChoP incorporation in the two species[4]. The importance of ChoP expression has been assigned to its recognition of the platelet activating factor receptor, which may help anchorage to mucosa. In addition, its recognition by C-reactive protein may lead to activation of complement or resistance to antimicrobial peptides[4]. To assess the significance of the elaboration of ChoP on the two distinct scaffolds in *Neisseriae*, we have studied various aspects of ChoP-associated phenomena using commensal as well as pathogenic *Neisseria* strains and variants expressing or lacking the expression of the moiety. These studies have shown both similarities and differences in ChoP-associated functions between neisserial spp.

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Identification of meningococcal outer membrane proteins that cross-react with sera raised against *Neisseria lactamica* using SELDI ProteinChip® technology

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Introduction: Nasopharyngeal carriage of the commensal bacterium *Neisseria lactamica* induces antibodies that are cross-reactive for epitopes exposed on the pathogen *Neisseria meningitidis*. This is thought to be involved in the development of natural immunity in young children since antibodies cross-reactive with a variety of meningococcal strains have been detected in sera from children colonised with *N. lactamica*. We have previously shown that experimental vaccines based on *N. lactamica* have been shown to protect against lethal meningococcal challenge in a mouse infection model[1].

Materials and Methods: IgG isolated from rabbit anti-*N. lactamica* outer membrane protein (OMP) serum was coupled to Dynabeads and incubated with *N. meningitidis* MC58 OMP preparations, solubilised in the detergent Empigen BB. After washing with water, the beads were placed on an H4 ProteinChip® and mass spectral profiles were determined using surface enhanced laser desorption ionisation-mass spectrometry (SELDI, Ciphergen). *N. meningitidis* MC58 serogroup B was chosen as the source of meningococcal OMPs since its entire genome sequence has been published.

Results and Discussion: We have previously shown that *N. lactamica* OMPs and fractions thereof, particularly proteins within the molecular weight range 25-35 kDa, protect mice against lethal meningococcal challenge[1]. Using sera raised against these and other groups of *N. lactamica* proteins, we have identified cross-reactive meningococcal proteins by SELDI ProteinChip® technology. These antibodies may have contributed to protection observed in mice immunised with *N. lactamica* OMPs. The cross-reactive proteins had molecular masses of approximately 11.2 kDa, 13.7 kDa, 26.8 kDa, 17.4 kDa, 28.1 kDa, 33.1 kDa, 53.2 kDa and 66.6 kDa. Using TagIdent (www.expasy.ch), several meningococcal proteins of unknown function and those that have been considered as vaccine antigens (PorB and TbpB) were putatively identified. The identity of the 66.6 kDa protein as TbpB was confirmed by comparison of *N. meningitidis* OMPs from wild type and TbpB knockout strains to *N. lactamica* sera.

Conclusion: SELDI ProteinChip® technology has been used to identify meningococcal proteins that are recognised by serum raised against the commensal *N. lactamica*. This cross-reaction may play a role in the protection against meningococcal challenge provided by *N. lactamica* vaccine preparations. The vaccine potential of these antigens is currently being investigated.

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Identification of potentially protective *Neisseria lactamica* proteins using a *N. lactamica* genomic library

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Introduction: Immunological and epidemiological evidence suggests that carriage of the commensal organism *Neisseria lactamica* is involved in the development of natural immunity against meningococcal disease. We have shown that outer membrane vesicle (OMV) preparations from *N. lactamica* protect against challenge with diverse meningococcal strains in a mouse model of meningococcal disease[1] and are now trying to identify the antigens responsible for this protection.
Methods: A phage display library was prepared using *N. lactamica* (strain Y92-1009) genomic DNA randomly digested with MboI. DNA fragments of 1-4kb were ligated to the ZAP Express vector (Stratagene). This library was screened 3 times using serum raised against *N. lactamica* outer membrane proteins. 18 persisting plaques were removed and the pBK-CMV phagemid excised. Sequencing of the inserts was performed from this vector by primer walking. DNA homology searches were carried out using the Institute for Genomic Research (TIGR) comprehensive microbial resource blast searches (tigrblast.tigr.org).
Results and Discussion: The antigens expressed by the recombinant phage were recognised by antibodies in meningococcal OMV sera and human convalescent sera using ELISAs but not by sera raised against unrelated bacteria. The inserts contained within these phage were sequenced and putative identities determined by sequence comparison with the published meningococcal genomes. Full sequence was obtained for 14 inserts, partial sequence has been obtained for 1 insert and the 3 remaining inserts were repeats of those already sequenced. The inserts showed homology to a number of meningococcal proteins, including CarB, and a number of hypothetical proteins. The translations of these sequences were determined and again putative identities of the proteins encoded were obtained. The conservation of these genes in a panel of diverse meningococcal and commensal *Neisseria* strains is currently being assessed by Southern blot analysis using DIG labelled probes.

Primers were designed to isolate and amplify open reading frames with restriction sites compatible with the p-GEX-6P-1 GST fusion protein expression vector. To date 9 open reading frames have been ligated into p-GEX-6P-1 and cloned into the *E. coli* protease free mutant BL-21, and expression studies are proceeding.

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A mannosyl transferase from *Neisseria gonorrhoeae* involved in pilin glycosylation

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Background: Pilin, an important adhesin of *Neisseria gonorrhoeae* (GC), is a polymer composed mainly of the pilin glycoprotein (PilE) subunit. Studies using X-ray crystallography have shown an αGal-1,3-GlcNAc O-linked with PilE of strain MS11C. However, the exact nature of the pilin glycans from most GC strains remain uncharacterized, and the current model (which is based on strain MS11C) may not represent pilin glycans from most other strains. For example, glucose was not observed in the crystal structure, but its presence was reported by several previous studies of *Neisserial* pilin glycosylation. Our High pH Anion Exchange – Pulse Amperometric Detection (HPAE-PAD) analysis of wild-type MS11A pilin further indicated the presence of mannose on these pilins. This led us to hypothesize that a mannosyl transferase may be involved in GC pilin glycosylation. A search of the GC strain FA1090 genome using known bacterial mannosyl transferases as queries identified a potential GC mannosyl transferase. This gene is subsequently referred to as *manT*.
Methods: To determine the role this putative mannosyl transferase may play in GC pilin glycosylation, an isogenic *manT* knockout mutant was constructed in strain MS11A (MS11A*manT*). In order to compare their molecular weights, pilin proteins from this mutant and its wild-type counterpart (PilE sequences were verified as identical) were run on a 10% Bis-Tris NuPage gel (Novex) using MES buffer (designed to resolve low MW proteins). Both pilins were then subjected to TFA and HCl hydrolysis and HPAE-PAD monosaccharide analysis was performed. NMR and MALDI-TOF analyses are currently being performed on this mutant and its wild-type counterpart.
Results: In PAGE, the migration of the MS111A*manT* pilin was significantly greater than that of the wild-type pilin. A clear loss of the Man peak was observed in the HPAE-PAD monosaccharide analysis of the *manT* mutant sample. Similar results were also obtained for an MS11A*pgtA* mutant, which lacks the transferase responsible for forming the bond between the α-galactosyl and the O-linked GlcNAc, thereby possibly indicating a biosynthetic link between these sugar epitopes.
Conclusions: The presence of mannose in GC pilin has been determined by several physiochemical methods, including HPAE-PAD and PAGE. Our analyses indicate that the *manT* gene likely encodes a mannosyl transferase that attaches Man to the GC pilin glycan. The presence of this previously unreported sugar indicates a more complex nature for the GC pilin glycan beyond the current model. The characterization of *manT* may further aid in elucidating the structure of GC pilin glycans and their role in pathogenesis.

Biochemical and genetic characterization of lipooligosaccharide in a cluster of serogroup C *Neisseria meningitidis* strains with 2a:P1.1,7 serotype

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Meningococcal disease can be life threatening. In North and South America and Europe, the disease is mostly caused by serogroups B and C. Most serogroup C disease isolates in Canada in the last decade belong ET-37 with serotype 2a and subtype P1.2,5 for class 2 and 1 outer-membrane protein antigens respectively. In 2001, many ET-37 serogroup C disease isolates from Quebec Province have been serotyped as 2a:P1.1,7. We have characterized the lipooligosaccharide (LOS) in eight representative isolates typed as C:2a:P1.1,7. Outer-membrane vesicles (OMV) that contain both protein and LOS antigens were prepared from the isolates were then analyzed by SDS-PAGE for outer-membrane proteins and LOS in 10% and 16% gels respectively. For the eight isolates, the 46 kDa proteins which contain the P1.1,7 epitope and the 41 kDa proteins which contain the 2a epitope were indistinguishable between strains but the size of class 5 proteins were variable around 28 kDa. For the LOS, all of them were quite heterogeneous in having five components. Seven LOS had a 4.1 kDa major component but one had a 3.7 kDa major component. Immunoblot analysis showed that the major components in the seven LOS had L2 immunotype and that of the other LOS had L5 immunotype. Genetic analysis of *lgt* genes responsible for the biosynthesis of the α-chain of LOS showed that all eight isolates had the same gene organization, *lgtABH*. Sequence analysis revealed only one base pair nonsynonymous mutation in *lgtH* of the L5 isolate compared to those from two L2 isolates. The seven C:2a:P1.1,7:L2 isolates apparently are the same clone and the other L5 isolate is closely related.

Unglycosylated hexa-acylated lipid A: expressed by meningococcal strains with defects in incorporation of 3-deoxy-D-manno-octulosonic acid (Kdo)

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Lipopoly(oligo)saccharide (LPS, LOS) or endotoxin is important in bacterial survival and the pathogenesis of gram-negative bacteria. A necessary step in endotoxin biosynthesis is Kdo glycosylation of lipid A, catalyzed by the Kdo transferase KdtA (WaaA). In enteric gram-negative bacteria, this step is essential for survival. A nonpolar *kdtA::aphA-3* mutation was created in *Neisseria meningitidis* via allelic exchange and the mutant was viable. Detailed structural analysis demonstrated that the endotoxin of the *kdtA::aphA-3* mutant was composed of fully acylated lipid A with variable phosphorylation but without Kdo glycosylation. In contrast to other gram-negative bacteria, tetra-acylated lipid IV_A did not accumulate. Other steps in Kdo assembly were also studied. The function of *kpsF* of *Neisseria meningitidis* was identified to be the arabinose 5-phosphate isomerase, an enzyme not previously identified in prokaryotes that mediates the interconversion of ribulose 5-phosphate and arabinose 5-phosphate. The arabinose 5-phosphate isomerase activity of KpsF was required for Kdo biosynthesis in *N. meningitidis*. Mutation of *kpsF* or the gene encoding the CMP-Kdo synthetase (*kpsU*/*kdsB*) in *N. meningitidis* also resulted in expression of a LOS structure that contained unglycosylated lipid A. In addition, mutations in *kpsF* and *kdsB* but not *kdtA* reduced capsule expression in all five major invasive disease associated meningococcal serogroups (A, B, C, Y, and W-135). Unlike meningococcal *lpxA* mutants, capsule expression was not required for the viability of Kdo defective mutants. Phospholipid composition analyses indicated increased short-chain fatty acids in both *lpxA* and *kpsF* mutants. In conclusion, lipid A biosynthesis in *N. meningitidis* can proceed without the addition of Kdo and Kdo glycosylation is not essential for survival of the meningococcus. In addition to LOS assembly, Kdo biosynthesis appears to be involved in meningococcal capsular polysaccharide expression.

Crystal structure of an Fab fragment in complex with a PorA P1.2 serosubtype antigen and a protein G domain

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Introduction: The porin protein PorA contains two loop regions, VR1 and VR2, which are hypervariable in sequence and which form the basis for serosubtyping of meningococci. Previous work has determined the crystal structures of antibody Fab fragments in complex with peptides corresponding to a VR1 serosubtype variant P1.7[1] and a VR2 variant P1.16[2]. We report here the determination of the crystal structure of a third Fab complex with a peptide derived from the sequence of a second VR2 variant, P1.2. Some common themes in the structures of all three PorA antigens are identified.
Materials and Methods: Crystals of anti-P1.2 antibody Fab fragment were obtained in complex with a single protein G domain and the 14-mer peptide HFVQQTPKSQPTLV. Data were collected to 2.2Å resolution at the SRS, Daresbury, U.K. and the structure solved by molecular replacment using the P1.7 Fab complex coordinates[1]. Electron density for 13 residues in the antigen was visible, starting from His1, although no density was detected for the sidechain of Leu13, which was built as Gly.
Results: The antigen binds in a shallow cavity on the surface of the Fab; the binding site is remarkably hydrophobic and interactions between the antigen and antibody are dominated by apolar residues. Pro7 from the antigen is located within a small hydrophobic pocket, lined by Tyr97H, Phe100^{d}H and Trp34L (Kabat numbering used for the antibody residues; H=heavy chain, L=light chain). Other residues contributing to the hydrophobic environment around the antigen include Tyr53H, Trp50H, Tyr99H and Tyr96L. The conformation of the antigen is most easily described as a distorted β-hairpin, with Pro7 and Lys8 at the tip forming a Type I β-turn. The antigen fits into the cavity 'side-on', so that most contacts are made with residues in the N-terminal half. The conformation is stabilised by two main-chain-main-chain hydrogen bonds between Val3 and Thr12, which form the base of the hairpin. Gln5 adopts an α_R conformation that distorts the β-hairpin by forcing the two β-strands apart. The side-chain of Gln5 forms the only hydrogen bonds between antigen and antibody, with hydrogen bonds to Thr53 and Thr30 on the heavy chain.
Conclusions: The conformation of the P1.2 antigen has some similarities with the structures of the P1.16 and P1.7 antigens described previously. All adopt hairpin loop structures, with a type I β-turn at the tip, although there is no significant sequence homology between the different serosubtype families. Identification of conserved structural motifs could play a role in the modelling of the structures of other PorA serosubtype antigens.

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The NalP autotransporter of N. *meningitidis* modulates the processing of secreted autotransporters

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The genome sequences of *Neisseria meningitidis* serogroup B strain MC58 and serogroup A strain Z2491 were systematically searched for ORFs encoding autotransporters[1]. Eight ORFs were identified, six of which were present in both genomes, whereas two were specific for MC58. Among the identified ORFs was the gene encoding the known autotransporter IgA1 protease. Putative functions for other autotransporters are based upon homology. Striking homology of 66 and 65% similarity was observed for the homologues of the *Haemophilus influenzae* colonization factors Hap and Hia, respectively. Western blotting using patient sera showed that the neisserial Hap and Hia homologues were expressed *in vivo*.

We report here a novel function for another autotransporter, which we named NalP (for Neisserial autotransporter lipoprotein), because it contains a lipoprotein box. We cloned the ORF from *N. meningitidis* serogroup B isolate H44/76 and detected the gene and its encoded protein in all clinical *N. meningitidis* isolates tested. The gene was, moreover, found to be phase variable through a mononucleotide G repeat in the coding sequence. In *N. gonorrhoeae* isolates (n=6), the *nalP* gene is interrupted by premature stop-codons. Homologous DNA was not detected in commensal *Neisseria* species (n=8). Western-blotting showed a very low amount of the 110 kDa mature unprocessed protein in whole cell lysates. The culture medium contained a processed form of 70 kDa. The protein was not recognized by patient sera.

We observed a striking change in the protein secretion profile of a *nalP* knock-out mutant. The major secreted proteins in the wild-type strain were identified as IgA protease and the Hap-homologue. The IgA protease is known to be processed into the active enzyme and an alpha peptide, which are separated by proteolytic cleavage at the cell surface[2]. We show that the presence of NalP negatively influences this proteolytic cleavage. Similarly, proteolytic cleavage of an alpha peptide from the Hap-homologue is prevented. Such an alpha peptide is absent in the *H. influenzae* Hap and represents a marked difference between these two homologous proteins. Finally, NalP also modulates a third autotransporter, AusI, by preventing its processing into a mature domain and two smaller peptides. In conclusion, our results show that the NalP protein negatively influences (auto)proteolytic cleavage of autotransporter proteins, presumably at the cell surface.

Furthermore, the C-terminal translocator domain of NalP, involved in outer membrane transport of the secreted domain, was purified and refolded in vitro. CD-measurements showed that the refolded protein consists primarily of ß-sheets and the domain functions as a pore in black lipid membranes. Crystallization of the refolded domain was successful and yielded diffracting crystals.

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Molecular characterisation of a novel ADP-ribosylating putative toxin of *Neisseria meningitidis*

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Introduction: By computer analysis on the *Neisseria meningitidis* (serogroup B, MC 58 strain) genome sequence, a protein with a feature similar to known bacterial ADP-ribosylating toxins (CT produced by V*ibrio cholerae*, LT by *Escherichia coli* and PT by *Bordetella pertussis*) has been identified. Enzymatic assay has shown that this protein (NM-ADPRT) possesses both NAD glycohydrolase and ADP-ribosyltransferase activity. In this study we describe the identification of the putative catalytic residues, their site-directed mutagenesis, and the resulting activity of the mutants.
Materials and Methods: The novel NM-ADPRT and the correspondent mutants, were expressed in *E. coli* as C-terminus His-tag protein fusions. Site-directed mutagenesis was performed using the Multi Site-Directed Mutagenesis Kit (QuikChange). Recombinant NM-ADPRT forms were purified from *E. coli* in their soluble form by metal chelate affinity chromatography.
Both the wild-type and the mutants were assayed for their ADP-ribosylation and NAD-glycohydolase activites, using [adenine –U-^{14}C] NAD and agmatine as ADP-ribose acceptor. Antisera against NM-ADPRT and the mutant derivatives were obtained by immunization of CD1 mice. 20μg of each recombinant protein were given i.p. together with CFA for the first dose and IFA for the second (day 21) and the third (day 35) booster doses. Blood sample were taken on days 34 and 49. Immune sera were used in western blot and tested in a bactericidal assay.
Results and Discussion: On the basis of sequence homology of NM-ADPRT with LT, CT and PT we have identified the putative residues involved in enzymatic activity. These residues have been changed by site-directed mutagenesis and the purified mutant toxins have been tested for both ADP-ribosylating and NAD-glycohydrolase activities. Interestingly, some of the mutants show reduced or abolished enzymatic activity indicating that the identified residues play a role in catalysis. Antisera against the wild-type and mutant toxins have bactericidal activity. The titers induced by two mutants were higher than those induced by the wild-type form. These data suggest that the mutations introduced could influence not only the enzymatic activity but also the *in vivo* stability of the toxin.
Conclusion: A novel ADP-ribosyltransferase has been identified in meningococcus B. Catalytic residues have been predicted by sequence homology and their role in catalysis has been confirmed by site-directed mutagenesis. These molecules are also able to induce a bactericidal response.

A new gene involved in pilin glycosylation ORF in *Neisseria meningitidis*; *pglI*.

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Introduction: Pili of pathogenic *Neisseria* are important colonisation factors, playing a major role in adhesion and contributing to host specificity. Pilin of *Neisseria meningitidis* is post-translationally modified by glycosylation at Ser63 with either a Gal(β1-4)Gal(α1-3)2,4-diacetamido-2,4,6-trideoxyhexose or the disaccharide Gal(α1-3)GlcNAc. We have identified potential pilin glycosylation genes from *N. meningitidis* strains MC58 and Z2491, as well as *N. gonorrhoeae* FA1090 genome sequencing projects. These genes include *pglABCDE* and *pglF*, all of which are implicated in the biosynthesis and glycosylation of pilin. However, not all the genes responsible for the biosynthesis and attachment of the sugar to the pilin have been elucidated. Recent work has concentrated on a new locus, *pglI*, which may be involved in the biosynthesis of the 2,4-diacetamido-2,4,6-trideoxyhexose sugar attached to the pilin of *N.meningitidis* strain C311. The gene *pglI* is homologous to acetyltransferases of *Pseudomonas aeruginosa* and *Salmonella typhimurium* involved in LPS biosynthesis. The *pglI* ORF contains a homopolymeric guanosine tract indicating that it is probably capable of phase-variation (high frequency on-off switching of expression). This is a common feature of glycosyltransferases genes of *Neisseria* (e.g. LPS biosynthesis genes such as *lgtC* and pilin glycosylation genes such as *pglE*).
Materials and Methods: The role of this gene in pilin biosynthesis is being studied by making a mutation in the *pglI*. As glycosylation genes can also be involved in the biosynthesis of LPS, the effect of these mutants on LPS was examined. The effect on pilin glycosylation was studied by analysing pilin migration by SDS-PAGE, western immunobloting and ELISA using defined antisera.
Results and Discussion: The LPS of the *pglI* mutant was like wild-type. The *pglI* mutant pilin displayed altered migration on SDS-PAGE. The *pilE* was sequence and found to be the same as the parent strain, confirming that the changed migration was due to alteration of the post-translational modification. Western immunobloting with different antibodies was used to analyse the C311 *pglI* mutant. Binding of mAb TEPEC-15 was the same as wild-type, confirming phosphorylcholine addition was unaffected. The pilin from the *pglI* mutant bound the poly-clonal anti-sera specific for the C311 trisaccharide, but with 2.7 fold reduction in affinity compared to that of the wild type. Suggesting the structure of the trisaccharide was altered. Homology with other acetyltransferases is consistent with a role for *pglI* in the biosynthesis of the basal 2,4-diacetamido-2,4,6-trideoxyhexose residue of the trisaccharide. The poly-guanosine repeat region was sequenced from many strains including patient isolates. This sequencing showed that there is variation in the number of repeats and the ORF is found both in and out of frame. These results suggest that *pglI* is capable of phase-variation. Phase-variation of *pglI* along with the known phase variation of *pglA* and *pglE* confirms that the trisaccharide can vary in all three sugars. Current efforts are concentrating on over-expression of this locus to help confirm its precise role in biosynthesis of the pilin linked trisaccharide.

Identification of lipopolysaccharide phosphoethanolamine transferases in *Neisseria meningitidis*

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Phosphoethanolamine (PE) is found at several positions within *Neisseria meningitidis* (Nm) lipopolysaccharide (LPS). PE is found at the 3-position of the β-chain heptose (HepII) of Nm inner core LPS in immunotypes L1, 3 and 7 [1,2,3], at the 6-position in immunotypes L2, 4 and 6 [4,3,1] and on lipid A [5]. The gene responsible for the addition of PE specifically at the 3-position of HepII has been identified as *lpt3* [6]. To investigate the genes responsible for addition of PE to the 6-position of HepII and to the lipid A, the amino acid sequence of Lpt3 was searched against the completed genome sequence of Nm serogroup B using the BLAST algorithm. Two open reading frames, ORFA and ORFB were identified with low levels of similarity to *lpt3* (e values of 5.2×10^{-13} and 4.8×10^{-9} respectively). ORFA is a member of the same protein family as Lpt3 and ORFB is a conserved hypothetical protein disrupted by an authentic frame shift. Alignment of the amino acid sequences encoded by *lpt3*, ORFA and ORFB showed a number of conserved residues across the entire length. Screening by the polymerase chain reaction with primers specific to ORFA and ORFB showed both genes to be present in all strains tested including representatives of all major serogroups and immunotypes.

ORFA and ORFB were inactivated by insertion of a kanamycin cassette then transformed into Nm immunotypes L2, strain 35E and L4, strain 89I. The transformants were screened with a monoclonal antibody (mAb) L2-16, which specifically recognises PE at the 6-position of the HepII. ORFA and ORFB mutants in both strains showed the same levels of reactivity as the parental strains.

Structural analysis of purified LPS from the L4 strain ORFA mutant showed an absence of PE on the lipid A moiety, whilst the PE on HepII remained. A similar result was obtained after analysis of LPS from an immunotype L3 strain, H44/76 with ORFA inactivated. When the double mutant of *lpt3* ORFA in strain H44/76 was analysed, PE was entirely absent. We conclude that ORFA is required for the addition of PE to lipid A and it has therefore been designated *lptA* (LPS phosphoethanolamine transferase on lipid A). Work is ongoing to identify the function of ORFB. From experimental studies it now seems likely that a further gene, not present in either of the Nm genome sequences is responsible for addition of PE at the 6-position of HepII. An alternative approach of *in vitro* mutagenesis [7] has been used to construct a random insertion library in DNA from a L4 strain, 89I. This DNA library is being used in conjunction with strain 89I-galE and mAb L2-16 to identify candidate genes.

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Epitope insertions into predicted loop 11 and globular plug domain of transferrin binding protein A show unexpected transferrin binding and surface exposure characteristics

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Neisseria gonorrhoeae can use human transferrin as a sole iron source. The gonococcus utilizes transferrin bound iron through the transferrin receptor, which is composed of two proteins TbpA and TbpB. *Neisseria gonorrhoeae* is an obligate human pathogen and its transferrin receptor shows exquisite specificity for human transferrin. Both TbpA and TbpB bind transferrin independently but only TbpA is required for utilization of transferrin as an iron source. TbpB shows a preference for ferrated transferrin and increases the efficiency of iron uptake. The ability of TbpA to internalize transferrin bound iron is dependent on the presence of an active TonB protein, which supplies the energy needed for this transport event. The hemagglutinin epitope (YPYDVPDYA) was inserted into the TbpA protein with the dual purpose of analyzing the cellular location of the insertion site, and analyzing the functional characteristics of these insertion mutants. The mutants were constructed using a two-step mutagenic PCR technique. The PCR products were cloned, sequenced, and transformed into the gonococcus replacing the wild-type TbpA gene by homologous recombination. A total of 12 epitope insertion mutants were constructed and allowed us to confirm the surface exposure of putative loops 2, 3, 5, 7, and 10. Mutants resulting from insertions into the putative globular plug domain and into putative loop 11 were chosen for further study due to their unexpected phenotypes. Both of these epitope insertions were analyzed for surface exposure of the epitope, transferrin binding, and ability to grow on transferrin as a sole iron source. Unexpectedly, the globular plug domain epitope insertion was exposed at the surface in a whole cell dot blot assay, while the loop 11 epitope insertion was not. The plug domain insertion mutant demonstrated an approximately ten-fold reduced affinity for transferrin in a liquid phase binding assay, and a concomitant loss of the ability to use transferrin as a sole iron source. The loop 11 epitope insertion, on the other hand, showed such high affinity for transferrin binding that we were unable to analyze the data by Scatchard analysis. This mutant, however, could not utilize the transferrin bound iron provided in a plate growth assay. These data conflict with the working model of gonococcal TbpA, which predicts the globular plug domain is not surface exposed while loop 11 is surface exposed. We also conclude that insertions into predicted loop 11 and globular plug domain have opposite but significant effects on transferrin binding, and propose a model to explain these contradicting results.

Lipid A – biosynthesis gene, *lpxA*, is an essential gene in *Neisseria meningitidis*

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During the septicemic phase of meningococcal disease, *Neisseria meningitidis* (Nm) interacts with components of the host immune system. Meningococcal components and in particular endotoxin released by the bacteria, are potent inductors of the inflammatory response. We have previously shown that adherent Nm induce the expression of tumor necrosis factor α (TNFα) not only in monocytes but also in endothelial cells. To attempt to identify the bacterial structures involved in the induction of TNFα we aimed to obtain a mutant of Nm lacking lipid A as this moiety of the meningococcal lipooligosaccharide (LOS) is known to be responsible for the endotoxin activity.
lpxA gene in Nm catalyzes an early step in the biosynthesis of lipid A. We constructed *lpxA* knockout mutants by inserting a kanamycin-resistance cassette (harboring the *aph-3'* gene) into the *Eco*RI site of the *lpxA* gene from two genetic and phenotypic different strains of Nm. As control, we constructed a knockout mutant in the *rfaD*-homologue gene encoding the ADP-L-glycero-D-mannoheptose-6-epimerase that is involved in the biosynthesis of LOS. The production of LOS in both *lpxA* and *rfaD* mutants was monitored by SDS-PAGE followed by silver staining. We observed that *lpxA* knockout mutants still showed full length LOS with reduced quantity compared to the parent strains. As expected, in the *rfaD* mutant, LOS corresponding band showed a lower molecular range than the parent strain and the *lpxA* mutants. We also demonstrated by RT-PCR analysis that both *lpxA* and *rfaD* mutants were able to induce transcription of TNF-∝ gene in the promyelocytic cell line HL60 confirming that endotoxin-like activity was still present.
We could demonstrate by Southern blot analysis that the insertional inactivation of *lpxA* gene was not possible and *lpxA/lpxA::aph-3'* heterodiploids were obtained. The reduction in the level of lipooligosaccharide in *lpxA* mutants is most likely due to a negative trans dominance effect of the *lpxA*-inactivated allele. All these data strongly suggest that, as in other bacterial species, *lpxA* is an essential gene in *Neisseria meningitidis*.

SESSION IV

Pathogenesis

X-ray crystal structure of *Neisseria gonorrhoeae* Gly1ORF1

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The *Neisseria gonorrhoeae* (GC) *gly1* locus was found in a plasmid library screened for conferral of a hemolytic phenotype upon transformation of *Escherichia coli*. It encodes two open reading frames, Gly1ORF1 and Gly1ORF2. ORF2 shows weak amino acid sequence homology to Uroporphyrinogen III cosynthase while ORF1 has no homologues in the GenBank database. ORF1 is found in outer membrane fractions of GC, is secreted when expressed in *E. coli,* and has a signal sequence that is cleaved. The *gly1* locus is found only in pathogenic *Neisseria* and is not found in commensal species. A null mutation of the GC (strain MS11) *gly1* locus shows no phenotype in adhesion or invasion of cultured cells. However, the *gly1* null mutant has increased toxicity to human fallopian tubes in organ culture, suggesting that g*ly1* may alter the amount or properties of toxic moieties produced by GC. A derivative of the Gly1ORF1 protein with a C-terminal His6-Tag, Gly1ORF1-H6, was expressed in *E. coli* and purified from culture supernatant, using nickel affinity chromatography [1].

Dynamic light scattering data of a Gly1ORF1-H6 sample had a baseline error of 1.001, 17% relative polydispersity and was modeled with a monomodal fit with a predicted molecular weight of 24 kDa, indicating that Gly1ORF1-H6 (MW 14.6 kDa) was a monodispurse dimeric complex. Crystals, space group P63, diffracting to 2.07 Å, were grown by hanging-drop vapor diffusion against ammonium phosphate and glycerol. The structure was solved by MAD phasing of a selenomethionine containing derivative. Topologically, the fold consists of four sequential antiparallel β-sheets (β1-β4), connected by an α-helix (α1) to another set of four sequential antiparallel β-sheets (β5-β8) that sandwich over, and are orthogonal to, the first set. The alpha helices associate with the β-turns between β5 and β6 and between β7 and β8 of the other subunit to form the dimer interface. A bipolar cavity is located at the dimer interface. The analysis of the structure of this putative binding site may allow prediction of what this site binds and reveal the function of the Gly1ORF1 protein and its role in pathogenesis.

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The role of the *ISPA/CBF* locus in *Neisseria meningitidis* pathogenesis

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Neisseria meningitidis is the most common cause of bacterial meningitis in the Western world and the second leading cause of mortality in 1-5 year-olds in the United Kingdom. A signature-tagged mutagenesis screen of approximately 3,000 insertional mutants of a serogroup B isolate of *N. meningitidis*, C311$^+$, identified 73 genes required for pathogenesis in an infant rat model of meningococcal septicaemia. Homology-based searches indicate that two of the genes identified, NMB0342 and NMB0345, have homologues in other pathogenic bacteria and may exist within an operon of seven genes (NMB0342-NMB0348). NMB0342 is a homologue of *ispA* (intracellular septation protein) in *Shigella flexneri*, required for effective intercellular spread and plaque formation in epithelial cells. NMB0345 is a homologue of *cbf*, a *Campylobacter jejuni* gene that encodes a 29 kDa protein which is a major antigenic peptide. PCR and Southern analyses of the genes in the NMB0342-NMB0348 locus show that they are conserved across a wide range of pathogenic isolates and serogroups of *N. meningitidis*. However, these genes are present in only a sub-set of commensal strains. Competition experiments demonstrate that *N. meningitidis* mutants with transposon insertions in NMB0342 and NMB0345 are highly attenuated when directly competed with the wild-type bacterium in the infant rat model. Mutants with transposon insertions in the NMB0343 and NMB0344 genes are significantly attenuated, while mutants with insertions in NMB0347 and NMB0348 are attenuated to a lesser degree. Complementation of the *ispA* mutation by ectopic chromosomal integration of a wild-type copy of *ispA* almost completely restores the virulence of the bacterium. Further genetic and phenotypic characterization including complementation of the *cbf* mutation, *in vitro* cell-culture analyses, and microscopic analysis of mutants in association with host cells are currently underway. These studies will provide a greater understanding of the involvement of the locus in the pathogenesis of *N. meningitidis* infections.

The effects of *Neisseria meningitidis*, meningococcal components and other bacterial pathogens on the inflammatory response of human meningeal cells

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Introduction: Meningitis is the most common infection of the central nervous system. Recent studies in our laboratory have demonstrated that cells of the meninges play an important role in initiating and sustaining an inflammatory response. We have extended these observations by investigating the nature of the components of *Neisseria meningitidis* that induce inflammation, and comparing the interactions of meningococci with other bacterial pathogens causing meningitis.
Materials and Methods: Cultures of human meningeal cells *in vitro* were challenged with i) viable *Neisseria meningitidis* H44/76, ii) an LPS-deficient mutant of the same strain (H44/76 pLAK33), iii) outer membranes (OM) prepared from both strains, and iv) other important bacterial causes of meningitis (*Streptococcus pneumoniae*, *Haemophilus influenzae* and *E. coli*). The inflammatory response was investigated by measuring cytokine production by meningeal cells.
Results and Discussion: When challenged with wild type H44/76, meningeal cells secreted a distinct group of chemotactic, pro-inflammatory and growth-factor cytokines. Challenge with the LPS-deficient mutant also induced cytokine secretion. In addition, OM from both strains induced cytokine secretion, demonstrating the presence of additional inflammatory modulins in the OM. These results clearly show that induction of inflammation occurs through both LPS-dependent and LPS-independent pathways in meningeal cells. Secretion of IL-6 was independent of LPS and pilus-mediated adherence, and was due to the activity of a secreted bacterial component. In contrast, secretion of IL-8 and MCP-1 was induced not only by the release of LPS by wild type meningococci but also by other components of the bacterium in the absence of LPS. Efficient stimulation of RANTES required pilus-mediated adherence that served to deliver increased local concentrations of LPS onto the surface of meningeal cells; however, GM-CSF secretion did not involve LPS but was dependent on pilus-mediated adherence. In preliminary studies to identify the nature of non-LPS modulins present in the OM, we have observed that purified class 1 porin specifically induced the secretion of chemokines by meningeal cells, and not pro-inflammatory or growth-factor cytokines. In studies with other pathogens causing meningitis, significant differences were observed in their interactions with meningeal cells, both in terms of adherence, invasion and their ability to induce host cell death. In addition, there were also significant differences in the patterns of cytokine and chemokine secretion induced by other meningeal pathogens compared with meningococci.
Conclusion: Our data demonstrate that secretion of cytokines by meningeal cells is stimulated not only by meningococcal LPS but also by other bacterial modulins, and that induction of inflammation occurs through both LPS-dependent and LPS-independent pathways. In addition, the major bacterial pathogens causing meningitis show significant differences in their interactions with meningeal cells. Taken together these findings may have significant bearing on the prognosis for patients with meningitis.

Detection of Opa receptor transcripts in human fallopian tube tissue

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Introduction: Gonococcal Opa proteins mediate attachment to and invasion of a variety of epithelial cell lines and human fallopian tube tissue. In various cell lines, some Opas are able to utilize a variety of carcinoembryonic antigen-like cell adhesion molecules (CEACAMs) to invade, while others prefer heparan sulfate proteoglycans (HSPGs), e.g., syndecans. In this work, reverse transcriptase/polymerase chain reaction (RT/PCR) techniques were used to detect mRNA for CEACAMs and HSPGs in human fallopian tube tissue.
Materials and Methods: Fresh human fallopian tube tissue was obtained from pre- or peri-menopausal women who were undergoing elective hysterectomy. The mucosal surface of each tube was washed with HEPES-buffered MEM. Trizol reagent was added to the mucosal surface to solubilize epithelial cell components, and RNA was isolated by standard methods. Established primer sets for specific amplification of CEACAM-related and control transcripts by RT/PCR included one set for all CEA-gene-family members along with individual sets for CEACAM1, 3, 5, 6, 8 and beta actin [Thompson et al, Int. J. Cancer, 1993]. Established primer sets for syndecan 1 and 4 were also utilized [Schofield et al, Biochem J, 1999]. Reaction products were analyzed via agarose gel electrophoresis.
Results and Discussion: The primer set for all CEACAMs amplified CEACAM transcripts in 3/3 fallopian tubes. CEACAM 1 was present in 1/3 tubes. CEACAM5 and CEACAM6 were present in 2/3 tubes. CEACAM3 and CEACAM8 were not detected in any tubes. CEACAM1 and CEACAM6 have been previously detected in both epithelial cells and granulocytes. However, CEACAM 5 is associated with epithelial cells. The absence of CEACAM3 and CEACAM8, which are produced by granulocytes, suggests that the detectable CEACAM1 and CEACAM6 transcripts were not derived from granulocytes in the tissue. Syndecan 1 and 4 transcripts were detected in 2/2 tubes tested. We previously detected CEACAM and HSPG expression in fallopian tube epithelium by immunofluorescence with monoclonal antibodies specific for CEACAM1/5 and heparan sulfate, respectively [Gorby, 11th International Pathogenic Neisseria Conference, 1998 p.383]. Immunohistologic studies by Fernandez et al [Human Reproduction, 2001], which utilized polyclonal antibodies detected both types of receptors in fallopian tube epithelium. However, other investigators failed to detect CEACAM expression in normal fallopian tube tissue by immunohistochemical staining with two monoclonal antibodies [Swanson et. al, Cell. Microbiol., 2001].
Conclusions: 1. Both CEACAM and HSPG transcripts are present in human fallopian tube tissue. 2. Additional immunohistologic studies with specific antibodies are needed to assess the anatomic localization of the receptors and identify the cell type/s that is/are expressing these putative Opa receptors. These studies are currently ongoing.

Type IV secretion system mutants of *Neisseria gonorrhoeae* are deficient in DNA secretion and show altered interactions with host cells

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Introduction: The gonococcal genetic island (GGI) contains eighteen genes with homology to type IV secretion system genes, with significant similarity to the conjugal transfer genes of *Escherichia coli* F plasmid. Type IV secretion systems are adapted conjugation machines that have the ability to secrete DNA as well as protein substrates. These secretion systems are essential to the virulence of a number of plant and animal pathogens, including *Agrobacterium tumefaciens* (oncogenic Ti plasmid injection), *Bordetella pertussis* (pertussis toxin secretion), *Legionella pneumophila* (secretion of factors for survival within macrophages), and *Helicobacter pylori* (secretion of *cag* gene products responsible for pseudopodia formation by host cell).
Results and Discussion: We have demonstrated previously that the type IV secretion system of *N. gonorrhoeae* secretes chromosomal DNA during exponential growth that is capable of transforming recipient gonococci. We made polar and non-polar insertion-duplication mutations in several type IV secretion genes of the GGI and an additional mutation in the putative pilin gene, *traA*, was constructed by in-frame deletion. Analysis of the exponential culture supernatants of these mutants using the DNA binding fluorescent dye PicoGreen revealed that they are deficient in DNA secretion. We are actively investigating the nature of the DNA secreted: its strandedness, size, and frequency or predominance of chromosomal locus secretion. Several of the type IV secretion system mutants have been analyzed in primary human cervical cell infection assays. In preliminary studies the type IV secretion mutants exhibit an aggregation phenotype as well as a delay in attachment to primary human cervical cells.
Conclusion: The gonococcal type IV secretion system secretes chromosomal DNA into the surrounding milieu. The phenotypes of the type IV secretion system mutants in cell infection assays suggest that secreted products or components of the secretion apparatus are involved in bacterium-bacterium or bacterium-host cell interactions.

Assessment of meningococcal phase variation *in vivo* by DNA extraction and sequencing

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Background: Capsule expression in serogroup B meningococci can be switched on or off by addition or deletion of a cytosine residue in a poly dC_7 repeat by a mechanism known as slipped-strand mispairing. We have previously shown that the meningococci resident in the skin lesions of patients with meningococcal sepsis express PorA, capsule and type IV pili in association with neutrophils, the vascular endothelium and the dermal interstitium. Using confocal laser scanning microscopy we have also shown that there is variation in capsule and pilin expression within these tissues. The aim of the present study was to develop a technique to extract meningococcal DNA from human tissue and then investigate the mechanism of the *in vivo* capsule variation observed by amplifying, cloning and sequencing of the poly dC repeat region of the *siaD* gene.
Methods: Five 30μm sections were made from frozen skin biopsies taken from the edge of petechial/purpuric lesions of children with purpura fulminans were obtained following informed consent. These were digested with proteinase K and then the DNA was isolated using a DNeasy mini column (Qiagen). The presence of meningococcal DNA was confirmed by PCR of the 16S rRNA gene specific for meningococci after which amplification of the *siaD* gene was undertaken. The gel-purified product was then ligated into a pCR®II-Blunt-TOPO vector (Invitrogen) with which One shot® chemically competent *E. coli* were transformed. Plasmids were extracted from positive clones and analysed by restriction enzyme digestion and sequencing.
Results: Meningococcal nucleic acid was successfully extracted and fragments of both the 16S rRNA and *siaD* genes were isolated. After sequencing of the cloned plasmids, it was found that capsulated (dC_7) as well as unencapsulated (dC_6 and dC_8) sequences were recovered.
Conclusions: These findings support the hypothesis that there is a heterogeneous population of meningococci existing *in vivo* with respect to capsule, and that at least some of this heterogeneity results from *siaD* phase variation mediated by single base changes in the $(dC)_7$ repeat. Whether this variation in the expression of capsule is a prerequisite to the interaction of meningococci with host cells within these environments *in vivo* remains to be established.

Differential expression of NMB1856 *(crgA)*, and the autotransporter homologues NMB1985 and NMB0992 by *Neisseria meningitidis* during early interactions with human epithelial cells

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Introduction: *Neisseria meningitidis* (Nm) asymptomatically colonises human nasopharyngeal epithelium in up to 30% of the population, and in a small number of carriers Nm traverses this epithelial barrier as a first invasive step in the pathogenesis of meningococcal septicaemia and meningitis. It is hypothesized that meningococcal differential gene expression occurs at the human epithelial surface between the colonising and invasive states. Recently it has been shown that a LysR-type transcriptional activator, *crgA*, controls intimate adhesion to human epithelial cells. Additionally, recent work has shown the presence of antibodies in the sera of patients and healthy carriers to the *Haemophilus* autotransporter homologues NMB1985 and NMB0992. The present study was conducted to address the possibility that these genes are differentially expressed by Nm during interaction with human epithelial cells.
Methods: An acapsulate piliated derivative of the serogroup B strain MC58 (h18:18 ¢13) was used to infect the human respiratory epithelial cell line A549. The number of bacteria that were adherent at 5 minutes were 0.75 cfu/cell and at 30 minutes were 2.2 cfu/cell. At these 2 time points all non-adherent h18:18 were removed by washing and meningococcal RNA stabilized by treatment with RNALater (Ambion Corp). Control bacteria were grown in the absence of human epithelial cells under the same conditions and for the same duration as the cell-associated organisms prior to RNA stabilization. Bacterial RNA was then extracted from the two Nm populations using a modified guanidinium isothiocyanate: phenol: chloroform extraction protocol following selective eukaryotic (A549) cell lysis and bacterial washing with RNALater. Contaminating DNA was removed by DNAseI treatment of the RNA extract. The extracted meningococcal RNA was reverse transcribed using gene specific primers for NMB1856, NMB1985 and NMB0992. Subsequent PCR amplification was carried out using 15 thermocycles.
Results: Relative RT-PCR performed on stabilized RNA from control and cell-associated organisms reveal the differential expression of NMB1856, NMB1985 and NMB0992 as early as 5 minutes and 30 minutes of interaction with human respiratory epithelial cells. There was no detectable PCR products for these three genes in the control bacterial RNA after reverse transcription and PCR. Positive controls included both bacterial 16S RNA and control RNA included in the SuperscriptII based RT-PCR kit (Invitrogen). A negative control underwent PCR amplification with 16S primers and bacterial RNA but with no reverse transcription. Eukaryotic RNA contamination was checked by the failure to detect a PCR product using 18S RNA primers (Classic II primers, Ambion Corp).
Conclusion: As expected from earlier studies, the LysR-type transcriptional activator *crgA* was expressed by meningococci within 5 minutes of human epithelial cell contact. In addition, the early expression of the *Haemophilus* hap and hia/hsf homologs, NMB1985 and NMB0992, further suggest that these autotransporters may play a role in host interaction.

Pathogenesis of experimental gonorrhea in the human male urethra: summary and update

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Experimental infection with *Neisseria gonorrhoeae* in the anterior urethra of male volunteers has proved to be a useful model for testing the importance of putative gonococcal virulence factors in men. Since the early 1990s, over two hundred subjects have been inoculated with *N. gonorrhoeae* at the University of North Carolina under the auspices of the Sexually Transmitted Diseases Cooperative Research Center. The presentation will provide a summary of the clinical and microbiological features of experimental infection with wild-type gonococci and the relative infectivity and attenuation of a number of mutant strains. Recent experimental infections with mixed inocula containing wild-type and mutant bacteria have provided an apportunity to examine the dynamics of competition between strains *in vivo*. Strengths and limitations of the model will be reviewed, and the potential for vaccine testing will be discussed.

Functional genomic and virulence analysis of an avirulent *phop* homologue mutant of *Neisseria meningitidis*

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Two component regulatory systems are important regulators of virulence genes in a number of bacteria. We previously generated and characterised a PhoP homologue mutant of *Neisseria meningitidis* (Mol Microbiol 2001, 39; 1345-55). This mutant showed many of the growth characteristics similar to the *phop* mutants of salmonella, but we were unable to demonstrate a change in virulence using a mouse intraperitoneal challenge model of meningococcal disease, as the parent strain was avirulent in this model. We therefore generated another mutant in a mouse-virulent C:2a:P1.2 (ST-11, ET-37 complex, UK) strain. This new *phoP* mutant has an altered growth morphology to the parent and again showed the characteristic inability to grow at low concentrations of magnesium. The mutant and parent strains were used to challenge groups of 5 female NIH mice at 3 doses, in the presence of human transferrin. The mice at all three challenge doses with the parent strain died within 2 days whereas all the mice injected with the mutant survived for the 4 weeks of the experiment.

We have therefore generated a *phop* mutant that is avirulent in the mouse model. Our hypothesis is that, similarly to the salmonella PhoP, the neisserial PhoP-homologue is involved in regulation of virulence genes. The identity and function of these PhoP-regulated genes is therefore of considerable interest. We have established that the *phoP* mutant expresses serogroup C polysaccharide and to identify proteins whose expression is deregulated in the mutant, we have performed proteomics analysis of *N. meningitidis* cell lysates. Wild-type and mutant strains were grown at 5mM magnesium and total proteins analysed by 2D electrophoresis. Several hundred protein spots were visible. The mutant showed a significantly different protein profile to the parent strain. The identity of these protein spots is currently being investigated by mass spectroscopy.

The genetics of glycosylation in *Neisseria meningitidis*

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Pili of *Neisseria meningitidis* are of major importance in virulence. They are the major adhesin of this capsulate organism and contribute to specificity for the human host. They have been reported to be post-translationally modified at Serine 63 by either the addition of an O-linked trisaccharide Gal(β1-4)Gal(α1-3)2,4-diacetimido-2,4,6-trideoxyhexose or the disaccharide GlcNAc(α1-3) Gal. We have identified a series of pilin glycosylation genes from the MC58, Z2491 and FAM18 genome sequencing projects. We have characterised genes with homology with genes involved in the biosynthesis and transport of oligosaccharide structures. Comparative analysis of available genomic data revealed a high degree of polymorphism in these glycosylation loci. This comparative analysis also revealed a large number of potentially phase variable genes involved in these loci. A strain survey was conducted to further characterise the nature of these polymorphisms and phase variable loci. *pglE* (Pilin glycosylation gene E) is a phase variable glycosyltransferase with a heptanucleotide repeat within its coding region. We present evidence that this phase variation mediates switching between a disaccharide and trisaccharide structure. Preliminary examination of the role of this variation in the association and invasion of the human bronchial epithelial cell line 16HBE14 by *Neisseria meningitidis* strain c311 has been conducted. Other defined glycosylation mutants were also examined. Further to these studies, non-piliated bacteria were created by insertional inactivation of the *pilE* gene. The *pilE* mutants exhibited extremely low levels of both associated and adherence with the 16HBE14. Electron microscopy studies have been conducted to further characterise the association and invasion process. These results highlight the variability and potential importance of pilin glycosylation in the pathogenicity of *Neisseria meningitidis*.

C4b-binding protein interactions with *Neisseria gonorrhoeae* porin is influenced by hexose substitutions on the heptose I chain of lipooligosaccharide

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Prior studies have shown that hexose substitutions of heptose I (HepI) of *Neisseria gonorrhoeae* lipooligosaccharide (LOS) modulates the ability of the organism to resist killing by nonimmune normal human serum (NHS). A pyocin-resistant mutant of gonococcal strain 1291 that lacked hexose substitutions on LOS HepI was fully killed (0% survival) by 10% NHS and did not process C4b to C4d (no C4b-binding protein [C4bp] cofactor activity on the bacterial surface), while parent strain 1291 (lacto-*N*-neotetraose substitution on HepI) fully resisted NHS (100% survival) and processed C4b. Prior work has demonstrated that the porin (Por) molecule of certain gonococcal strains binds C4bp, which results in C4b processing and serum resistance. We examined the effect of HepI hexose substitutions on C4bp-Por interactions. *L*OS *g*lycosyl *t*ransferase E *(lgtE)* mutations (that resulted in Hep substitution of Glc→HepI) decreased C4bp binding to 1291 (Por1B-3), FA1090 (Por1B-3), did not alter C4bp binding to FA19 (Por1A-1), and enhanced C4bp binding to MS11 (Por1B-9). *lgtF* mutants (HepI unsubstituted) decreased C4bp binding to all 4 strains. Serum resistance correlated well with C4bp binding. Replacement of 1291 *lgtE* Por with MS11 Por enhanced C4bp binding, suggesting that Por remained the acceptor for C4bp when LOS was truncated. Strain background did not influence C4bp-LOS-Por interactions, as shown by introducing MS11 Por and 1291 Por separately into an F62 background, followed by creating *lgtE* and *lgtF* mutants. LOS mutations in an F62/Por MS11 background simulated observations with corresponding LOS mutants of MS11. Rather unexpectedly, F62/Por 1291 did not bind C4bp, and was serum-sensitive. Creation of an *lgtA* mutant (Gal→Glc→HepI) of F62/ Por1291 restored C4bp binding (and serum resistance), suggesting that a GalNAc extension beyond the lacto-*N*-neotetraose LOS structure (as seen with strain F62) might decrease C4bp binding to certain Por molecules (such as 1291 Por). This is the first demonstration of a microbial surface component modulating binding of a complement regulatory protein to a distinct target, and might provide a molecular basis for the enhanced serum sensitivity of *N. gonorrhoeae* with truncated LOS structures.

Variation of human proinflammatory cytokine genes influences likelihood and severity of meningococcal disease

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Introduction: Genetically determined variation of human cytokine release influences the severity of meningococcal disease (md); first degree relatives of people who die of md exhibit altered stimulated cytokine release by peripheral blood mononuclear cells. TNF and IL-1β (encoded by genes *TNF* and *IL1B* respectively) are central, early and highly inflammatory mediators of the innate immune response. There is a single nucleotide polymorphism (SNP) within the *TNF* promoter 308 nucleotides upstream of the transcriptional start site [*TNF*(–308)]; likewise there is a SNP at *IL1B* (-511) and each of these is associated with aberrant gene transcription.
Objective: To ascertain the relative frequencies of *TNF* (–308) and *IL1B* (-511) amongst survivors and fatal cases of md and a control population of blood donors.
Methods: We conducted automated genotyping under blinded conditions of 1106 consecutively-referred blood samples from patients with md, amongst whom 91 died. Patients presented throughout England and Wales during the period July 1998-November 1999. We also genotyped 839 northern English blood donors. The study was approved by the ethics committee of the Public Health Laboratory Service.
Results: The death rate was influenced significantly by age. The genotyping results are shown in the table. The common and rare alleles are denoted ‘1` and ‘2` respectively; homozygotes are denoted ‘11` or ‘22` and heterozygotes ‘12`.

***IL1B*(-511)**	**11**	**12**	**22**
Survivors	467	376	113
Fatal cases	42	27	18
Blood donor controls	355	346	96

In the case of *IL1B* (-11), no difference was observed between cases and blood donor controls. Logistic regression analysis with age included in the model indicated that patients carrying the common allele at *IL1B* (-511) were more likely to survive their disease (OR 2.32[CI 1.21-4.46]).

***TNF* (-308)**	**11**	**12**	**22**
Survivors	654	301	43
Fatal cases	62	19	3
Blood donor controls	598	285	12

In the case of *TNF* (-308) there was no significant difference between survivors and fatal cases, indicating no effect of this SNP upon severity of the disease. However, homozygotes for the rare allele (22) were significantly more frequent amongst patients with meningococcal disease compared with blood donor controls (OR 3.27 [CI 1.72-6.21]). The genotype distribution amongst this group of controls is highly consistent with data in other series.

Conclusion: We confirm that genetic variation of cytokine genes does influence likelihood and severity of meningococcal disease. *TNF* (-308) is associated with susceptibility to md, whilst *IL1B* (-511) is associated with fatal outcome.

Mapping of molecular events during interaction between *Neisseria* and host target cells

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Our hypothesis was that *Neisseria* organisms adhering to human epithelial target cells would upregulate certain genes required for optimal interaction with target tissue. By using two-dimensional gel electrophoresis and silver/coomassie blue staining, we have compaired the protein expression pattern between *Neisseria* adhering to a human epithelial cell line, ME180, and non-adherent bacteria. Several proteins showed increased staining when the bacteria were attached to the cells, indicating that expression of these proteins were upregulated. Protein spots occurring in adherent bacteria, but not in bacteria alone were selected and analysed by MALDI-DOF. The *Neisseria* genome was searched for gene products with matching mass values using MS-Fit. A number of novel proteins involved in the initial bacteria-host cell interaction have been identified. Upregulation of proteins was confirmed by RT-PCR. Further, genes of interest are currently being insertionelly inactivated and analysed for their ability to interact with human epithelial cells. This approach has identified novel virulence-associated proteins expressed during contact with human target epithelial cells.

Opa proteins of *Neisseria gonorrhoeae* bind Thyroid Hormone Receptor Interacting Protein 6, a focal adhesion plaque-associated host cell protein

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The Opa proteins of *Neisseria gonorrhoeae* (gonococci, GC) are outer membrane proteins involved in adherence to and invasion of epithelial cells. Previously our lab used the yeast two-hybrid system to identify several intracellular host cell proteins that appear to interact with Opa proteins, indicating that Opa may play an important role in the intracellular life of GC. One of the proteins identified by the yeast two-hybrid system was Thyroid Hormone Interacting Protein 6 (Trip6), a poorly characterized member of the Lim domain-containing family of proteins. The work described here was designed to confirm the results of the yeast two-hybrid system and to determine the physiologic significance of the Opa-Trip6 interaction in the pathogenesis and intracellular lifestyle of GC. We used immunofluorescence microscopy to characterize the distribution of Trip6 in several cervical epithelial cell lines. We also infected these cells with both GC and *E. coli* expressing recombinant Opa proteins. Our results show that intracellular bacteria expressing Opa proteins bind Trip6, confirming the yeast two-hybrid system results. To our knowledge, this is the first observation of intracellular bacteria binding Trip6. We have also used chimeric Opa proteins and the yeast two-hybrid system to identify the specific region(s) of Opa and Trip6 involved in the Opa-Trip6 interaction.

Gonococcal genital tract infection in estradiol-treated mice

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Our understanding of the pathogenesis and immune biology of *Neisseria gonorrhoeae* is hindered by the lack of animal models for gonococcal infection. Previously, we described a mouse model of long-term gonococcal genital tract infection. Here, we further characterized this animal model by analyzing the tissue and cellular distributions of gonococci and the local inflammation in infected mice.

Female BALB/c mice were treated with 17-β-estradiol and inoculated intravaginally with wild type *N. gonorrhoeae* strain FA1090 or saline. The genital tissues were collected and processed for immunohistochemistry and electron microscopy two and five days after the inoculation. The number of gonococci from vaginal swabs and the number of polymorphonuclear leukocytes from vaginal smears of infected mice were determined daily to ensure the establishment of infection. The number of gonococci and polymorphonuclear leukocytes in different regions of mouse genital tissue was determined using immunohistochemistry. The cellular distribution of tissue-associated gonococci was further analyzed using electron microscopy.

Gonococci were primarily detected on the surface of the vaginal mucosa two days post-intravaginal inoculation. At five days, besides the vaginal mucosa, gonococci were also detected in the cervix and uterine horns. Gonococci not only colonized the surface of the epithelial cells in these body sites, but also invaded into the tissues. Compared to placebo controls, mice that were inoculated intravaginally with *N. gonorrhoeae* had an increased number of polymorphonuclear leukocytes, especially neutrophils, extending into the lamina propria of vaginal and cervical tissue. This increase was sustained for at least 5 days after infection.

Our results show that intravaginal inoculation of mice with *N. gonorrhoeae* results in colonization of the apical surface of the vagina, cervix and uterine horns, and invasion into the genital tissue. A localized inflammatory response was also induced. This study supports the 17-β-estradiol-treated mouse model as a valuable tool for studies on gonococcus-host interactions.

Human lipoproteins (LDL, HDL or VLDL) inhibit the cytokine induction by *E. coli* LPS but not by *N. meningitidis* LPS

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Introduction: LPS-induced cytokine production is considered pivotal in the pathogenesis of Gram-negative septic shock. Therefore various therapeutic strategies aimed to inhibit this cytokine production have been explored. One of these is the application of (human) lipoproteins, as it has been shown that pre-incubation of LPS with lipoproteins diminishes the amount of TNFα and IL-1β production. The candidate infection for such a therapeutic strategy is fulminant meningococcal sepsis (FMS), the prototype LPS driven Gram-negative sepsis. However, all experiments so far have been done with LPS derived from enterobacteriaceae which differs in many aspects from meningococcal LPS. In order to evaluate the possible efficacy of LP-therapy in FMS, we compared quantitatively the inhibitory effect of human lipoproteins on the cytokine induction by enterobacteraceal LPS and on that by meningococcal LPS.
Methods: Cytokine (TNFα, IL-1β and IL-10) production was measured *in-vivo* in human PBMC-cultures stimulated with equimolar concentrations of *Salmonella minnesota* LPS, *E. coli* LPS (wild type and a Re-mutant) or meningococcal LPS (wild type H44/76 or the H44/76-*rfaC* mutant*).* All types of LPS were preincubated for various periods either with lipoprotein depleted plasma (LPDP) or with LPDP enriched with human LDL, HDL and VLDL lipoproteins in various concentrations.
Results: Cytokine production induced by all types of enterobacteraceal LPS was significantly inhibited by LDL and - although to a slightly lesser extent - by HDL and VLDL, even after a relative short period (1 to 2 hrs) of preincubation, and was further decreased to 20% after more than 4 hrs of preincubation. The pattern for *E. coli* wild type and *E. coli* Re LPS was similar. The cytokine production induced by meningococcal wild type LPS and by the polysaccharide deficient mutant *rfaC*-type LPS was hardly inhibited. Significant inhibition in these case occurred only after 24 hrs of preincubation with lipoproteins.
Discussion and Conclusion: Meningococcal LPS interacts much more slowly with human lipoproteins than *E. coli* LPS. This slow interaction precludes the application of lipoproteins as a therapeutic strategy in FMS. Based on the observation of similar pattern for the wild-type LPS and for the polysaccharide deficient mutant LPS-types, we presume that the lipid A part of the LPS-molecule is the main determinant depicting the interaction with human lipoproteins.

The «kinetics of dying» in meningococcal septic shock; implications for prospective and retrospective therapeutic studies

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Introduction: Fulminant meningococcal septic shock (FMS) is characterised by a high number of early deaths. This rapidly fatal course limits the value of therapeutic strategies that require time before they can be installed. In addition, for clinical studies this «kinetics of dying» limits the value of retrospectively obtained results, and may form an obstacle in the design of prospective therapeutic trials.
Methods: To quantify the effect of these «kinetics of dying» for the analysis of retrospective studies and the design of prospective trials, we analysed the data of 25 fatalities in the University Hospital Nijmegen and 191 deaths accurately reported in the literature.
Results: The estimated shape of the «time curve of dying» was described by the formula:

$$\% \text{ of deaths} = 100x \frac{t}{t+12}\%$$

(t = hrs after admission)

Discussion and Conclusion: From this formula it can be calculated that the mortality of a group of patients included 6 or 12 hrs after admission will be 67% or 50% of the mortality estimated from admission parameters. As, to our knowledge, none of the so far reported retrospective therapeutic studies incorporate this «kinetics of dying» in their analysis, all these studies should be interpreted with caution. For prospective studies the formula indicates that the study size will be determined by the moment the study-therapy can be started. For instance, assuming an expected mortality at admission of 30% and a reduction by the study-therapy of 33%, the required study size of a placebo-controlled trial will be 2 x 313 (*p*-value of .05, power 0.80). However, when the study-therapy is started after 6 or 12 hrs, at least 2 x 458 or 2 x 604 patients are needed.

Neisseria outer membrane proteins direct the traffic of Lamp1 exocytic compartments

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The *Neisseria gonorrhoeae* porin and pili induce a series of calcium fluctuations in the target host cell. The former causes an influx of extracellular calcium, while the latter releases calcium from intracellular stores. These calcium transients trigger the exocytosis of hLamp1 compartments, bringing hLamp1 to the epithelial cell surface, where it is cleaved by the secreted *Neisseria* IgA1 protease. Consequently, fewer lysosomes are present within the cells of infected cultures. This sequence of events enables bacterial intracellular survival, presumably because a cell with fewer functional lysosomes is less able to destroy the intracellular pathogen.

We wished to determine whether the exocytic events in response to the *Neisseria*-induced calcium signals are specifically directed at the site of bacterial attachment or whether they are a randomly distributed to the plasma membrane. We observed that beads coated with bacterial membranes containing porin and pili triggered the redistribution of cellular hLamp1 to the site of contact between bead and plasma membrane. In the presence of extracellular calcium, beads coated with porin-containing preparations caused hLamp1 to concentrate in vesicles at the cell cortex. Fusion of these vesicles with the plasma membrane, however, required the presence of pili and release of calcium from intracellular stores. To visualize these exocytic events, we constructed an hLamp1-EGFP fusion and stably expressed it in the A431 human epithelial cell line. Live-cell microscopy of beads contacting these cells enabled dynamic visualization that supported our observations. Together, our results indicate that hLamp1 exocytosis occurs in a stepwise manner upon contact of *Neisseria* membranes with epithelial cells and is dependent on pilus-induced calcium transients. In concert with the known activity of the IgA1 protease, these results illustrate the ability of pathogenic *Neisseria* to manipulate hLamp1 trafficking to its advantage.

Neisseria porin P1.B induces endosome exocytosis and a redistribution of Lamp1 to the plasma membrane

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The IgA protease secreted by pathogenic *Neisseria* cleaves Lamp1, thereby altering lysosomes in a cell and promoting bacterial intracellular survival. We sought to determine how the IgA protease gains access to cellular Lamp1 in order to better understand the role of this cleavage event in bacterial infection. In a previous report, we demonstrated that the pilus-induced Ca^{2+} transient triggers lysosome exocytosis in human epithelial cells. This, in turn, increases the level of Lamp1 at the plasma membrane where it can be cleaved by IgA protease. Here, we show that porin also induces a Ca^{2+} flux in epithelial cells. This transient is similar in nature to that observed in phagocytes exposed to porin. In contrast to the pilus-induced Ca^{2+} transient, the porin-induced event does not trigger lysosome exocytosis. Instead, it stimulates exocytosis of early and late endosomes and increases Lamp1 on the cell surface. These results indicate that Neisseria pili and porin perturb Lamp1 trafficking in epithelial cells by triggering separate and distinct Ca^{2+} dependent exocytic events, origing Lamp1 to the cell surface where it can be cleaved by IgA protease.

SESSION V

Host defence and response to infections

Cellular responses to type IV pilus-mediated adherence by *Neisseria gonorrhoeae*

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Colonization of mucosal surfaces by pathogenic *Neisseria* elicits multiple responses in host cells, including cellular signalling events, cytokine production, and modulation of the eukaryotic cell surface. Using a microarray approach, we have assessed the respective involvement of 375 human genes during adherence of piliated and non-piliated *Neisseria gonorrhoeae* strain MS11 to the epithelial cell line ME180. Genes targeted by the array encode proteins likely to be involved in inflammatory and adherence processes, e.g. adhesion molecules, integrins, interleukins, cytokines, chemokines, and growth factors. Thirty-eight genes were differently expressed. Out of these, 27 genes were upregulated, nine were downregulated, and the expression levels of two genes fluctuated during the assay. Several of the upregulated genes showed pilus-specific induction, i.e. induction of expression was stronger by piliated than by non-piliated bacteria. Further studies will focus on selected pilus-induced genes, most of which are integral membrane proteins. We are using quantitative real-time polymerase chain reaction (QRT-PCR) and immunoblots to confirm the microarray data and to quantify up-/downregulation in more detail. In addition, we are currently using an RNA interference system to knock down the expression of each selected pilus-induced gene in order to determine the possible requirement of these proteins for adherence or invasion by *Neisseria gonorrhoeae*.

Upregulation of anti-apoptotic factors in primary human urethral epithelial cells following infection with *Neisseria gonorrhoeae*

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Following invasion of host epithelium, the intracellular fate of *Neisseria gonorrhoeae* is still in question. In order to better understand the gene expression profile in human urethral epithelial cells following infection with *N. gonorrhoeae*, microarray analysis was performed. Analysis of data from three independent experiments utilizing the Affymetrix GeneChip™ revealed the differential expression of hundreds of host genes in response to infection with the gonococcus. Of interest, several host genes involved in blocking apoptosis were upregulated following infection. Past studies have demonstrated that primary human urethral epithelial cells infected with *N. gonorrhoeae* for 72 hours maintain a viability of greater than 90%. This suggests that the gonococcus may interfere with cell death during epithelial cell infection. Several reports have indicated that certain bacterial factors, including Neisserial porin, may induce the apoptotic cascade. In contrast, other reports have demonstrated the anti-apoptotic effects of these same bacterial factors. The results of the microarray analysis performed by our laboratory revealed a 10-fold increased expression of the anti-apoptotic factor, *bfl-1*, in urethral epithelial cells infected with *N. gonorrhoeae*. Bfl-1 is a host protein that blocks programmed cell death by binding to pro-apoptotic factors, thereby inhibiting their function. Subsequent assays confirmed the increased transcript levels of *bfl-1* in infected cells. Furthermore, RT-PCR and RNase protection assays indicated that while *bfl-1* is increased in expression in response to infection, host factors that act to promote apoptosis do not change in expression.

Analysis of host cell apoptosis by propidium iodide staining and flow cytometry indicated that infection with *N. gonorrhoeae* does not induce programmed cell death. Furthermore, infection of urethral epithelium with the gonococcus for 24 hours rescues host cells from staurosporine-induced apoptosis. The bacterial factors responsible for the increased expression of host anti-apoptotic genes, and the subsequent suppression of apoptosis, are currently under investigation. Preliminary evidence suggests that signaling through the host cell receptor (ASGP-R) to which the gonococcus binds may be responsible. The upregulation of anti-apoptotic factors could be a means by which the gonococcus prevents host cell death following invasion. This may represent a mechanism utilized by *N. gonorrhoeae* to survive and proliferate in host epithelium.

Investigations into the factors that determine increased susceptibility to meningococcal invasion: the roles of cytokines and target receptors

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Invasive meningococcal strains are universally encapsulated yet capsule may mask key adhesins required for bacterial-host cell interactions. In previous studies we have reported that capsulate meningococci may effectively interact with transfected target cells that have high receptor densities[1]. Such a situation may occur *in vivo* if such receptors are upregulated as a result of prior viral or bacterial infection. The aim of the present study is to formally address the hypothesis that at high receptor densities, interactions of outer membrane adhesins may occur in capsulate phenotypes.

Previously, IFN-γ was shown to increase CEACAM expression on Chang conjunctival epithelial cells but no increase in CD46 expression was observed. Subsequent work has confirmed these observations on several other epithelial cell lines including Hep-2 (laryngeal), Detroit 562 (pharyngeal), A549 (lung pneumocyte) and NCI-H292 (lung mucoepidermoid). In addition to IFN-γ, TNF-α stimulated the expression of CEACAMs on these cell lines. Concurrently with the cytokine induced increase in CEACAM expression, there was a corresponding increase in Opa-dependent meningococcal association.

To enable direct comparison of the receptor density with the level of meningococcal attachment, a flow cytometry method has been developed. Initial experiments have used CEACAM1 transfected Chinese Hamster Ovary (CHO) cells expressing distinct levels of the receptor (covering a 300-fold range as measured by FACS). As may be expected, these experiments show that greater the level of CEACAM expression, the higher the number of attached acapsulate meningococci that expressed Opa proteins. Adherence of the capsulate meningococcal strain MC58 (Opa^+) also increased significantly. Further, we observed that receptor density affected the rate of attachment of both capsulate and acapsulate bacteria. The adhesion of capsulate meningococci to CHO cells expressing high levels of CEACAM1 was confirmed using a viable count assay and was dependent on Opa expression alone.

These results have confirmed that receptor densities dictate interactions of capsulate bacteria and imply that *in vivo* conditions that affect receptor expression on target tissues may lead to increased susceptibility to meningococcal colonisation and invasion.

Reference

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TonB confers a survival advantage to *Neisseria gonorrhoeae* in the genital tract of female mice, but is not required for experimental murine infection

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Neisseria gonorrhoeae possesses a variety of receptors for acquiring iron from the host, including receptors specific for human lactoferrin, human transferrin and hemoglobin. A putative siderophore receptor, FetA, has also been described. These receptors require the TonB-ExbB-ExbD inner membrane complex to energize transport through the outer membrane. The existence of three other TonB-dependent outer membrane proteins was predicted recently from the gonococcal genome database; the functions of these proteins, however, have not yet been definitively determined. The iron sources available to *N. gonorrhoeae* during infection are likely to differ with respect to body site. The redundancy of the iron acquisition systems in *N. gonorrhoeae* may therefore provide this pathogen with the ability to proliferate in different mucosal and systemic niches of infection. Male volunteer studies have implicated transferrin as a critical iron source for *N. gonorrhoeae* in the male urethra. The male urethra differs substantially from the female genital tract with regard to potential iron stores, however, and therefore sources of iron other than transferrin may also be sufficient to promote gonococcal infection in women. For example, the low pH of the female genital tract may promote solubilization of iron or the release of iron from transferrin and lactoferrin. Commensal flora in the vagina and endocervix may produce siderophores or metabolic intermediates that can form complexes with iron that the gonococcus then can utilize. Additionally, the high turnover of genital epithelial cells that occurs under the influence of estrogen may result in the release of large amounts of iron from intracellular stores. We recently reported that *N. gonorrhoeae* can persist in the genital tract of estradiol-treated mice in the absence of human transferrin for as long as 40 days, and that the gonococcal transferrin and hemoglobin receptors were not required for experimental murine infection. To further explore the iron sources that may be available to *N. gonorrhoeae* in the female lower genital tract, we tested the capacity of a *tonB* mutant of strain FA1090 (kindly provided by P. F. Sparling) to infect estradiol-treated BALB/C mice. Mice were inoculated intravaginally with 10^6 CFU of wild type or *tonB* mutant FA1090 and vaginal mucus was cultured daily for 10 days. The duration of recovery was similar for the wildtype FA1090 and the *tonB* mutant (average 7.1 and 8.1 days, respectively). In competitive mixed inoculum experiments, however, the *tonB* mutant was dramatically attenuated compared to the wild type strain. These results suggest that although gonococci can acquire sufficient iron for growth in the murine lower genital tract in the absence of TonB, one or more TonB-dependent receptors may give *N. gonorrhoeae* a competitive advantage in this body site.

The cellular responses of human dendritic cells to outer membranes of *Neisseria meningitidis*

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Introduction: Serogroup B *Neisseria meningitidis* is a major cause of life-threatening meningitis and septicaemia world-wide, and no effective vaccine is available. Initiation of innate and acquired immune responses to meningococci is likely to be dependent on the cellular responses of dendritic cells to antigens present in the meningococcal outer membrane (OM). In this study, the responses of monocyte-derived dendritic cells (mo-DC) to OM from parent meningococci and a mutant deficient in LPS were investigated.
Materials and Methods: Human mo-DC were challenged with the parent strain H44/76, the LPS-deficient mutant H44/76 pLAK33, and also with their corresponding OM and pure LPS. Viability of bacteria in the presence of mo-DC was monitored over time. Maturation of mo-DC; production of nitric oxide, cytokines and chemokines; and modulation of TLR receptor status, receptor-mediated endocytosis and antigen-specific T-cell proliferation were all used as markers of the biological function of dendritic cells.
Results: Viability of parent meningococci was unaffected in the presence of mo-DC: conversely, growth of the LPS-deficient mutant was inhibited and this correlated with the production of high levels of nitric oxide by mo-DC. Expression of Toll-like receptors (TLRs) was differentially regulated by the presence of meningococcal LPS in OM. Parent OM selectively up-regulated TLR4 mRNA expression and induced mo-DC maturation, reflected by increased production of chemokines, pro-inflammatory cytokines and CD83, CD80, CD86, CD40 and MHC class II molecules. In contrast, LPS-deficient OM selectively up-regulated TLR2 mRNA expression, and induced moderate increases in both cytokine production and expression of CD86 and MHC class II molecules. Pre-exposure to OM, with or without LPS, augmented the allo-stimulatory properties of mo-DC and resulted in the proliferation of naïve $CD4^+CD45RA^+$ T-cells. In contrast, LPS-replete OM induced a greater interferon-γ:IL-13 ratio by naïve T-cells, whereas LPS-deficient OM induced the reverse profile.
Discussion: The current study demonstrates that OM from *N. meningitidis* are immuno-modulatory and exert distinct effects on human mo-DCs. OM induced changes in mo-DC phenotype that resulted in the DCs acquiring potent T cell-activating properties. Meningococcal LPS played a major role in activation of mo-DC; however, components of the OM, other than LPS, also influenced the levels of DC activation and the subsequent balance between Th1 and Th2 cells. Our data suggest that the appropriate response mounted against meningococci *in vivo* is likely to involve a combination of several factors, including the presence of distinct pattern-recognition receptors on DC subsets; the duration/levels of antigen encountered; and the presence of cytokines within the microenvironment. Modulation of these biological activities presents the possibility to induce or manipulate the desired innate and/or adaptive immune responses with appropriately designed vaccines.

Immunostimulatory properties of *Neisseria meningitidis* class 1 porin on human dendritic cells

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Introduction: Dendritic cells (DC) are professional antigen presenting cells that play critical roles in the regulation of innate and adaptive immunity. The class 1 porin of *Neisseria meningitidis* is a major protein component of the outer membrane (OM), which has been reported to exert immunostimulatory effects on B-cells. In this study, the effects of purified recombinant class 1 porin on human monocyte-derived dendritic cells (mo-DC) were examined.
Materials and Methods: Class 1 porin from strain H44/76 was expressed as a recombinant protein in *E. coli* and purified with nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography. The recombinant protein was solubilized in water containing 0.04% SDS (wt/vol), and the levels of contaminating *E. coli* endotoxin was quantified at 0.2pg/ml. Mo-DCs were stimulated with various concentrations of purified class 1 porin, endotoxin and SDS vehicle. Maturation of mo-DC; production of cytokines and chemokines; and modulation of TLR receptor status, receptor-mediated endocytosis and antigen-specific T-cell proliferation were all used as markers of the biological function of dendritic cells.
Results: Recombinant class 1 porin induced marked mo-DC maturation, reflected by increased expression of CD83, CD80, CD86, CD40 and MHC class II molecules and reduced capacity to take up antigen as observed by flow cytometry. Expression of Toll-like receptor (TLR)4 mRNA in mo-DCs was significantly up-regulated and a trend ($p>0.05$) showing down-regulation of TLR2 mRNA was observed following stimulation with class 1 porin. Moreover, porin-treated mo-DCs selectively increased secretion of IL-1β, IL-6, IL-10, TNFα, IL-12p40 (but not biologically active IL-12p70), MIP-1α, MIP-1β, RANTES and IL-8. A strong allo-stimulatory capacity to drive $CD4^+CD45RA^+$ T-cells was observed by mo-DCs pre-exposed to class 1 porin. In addition, these mo-DCs augmented the ability to stimulate proliferation of autologous tetanus-toxoid specific short term $CD4^+$ lines compared to mo-DCs pulsed with tetanus toxoid alone. In all studies, effect of treating mo-DCs with contaminating endotoxin LPS levels was comparable to mo-DCs treated with SDS vehicle alone.
Discussion: The current study demonstrates that purified meningococcal class 1 porin exerts stimulatory effects on mo-DCs by modulating their antigen presenting function, in part by increasing co-stimulatory molecule expression, to stimulate potent allogeneic T-cell proliferation. The observed ability of porin-treated mo-DCs to markedly augment tetanus-specific T-cell demonstrates the potent adjuvant properties exhibited by the class I porin. These data suggest that the meningococcal class 1 porin plays a significant role in the overall activation of mo-DCs by whole OM.

Inflammatory response to proteins of pathogenic *Neisseria*

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Introduction: Infections by *Neisseria gonorrhoeae* and *N. meningitidis* are characterised by the induction of intense inflammation. Several bacterial factors appear to be involved in the inflammatory response. These include the classical inducer of inflammatory responses, LPS, and some proteins like pili, Opa and IgA protease. This work was initiated to find out whether other proteins could contribute to the inflammatory process.
Methods: Protein fractions of *N. gonorrhoeae* were obtained from a whole cell lysate after separation by SDS-PAGE followed by slot elution. The fractions were used to stimulate the human cell line U937 differentiated by incubation with PMA for 72 h. At this stage, cell surface CD14 expression was demonstrated by FACS analysis. A total of 20 fractions were used, as well as whole cell lysates and purified LPS as a positive control, to stimulate cells in triplicate wells. Supernatants were obtained at time zero and at intervals up to 48 h and tested by ELISA for the presence of pro-inflammatory cytokines. Whole cell lysates and purified porins were used to stimulate polymorphonuclear cells (PMN) separated by sequential density gradient centrifugation from freshly obtained blood of normal human volunteers.
Results: Several fractions induced production of TNFα, IL-1β, Il-6 and Il-8. Induction of cytokine production by some but not all fractions was reduced by incubating the cells with anti CD14 antibodies prior to stimulation, indicating that the effect may have been due to LPS contamination of the proteins. Some fractions, particularly those containing the porins PorB and Rmp and the Gro-EL homologue Hsp60, remained active, suggesting that certain proteins may be stimulatory and probably use a CD14 independent pathway.

The pro-inflammatory activity of the gonococcal porin was confirmed by using purified Porb1b in the same system. Porb1b induced a vigorous cytokine response that could not be inhibited by treatment with Polymixin B or by blocking the CD14 receptor with concentrations of specific antibody sufficient to block the effect of LPS at a high dose. A similar effect was seen when using a porin from *Neisseria meningitidis*. The meningococcal porin PorB3 from strain 44/76-M14 was shown to induce TNFα, IL-1β and Il-8 but at a lower level than the gonococcal porin Porb1b.

Whole cell lysates and the two porins Porb1b and PorB3 induced production of IL-8 from PMN. The IL-8 response was detected at 6 h after stimulation, was particularly intense at 24 h and remained high for the duration of the experiments.
Conclusions: The porins Porb1b of *N. gonorrhoeae* and PorB3 of *N. meningitidis* induce production of pro-inflammatory cytokines in PMN and macrophages. This activity may contribute to the intense inflammation characteristic of infections by these pathogens and has implications in the development of porin containing vaccines.

Two major outer membrane proteins of *Neisseria meningitidis*, opacity protein and PorB, but not lipooligosaccharide (LOS) bind human mannose-binding lectin (MBL)

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Human MBL is a serum collectin that has been shown to activate complement in an antibody-independent manner when bound to repeating sugar moieties on several micro-organisms. Genetic MBL deficiency has been associated with an increased risk of meningococcal disease. We have previously reported that MBL binds to and activates complement on encapsulated serogroup B and C meningococci including strain 8026, that leads to increased bacterial killing. The major determinant of MBL binding was LOS sialylation. In the present study, we sought to identify the meningococcal cell surface components to which MBL bound and to characterize this binding. Purified LOS and outer membrane complex (OMC) containing proteins and LOS from strains known to bind MBL were examined for MBL binding by dot blot and ELISA. OMC bound MBL but LOS did not, although an anti-LOS monoclonal antibody (Mab) bound strongly. Preliminary experiments showed that MBL bound strongly to bovine serum albumin (BSA). On ELISA, the binding of MBL to OMC did not require calcium and was not inhibited by 200 mM *N*-acetyl-D-glucosamine when BSA was removed from the experimental conditions. With the use of SDS-PAGE and immunoblot analysis and Mabs specific for meningococcal opacity (opa) proteins and porin proteins, we found that MBL did not bind to LOS but did bind to opa and porin (PorB). We confirmed this by determining the *N*-terminal amino acid sequences of the two MBL binding proteins on strain 8026. A BLAST search matched the proteins to *N. meningitidis* opa and *N. meningitidis* PorB. On immunoblot, the binding of MBL to opa and PorB did not require calcium but was inhibited in 0.5 M NaCl. MBL bound to purified PorB on ELISA and immunoblot and this binding was confirmed by surface plasmon resonance (BIACORE). To determine if MBL bound to whole organisms suspended in human serum, meningococci were incubated with 50% hypogammaglobulinemic serum for 30 minutes at 37^0C. After washing, the bacteria were resuspended in buffer and allowed to coat microtiter wells. Serum MBL bound to the whole organisms as detected by an anti-MBL Mab. We conclude that MBL binds to two major meningococcal OMC proteins, opa and PorB but not to LOS. Meningococcal opa and Porins are not glycosylated and the binding of MBL to the meningococcal strains tested did not appear to be mediated by its lectin domain. Binding of MBL to whole meningococcal organisms occurs in human serum.

The interactions of bacterial pathogens causing meningitis induce significant differences in the inflammatory response of human leptomeningeal cells

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Introduction: An *in vitro* cell culture model of the human leptomeninges, based on cells derived from human meningiomas, has been previously described. This model has been used to identify the significant contribution of meningeal cells to the acute intracranial inflammatory response characteristic of meningococcal meningitis. Recently, we have extended the use of this model to investigate the interactions of other bacterial pathogens causing pyogenic meningitis, comparing both their cellular interactions and induction of cytokines and chemokines.
Materials and Methods: Human meningothelial meningioma cells were cultured *in vitro* to confluence and challenged with various concentrations of *Neisseria meningitidis*, *Escherichia coli* K1, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *N. lactamica*. At intervals up to 48 hours, attachment and internalisation of bacteria were quantified by colony counting following saponin lysis and gentamicin treatment. In addition, secretion of cytokines and chemokines were detected by RT-PCR and immunoassay.
Results: All of the bacteria showed a specific predilection for adhering to meningioma cells, association increased over time, although significant differences in the dynamics of their adhesion were observed. *N. meningitidis* and *E. coli* K1 demonstrated equally high adhesion rates, which were significantly greater than *H. influenzae* and *S. pneumoniae*. In contrast, the commensal *N. lactamica* demonstrated the lowest levels of adhesion. However, of all the meningeal pathogens, only E.coli K1 invaded meningioma cells, an observation that was confirmed with confocal and transmission electron microscopy. In addition, *E. coli* and *S. pneumoniae* were able to induce host cell death, an observation not seen with meningococci, *N. lactamica* or *H. influenzae*.

Meningeal cells secreted large amounts of IL-6, IL-8 and MCP-1, and lesser amounts of RANTES and GM-CSF in response to challenge with the different bacterial pathogens. Notably, significant differences were seen in the type and concentration of these inflammatory mediators following challenge.
Discussion: Invasion of the CSF by meningococci, *E. coli* K1, *H. influenzae* and *S. pneumoniae* results in the production of an acute inflammatory response. Our data demonstrate that the interactions of these pathogens with meningeal cells differ, and that this is likely to be dependent on the expression of surface adhesins / invasins. The observed cell death induced by *E. coli* K1 and *S. pneumoniae* maybe toxin-mediated; indeed, the rapid cell death observed with *E.coli* K1 is consistent with the high rates of mortality for the neonate.

In this study, there were also significant differences between the pathogens in the secretion of inflammatory mediators, which is likely to be dependent on the nature of the interactions of bacterial modulins and meningeal cell surface receptors and the signal transduction pathways involved.

Piliated *Neisseria gonorrhoeae* down-regulate membrane cofactor protein (MCP, CD46) on ME180 cells

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Introduction: Human CD46 is a widely expressed cell surface glycoprotein that protects host cells against autologous complement attack. This protein is expressed as four major isoforms; BC1, C1, BC2, and C2. MCP is a cellular pilus receptor for *Neisseria gonorrhoeae* (NG). It also interacts with several other human pathogens including *Streptococcus pyogenes*, Herpesvirus 6, and some strains of the measles virus. Measles virus infection of cultured mammalian cells induces a down-regulation of MCP expression. In this work, we describe down-regulation of MCP in response to the adherence of NG strain MS11 to ME180 cells.
Materials and Methods: The ME180 human cervical cancer cell line was infected with NG strain MS11 for up to 24 hrs. Cells harvested over this time course were evaluated for expression of MCP and other surface markers by FACS, Western Immunoblot, and RT-PCR.
Results and Discussion: Within 6 hrs of infection of ME180 cells with P^+ Opa^- NG, but not P^- Opa^- NG, a nearly 80% reduction in MCP surface expression was noted which persisted for at least 24 hours. NG adherence did not influence the expression of HLA and up-regulated the expression of the cell adhesion molecule E-cadherin. MCP down-regulation is influenced by the MOI, being maximal at an MOI of 1000 and undetectable at an MOI of 1. In addition, down-regulation of MCP is not blocked by antibodies specific to MCP, suggesting that the initiation of this effect is not reliant upon a direct interaction of the bacteria with the ectodomain of MCP. MCP down-regulation is not initiated by heat-killed or chloramphenicol-treated bacteria. Analysis of mutants altered in pilus expression revealed a likely role for PilT and pilus retraction in inducing this response. RT-PCR analysis of MCP expression does not show a decrease in the amount of MCP being transcribed, indicating that the loss of MCP from the cell surface is not due to decreased transcription. However, the MCP isoform pattern at the RNA level changed from predominantly BC2 to predominantly C2 by 24 hrs postinfection.
Conclusions: Adherence of piliated NG to ME180 cells results in a decrease in the quantity of MCP on the cell surface and a shift in the isoform distribution pattern at the RNA level. Future investigations will focus on determining if these changes in MCP expression are part of a defense response of the cell and/or if they contribute to the survival and pathogenesis of the gonococci.

Regulation of the mannan-binding lectin pathway of complement on pathogenic *Neisseria* by C1-inhibitor and α-2 macroglobin

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We examined complement activation by *Neisseria gonorrhoeae* via the mannan-binding lectin (MBL) pathway in normal human serum (NHS) . Maximal binding of MBL complexed with MBL-associated serine proteases (MASPs) to *N. gonorrhoeae* was achieved at a concentration of 0.3 μg/ml. Preopsonization with MBL-MASP at concentrations as low as 0.03 μg/ml resulted in ~60% killing of otherwise fully serum-resistant gonococci. However, MBL-depleted serum (MBLdS) reconstituted with MBL-MASP prior to incubation with organisms (postopsonization) failed to kill at a 100-fold higher concentrations. Preopsonized organisms showed 1.5-fold increase in C4, 2.5-fold increase in C3b and ~25-fold increase in factor Bb binding; enhanced C3b and factor Bb binding was classical pathway-dependent. Preopsonization of bacteria with a mixture of pure C1-inhibitor and/or α-2 macroglobulin (α-2M) added together with MBL-MASP, all at physiologic concentrations prior to adding MBLdS, totally reversed killing in 10% reconstituted serum. Reconstitution of MBLdS with supraphysiologic (24 μg/ml) concentrations of MBL-MASP partially overcame the effects of inhibitors (57% killing in 10% reconstituted serum). We also examined the effect of sialylation of gonococcal lipooligosaccharide (LOS) on MBL function. Partial sialylation of LOS did not decrease MBL or C4 binding, but did decrease C3b binding by 50%, and resulted in 80% survival in 10% serum (lacking bacteria-specific antibodies) even when sialylated organisms were preopsonized with MBL. Full sialylation of LOS abolished MBL, C4 and C3b binding, resulting in 100% survival.

Similarly, only *N. meningitidis* expressing LOS that terminated in a GlcNAc- residue bound MBL when incubated with NHS, and bactericidal activity was demonstrated only when the strain was «preopsonized» with MBL-MASP, followed by addition of NHS lacking bacteria-specific antibody. Menningococci that expressed the lacto-*N*-neotetraose LOS did not bind MBL.

Our studies indicate that MBL does not participate in complement activation on *Neisseria* in the presence of «complete» serum that contains C1-inhibitor and α-2M.

Sibling familial risk ratio of meningococcal disease in UK Caucasians

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Background: Despite evidence indicating a host genetic component to meningococcal disease (MD) susceptibility, to date this has not been quantified. The sibling risk ratio of disease, λ_S, is a standard parameter used in genetic analysis to indicate the increased risk of disease in siblings of affected cases compared with the risk of disease in the general population. An increased risk of disease in siblings indicates a host genetic component to susceptibility. Unbiased estimates of λ_S are generally difficult to calculate for infectious diseases for two reasons: 1) estimates of λ_S for infectious diseases will overestimate the importance of the host genetics because of the increased risk of exposure to infection in family members compared to the general population 2) the risk of disease in the general population is generally not accurately known as this may vary with age and calendar year. We have calculated λ_s taking into account these two factors.
Methods: Questionnaires were administered to 443 UK MD Caucasian cases identified between 1995 and 2001. Age-calendar year specific MD incidence rates in the general population of England and Wales were calculated using notification information from the Public Health Laboratory Service and population information from the Offices for National Statistics. λ_s was calculated as the ratio of observed MD cases among the 845 siblings of these index cases to the number expected, calculated from the age-calendar specific MD incidence rates. To minimise the effect of overestimating λ_s due to increased exposure to *N meningitidis*, λ_s was re-calculated using 1 week, 1, 3, 6, 9 and 12 months as cut offs between the onset of MD in affected cases and their affected sibling(s).
Findings: 27 siblings contracted MD. The expected number of MD cases among the 845 siblings was 0.89, generating a λ_s of 30.3 (95% CI 20-44). If siblings contracted MD more than 1 month after onset in the index case, the λ_s was 11, decreasing slightly to 8.2 (95% CI 3.3-17) for risk of MD more than 12 months after onset in the index case.
Conclusions: This study is the first to calculate λ_s for MD (λ_s=30.3). Excluding cases occurring within one month of index case onset, the value of λ_s is lower, which is likely due to the effect of increased *Neisseria meningitidis* exposure at the time of index case disease onset. Using a one-month cut off, host genetic factors were estimated to contribute to at least one third of the sibling risk ratio.

Death from meningococcal disease is associated with possession of the TNF2 allele

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Background: Disease severity and outcome in meningococcal disease (MD) have been correlated with circulating levels of cytokines, particularly tumour necrosis factor-alpha (TNF-α). A biallelic polymorphism that may have a role in regulating the expression of the TNF-α gene is located at position –308 in the promoter region. Possession of the TNF2 allele, (involves a guanosine to adenosine substitution), is associated with higher constitutive and inducible levels of transcription than possession of the TNF1 allele. We have previously described an association between MD mortality and possession of the TNF2 allele in 98 children. This finding has been questioned. To investigate further, two additional association studies on two independently collected, separate cohorts of MD patients were carried out plus a meta-analysis on all samples including our previously published cohort.
Methods: DNA was collected from 2 populations between 1995 and 2000: 295 children ≤16 years of age admitted to the Paediatric Intensive Care Unit (PICU) at St Mary's Hospital, London, UK and 258 patients (age range at MD onset: 0-82 years: 220 ≤16 years and 38 >16 years) from the Manchester Reference Unit (MRU). Genotyping was carried out using restriction enzyme digest. PCR amplification of the area containing the G-A substitution was followed by digestion with *Nco* I which cuts the TNF1 allele. Digest products were run out on a 4% agarose gel.
Statistical Analysis: Risk ratios (RR) and 95% confidence intervals (CI) were calculated on the PICU and MRU cohorts plus a meta-analysis on all samples (n=651). Meta-analysis is recommended to take into account any inter-study bias/diversity between multiple cohorts. Chi-squared test was used to calculate p values.
Results: The meta-analysis confirmed the association between MD mortality and TNF2 possession (RR=1.46 [1.05-2.04], p=0.03) with higher possession of TNF2 than TNF1 in non-survivors in the PICU (9.2% vs 4.1% p=0.08; RR=2.26 [0.9-5.7]) and MRU (37% vs 28.6% p=0.2 RR=1.29 [0.9-1.9]) cohorts. MD mortality was also associated with a higher TNF2 allele frequency in the meta-analysis (0.22 vs 0.16, p=0.04) and PICU cohort (0.32 vs 0.17, p=0.02) with a higher but not significant TNF2 allele frequency in the MRU cohort (0.18 vs 0.15).
Interpretation: This study has re-investigated the role of the TNF2 allele and mortality from MD. The overall association in the individual groups studied is seen to vary. This is not surprising as multiple factors will be contributing to the overall risk of mortality from MD and thus the degree of association will vary between studies. Meta-analysis overcomes this inter-study bias/diversity and confirms that TNF2 possession is associated with mortality from MD.

The role of the E-selectin polymorphism S128R in meningococcal disease

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Objectives: The A to C polymorphism within the E- selectin gene results in a serine (S) to arginine (R) change at codon 128. Its location within the EGF-domain and *in vitro* data has suggested that the R allele may cause higher affinity binding and migration of leukocytes to and through activated endothelium, with increased cytokine production, endothelial cell damage and capillary leak. As endotoxin from invading *Neisseria meningitidis* activates leukocytes, causing migration to the endothelium, the R allele may have a role in susceptibility and/or severity of meningococcal disease (MD).
Methods: DNA from MD cases (n=155) and ethnically matched controls (n=80) was amplified using PCR across the region containing the S128R polymorphism and digested with the restriction enzyme *PST* I to genotype alleles. All MD cases were given "PRISM" predicted mortality (PM) scores as an indication of disease severity.
Results: No difference in genotype frequencies were found between: cases and controls (χ^2=0.6 p=0.8); disease outcome (survival vs non- survival χ^2=1.6 p=0.5) and PM scores (p=0.41-0.66).
Conclusions: The S128R polymorphism is not associated with MD susceptibility and severity in this study. This does not exclude a role for E-selectin in MD as other polymorphisms within the gene may be functionally important.

Mechanisms of platelet-neutrophil interactions in meningococcal disease

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Background: There are multiple interactions between the inflammatory and coagulation pathways in fulminant sepsis. We have previously shown that platelet-neutrophil complexes (PNCs) represent a subpopulation with a more activated adhesion molecule profile, a greater capacity for phagocytosis and ability to produce toxic oxygen metabolites. We set out to determine whether PNCs increase in meningococcal sepsis and explore the mechanisms that underlie this process.
Patients and Methods: Blood and skin biopsies were obtained at presentation from 27 children with meningococcal sepsis following informed consent. Circulating neutrophil (L-selectin, CD11b, Mab 24) and platelet activation (activated CD41) were measured by standard whole blood flow cytometry. PNC formation was assessed by dual immunostaining with antibodies against platelet antigen CD42b and neutrophil CD11b. Biopsy material was immunostained for platelets (CD41), neutrophils (neutrophil elastase) and monocytes (CD68). The mechanisms of PNC formation were then explored using an *ex-vivo* model of meningococcal bacteraemia.
Results: Marked circulating neutrophil activation (increased CD11b expression and Mab24 binding) was seen in all subjects at presentation. Platelet activation (activated CD41 binding) was also observed in several subjects but rarely those with normal or extremely low platelet counts. A significant decrease in the %PNC's ($CD11b^+$ $CD42b^+$) was observed in the majority of meningococcal sepsis patients compared to controls, and returned to normal in convalescence. Leucocyte-platelet complexes (CD41 positive) were observed in all skin biopsies studied. Ex-vivo experiments revealed the absence of free platelet activation or PNC formation in a whole blood model in response to *N. meningitidis*, despite marked neutrophil activation. In contrast, platelet pre-activation with ADP resulted in a marked increase in PNC formation following inoculation of whole blood with meningococcus. PNC formation in this context appears to be CD62P but not neutrophil integrin dependent. Stimulation experiments undertaken with a *lpxA*$^-$ strain and a range of bacterial components including purified LPS, and the bioactive peptide fMLP failed to activate free platelets or increase PNC formation alone. All potentiated PNC formation in response to ADP.
Conclusions: In the context of platelet and neutrophil activation, PNC disappear from the circulation early in the course of meningococcaemia and may be detected deep in the skin lesions of these patients. Platelet activation does not appear to occur through direct interaction with *N. meningitis* or its bacterial products. However, PNC formation is potentiated by neutrophil activation by meningococci through both LPS-mediated and non-LPS mediated pathways.

Meningococcal interactions with human blood components

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A serious complication of meningococcal (Nm) disease is septicaemia with intravascular coagulation and thrombocytopaenia. Amongst the factors that may affect the outcome of bloodstream infection is the interaction of bacteria with blood cells including platelets. Several microbial pathogens have been shown to interact with platelets, which may lead to internalisation and killing within platelets or result in platelet activation, augmented aggregation and coagulation. Platelets express receptors analogous to several receptors on epithelial, endothelial and haemopoietic cells (including integrins and CEACAMs), which have been shown *in vitro* studies to be utilised by meningococci for subversion of host cell signalling and manipulation for colonisation, invasion and evasion of immune functions. Potential roles of such platelet receptors in meningococcal interactions have not been reported.

We have previously shown that vitronectin receptor (VNR, $\alpha v\beta 3$) and the fibronectin receptor (FNR, $\alpha 5\beta 1$) on endothelial cells support Opc-expressing bacterial interaction via serum components vitronectin and fibronectin in a trimolecular complex at the apical surface of endothelial cells[1,2]. Platelets express the integrin $\alpha_{IIb}\beta_3$, a fibrinogen receptor, related to VNR, which plays a crucial role in platelet aggregation, normal haemostasis and pathological thrombus formation. Most ligands that bind to $\alpha_v\beta_3$ e.g. fibrinogen, von Willebrand factor, and vitronectin also bind to $\alpha_{IIb}\beta_3$. It is therefore possible that Opc may interact with this integrin.

To study the extent of association of distinct Nm phenotypes with multiple serum and matrix components (SMC), using both immobilised and soluble matrix assays, we investigated the potential of Nm to interact with a number SMC including fibrinogen, cellular and soluble forms of fibronectin, laminin and collagen. We have confirmed and extended the observations that Opc mediates interactions with multiple SMC over and above vitronectin and fibronectin reported previously, whereas Opa proteins primarily target fibronectins[1,2]. In competitive experiments, we have determined the relative affinities of the opacity proteins for fibronectins.

We have assessed the roles of various SMC in supporting bacterial interactions with integrins, initially using human umbilical vein endothelial cells. These studies show that a number of SMC support Nm interactions with the endothelial cells. These are often but not always RGD-dependent integrin interactions.

Interestingly, Opc interactions with endothelial integrins require cytoskeletal activity and even the adhesion is decreased in the presence of cytochalasin D or metabolic inhibitors. These requirements are also noted in inside/out signalling events of $\alpha_v\beta_3$ integrins and suggest that Opc-dependent binding to such integrins may also require receptor activation. $\alpha_{IIb}\beta_3$ is a well-recognised receptor requiring inside-out signalling for activation and binding to its ligands. To investigate its potential in supporting the interaction of Opc expressing bacteria, we have employed human platelets. Using flow cytometry and solid phase assays, we have demonstrated Opc-dependent binding of Nm to platelets, the precise molecular details are under investigation.

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The H.8 lipoprotein from pathogenic *Neisseria gonorrhoeae* stimulates NF-κB and cytokine release by epithelial cells in TLR2-dependent manner

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Neisseria gonorrhoeae is the second most commonly reported transmissible infection in the United States. While this organism primarily infects the lower genital tract, it can ascend to the upper genital tract in women resulting in pelvic inflammatory disease, and certain strains are capable of dissemination. Early events in the establishment of infection involve interactions between *N. gonorrhoeae* and cells present in the genital tract. Here, surface antigens on the gonococcus trigger the innate immune response that results in the release of cytokines, prostaglandins, and other inflammatory mediators. Because of this, it is important to identify interactions between bacterial virulence factors and host cell components that contribute to local inflammation. Previous efforts have focused on defining the immunologic responses to protein antigens and lipooligosaccharide (LOS) on the surface of *N. gonorrhoeae*. In contrast, little attention has been paid to the pro-inflammatory effects of Gram-negative lipoproteins.

We identified a significant lipoprotein contaminant in a preparation of *N. gonorrhoeae* strain F62 LOS as H.8. H.8 was originally identified almost 20 years ago as a surface antigen common to pathogenic *Neisseria* species. Although the size and amino acid sequence of H.8 can differ between *Neisseria* species, it consists of primarily 13-19 pentameric amino acid repeats, a lipid modified N-terminal cysteine residue, and lacks any aromatic residues. This latter property makes it undetectable by most protein assay and staining methods. As a result, the purified preparation of H.8 lacked any discernible protein bands when analyzed by gel electrophoresis and silver-staining. However H.8 can be detected as an 18 kD protein by immunoblotting using the anti-H.8 antibody 2C3.

In order to study the biological activity of H.8 further, we purified it from gonococcal membranes using a series of column chromotography steps. In human embryonic kidney (HEK) 293 epithelial cells, purified H.8 stimulated the activation of the transcription factor NF-kB, which regulates the inducible expression of many cytokines, chemokines, costimulatory proteins, and adhesion molecules. This NF-kB activation by H.8 could be removed by immunoprecipitation with 2C3 and was dependent on the expression of toll-like receptor 2 (TLR2), a member of a family of receptors that recognize conserved molecular patterns shared by a broad range of pathogens. The purified H.8 preparation also stimulated the release of cytokines by HEK 293 cells transfected with TLR2 and by immortalized human endocervical epithelial cells, a cell line that expresses TLR2, and maintains the morphological and immunocytochemical characteristics of endocervical columnar epithelial cells.

These observations provide evidence that H.8 can trigger the release of inflammatory mediators from cervical epithelial cells and may be an important factor in the pathogenicity of *Neisseria* infection. Furthermore, the difficulty in detecting H.8 by routine staining makes it a potential contaminant in preparations derived from Neisserial membranes.

Mannose-binding lectin increases phagocytosis of *Neisseria meningitidis* by an enhancement of internalisation dependent on actin rearrangement and Rho GTPases

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Introduction and Methods: We have shown that the humoral innate immune component, mannose-binding lectin (MBL), enhances phagocytosis of meningococci. We studied the mechanisms by which this occurs using a human monocyte-derived macrophage (MDM) model with fluorescent staining to discriminate internalised from bound organisms and to measure the incorporation of the late endosome marker, lysosome-associated membrane protein-1 (LAMP-1).
Results: Mid-log *N. meningitidis* strain B1940*cpsD*- were incubated with MBL (5µg/ml) and then added to MDMs at a multiplicity of infection (MOI) of 250:1 or 500:1 for 30 min. At both MOIs, MBL significantly increased the number of organisms associated with 100 MDMs by 28% (250:1) or 41% (500:1). This was related to an increase in the proportion of cells taking up cells by 46% (250:1) or 15% (500:1). The number of organisms per cell was significantly increased only at 500:1 by 23%. MBL increased the number of organisms internalised by 140% (250:1) and 63% (500:1). Internalisation, the percentage of organisms bound that were internalised, was increased by 117% (250:1) but not significantly at 500:1. The influence of MBL on binding and internalisation changes with MBL was inhibited with N-acetyl-glucosamine but not mannitol, consistent with carbohydrate dependent binding to the organism. MBL increased the number of organisms in LAMP-1 positive endosomes by 130%.

To analyse the stages of phagocytosis influenced by MBL, MDMs were cooled to 4ºC to prevent internalisation and then incubated with organisms. MBL did not influence association of meningococci with MDMs, but when the cells were warmed to allow internalisation, MBL enhanced internalisation at 60 min from 28% to 44%. This effect was dose dependent and maximal at 1.5µg/ml MBL.

Latrunculin B was added to inhibit actin polymerisation and abolished any increase in phagocytosis with MBL. *C. difficile* toxin B was added to inhibit the Rho GTPases necessary for actin rearrangement and this also inhibited any increase in phagocytosis with MBL. Scavenger receptor-A (SR-A) is a known internalisation receptor for meningococcus which can be inhibited using polyinosinic acid. However, the addition of polyinosinic acid, whilst inhibiting phagocytosis in the absence of MBL, did not alter the level of phagocytosis in the presence of MBL.
Conclusion: MBL enhances phagocytosis by enhancing internalisation, but it does not mediate binding of organisms to phagocytes. Internalisation is dependent on actin rearrangement by Rho GTPases, but is independent of the internalisation receptor, SR-A.

Neisseria meningitidis LPS dependent and independent activation of endothelial cells

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Background: Vascular damage and dysfunction are responsible for many of the pathophysiological features of severe meningococcal disease. This is largely mediated by activated leukocytes. The expression of cell adhesion molecules on the surface of endothelial cells is a critical step in leukocyte attachment and transmigration. We have previously shown that meningococci are potent inducers of the endothelial adhesion molecules CD62E, ICAM-1, and VCAM-1. In this study we have explored the relative importance of LPS and non-LPS components of *Neisseria meningitidis* in the modulation of endothelial cell adhesion molecule expression.
Materials and Methods: HUVEC (Human Umbilical Vein Endothelial Cells) were exposed to a serogroup B *Neisseria meningitidis* (strain H44/76- WT), purified LPS, and an LPS deficient isogenic mutant (*lpxA*-) at different concentrations and variable exposures. CD62E, ICAM-1, and VCAM-1 were detected by flow cytometry. Further studies were performed to investigate the transcriptional regulation of CD62E expression by analysing CD62E mRNA by RT-PCR.
Results: At high concentrations (10^8 organisms per ml), all stimuli induced high levels of CD62E, ICAM-1 and VCAM-1. However it was apparent that the expression of CD62E was much higher following exposure to the intact bacteria when compared to LPS. Interestingly, at lower concentrations of organisms, (10^6 organisms per ml), CD62E expression was still elevated in response to the WT strain but little expression was seen with the LPS deficient bacteria. mRNA analysis indicated that CD62E expression was largely related to changes in transcriptional activity.
Conclusion: These results show that at high concentrations of organisms, the absence of LPS did not have a major influence on endothelial adhesion molecule expression. However in contrast, LPS alone, even at very high concentrations, was always less potent than the bacteria. This appears to be the result of enhanced CD62E transcription in response to the intact organisms, and may involve multiple signal transduction pathways. These results may shed light on why patients with high levels of bacteraemia are more at risk of severe vascular injury.

Infection of transgenic CD46 mice with *Neisseria meningitidis*

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Attachment of pathogenic *Neisseria* to human host cells is mediated by pili. CD46 acts as a cellular pilus receptor (1). We have evaluated CD46 transgenic mice as an experimental infection model system for *Neisseria*.

Expression of CD46 in the transgenic mouse line closely mimicked distribution and pattern found in humans (2). Piliated, but not nonpiliated, *N. gonorrhoeae* bound to tissue sections from transgenic mice. CD46 transgenic mice were infected intraperitoneally (ip) with two different doses of piliated *Neisseria meningitidis* serogroup C FAM20. Ip infection with 10^8 bacteria resulted in 100% mortality, whereas infection with 10^6 bacteria resulted in 16% mortality. Bacteria were recovered from blood at 10^6 cfu/ml for the higher challenge dose. Moreover, bacteria were identified in histological brain tissue sections of infected CD46 transgenic mice. *Neisseria* were also found in the bloodstream of non-transgenic mice shortly after infection, but were cleared out after a few days. Both transgenic and nontransgenic mice showed significant increase in serum levels of TNFα, IL-6 and IL-10 three hours post infection. Ip infection with *Neisseria lactamica*, heat inactivated FAM20 or LOS deficient FAM20 did not result in bacteraemia or symptoms of disease.

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Passive protection by anti-protein and -polysaccharide monoclonal IgG antibodies in complement component C6 deficient and sufficient rat pups

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Introduction: We have established an infant rat passive protection (IRPA) model for studying the protection against meningococcal bacteremia and meningitis afforded by human (1) and murine (2) antibodies of different specificity. Antibodies to PorA and capsular polysaccharide (PS) have proven protective in that model. The relative contribution of intravascular bacteriolysis and complement/Fc mediated opsonophagocytosis to host immunity against meningococcal infections is currently unclear. We have used C6 deficient and sufficient rat pups to study the role of the terminal part of the complement pathway (generation of MAC) in the protection afforded by murine and V-gene matched human chimeric monoclonal antibodies (Mab) in the IRPA.
Materials and Methods: Inbred C6 sufficient (PVG/OlaHsd) and deficient (3; PVG/c+ and PVG/c-, respectively) and outbred HsdBrlHan:Wist rat pups were used for studying IRPA. 0.02 to 20 μg/pup of human chimeric monoclonal anti-PorA (HMN12H2; IgG1) (4), murine anti-PorA (MN12H2, IgG2a) and murine anti-group B PS (NmB735; IgG2a) were given i.p. resulting in approximately 0.01 to 10 μg/ml serum antibody concentrations, respectively. The bacterial (B:15:P1.7,16:L3,7,9; 44/76-SL) challenge (10^6/pup) was given i.p. after 1 h. Bacterial counts in the blood were measured after 6 h. Bactericidal activity of the Mabs was determined using human, infant rat or baby rabbit serum as a complement source.
Results: All Mabs were bactericidal with the three complement sources used. The same challenge dose (10^6/pup) was needed for the PVG/c+, PVG/c- and HsdBrlHan:Wist rats for development bacteremia and meningitis. >100-fold (2logs) reduction in blood bacterial density was achieved by a dose of 20, 0.5 and 0.5 μg/pup of MN12H2 (IgG2a) in the PVG/c-, PVG/c+ and HsdBrlHan:Wist rats, respectively. A dose of HMN12H2 (IgG1) needed for >2log decrease in the bacterial density was >20, 5 and 0.5 μg/pup, respectively. The dose of 0.2 μg/pup of NmB735 (IgG2a) was needed both in complement sufficient and deficient pups.
Conclusions: The C6 deficient and sufficient rat pups developed bacteremia and meningitis similarly. A ten-fold higher dose of HMN12H2 was needed for protection in the PVG/c+ pups as compared to HsdBrlHan:Wist pups; MN12H2 was equally protective in both rat strains. Higher concentration of anti-PorA antibodies were needed for protection in the PVG/c- than in the PVG/c+ pups, suggesting that complement mediated lytic bactericidal activity plays a role in the protection. Equal protection afforded by anti-MenB PS in both C6 deficient and sufficient animals suggests that complement/Fc mediated opsonophagocytosis plays an important role in protection given by anti-MenB PS IgG.

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The effect of hydrogen peroxide-producing lactobacilli on wild type and catalase-deficient *Neisseria gonorrhoeae* in a murine model of gonococcal genital tract infection

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Introduction: Gonorrhea is a major women's health concern due to the resultant complications of involuntary infertility and chronic pelvic pain and the high incidence of pelvic inflammatory disease. The endocervix is the most common site of gonococcal infection in women of reproductive age. Survival of the gonococcus in this body site is challenged by a variety of host defenses including inhibitory commensal flora. Lactobacilli, the predominant facultative anaerobic microflora in the female lower genital tract, produce a variety of potential inhibitory products (i.e., H_2O_2, bactericins, organic acids, biosurfactants). H_2O_2-producing lactobacilli, in particular, have been hypothesized to protect against gonorrhea based on clinical surveys. We previously showed that *L. crispatus* and *L. jensenii*, the predominant *Lactobacillus spp.* of the female genital tract, inhibit *N. gonorrhoeae* in vitro. H_2O_2 was determined to be the primary mediator of the inhibition, and as expected, a genetically defined catalase mutant was more sensitive to H_2O_2-producing lactobacilli. As an extension of these studies, here we assessed the capacity of H_2O_2-producing lactobacilli to protect against *N. gonorrhoeae* in vivo using a murine model of gonococcal genital tract infection developed in our laboratory.
Methods and Results: Three lactobacillus strains were screened for the potential to protect against experimental murine genital tract infection. *L. crispatus* 33197 was selected for further study due to its ability to inhibit *N. gonorrhoeae* strain FA1090 in a gel overlay assay at both neutral and acidic pH. A streptomycin resistant derivative of *L. crispatus* 33197 was isolated, and shown to colonize the lower genital tract of estradiol-treated BALB/c mice for greater than 7 days following intravaginal inoculation. To test the effect of *L. crispatus* on *N. gonorrhoeae* in vivo, groups of estradiol-treated BALB/c mice were inoculated intravaginally with *L. crispatus* 33197 or left untreated, and challenged with 10^6 (high dose) or 10^5 CFU (low dose) of wild-type or catalase mutant FA1090 four hours later. Vaginal mucus was cultured quantitatively for *L. crispatus* and/or *N. gonorrhoeae* for 14 days. No difference in the duration of recovery of wild-type or catalase mutant gonococci from mice with or without *L. crispatus* at either challenge dose was detected (average duration of recovery for all groups 12-14 days). However, fewer numbers of the catalase mutant were recovered from mice colonized with *L. crispatus* following challenge with the lower dose.
Conclusions: The increased sensitivity of the catalase mutant to *L. crispatus* inhibition coupled with the ability of wild-type gonococci to persist in mice colonized with *L. crispatus* suggests catalase is an important gonococcal protective mechanism against H_2O_2-producing bacteria. Additional, these data suggest many factors may be involved in the complicated interaction between lactobacilli and wild-type gonococci *in vivo*.

The endothelial nitric oxide synthase gene and severity of meningococcal disease

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Introduction: Meningococcal disease is frequently characterised by inflammation of the endothelium. The presence of bacteria on the endothelium is thought to initiate a localised host response that includes both a free radical bacterial killing process and a recruitment of immune cells. Possible consequences of over stimulation of this process include damage of the endothelium that may lead to the capillary leak syndrome, vascular constriction, and the production of large amounts of cytokines, which are all thought to contribute to disease severity. We have investigated the role of a possible mediator of this endothelial damage, nitric oxide (NO), by comparing the consequences of different polymorphisms in the endothelial nitric oxide synthase (eNOS) gene and measuring indirect indicators of NO levels nitrate and nitrite (Nox).
Subjects and Methods: Caucasian patients from the paediatric intensive care unit at S[t] Mary's Hospital (n = 256 including 25 deaths) and 108 Caucasian controls were investigated. An insertion/deletion (A/B) polymorphism within intron 4 of the gene was typed by PCR and agarose gel electrophoresis. Nox levels in patient serum samples (n = 46), taken on entry to St Mary's, were determined by Griess Assay and colourimetry.
Results: The control population genotype frequencies were similar to previously published studies. In contrast, the St Mary's patient population showed increased frequency of the A/A genotype that did not reach significance (3% vs 0% in controls, p = 0.1). Investigation of severity of disease using outcome showed that patients with the A/A genotype had a higher PRISM predicted mortality than other genotypes (53% vs 30%, p = 0.1) and a higher actual mortality (43% vs 9%, p = 0.03). Analysis of the serum samples showed a significant increase in Nox concentration in those patients with an increased predicted mortality (p = 0.007).
Discussion: These results suggest that variations in the eNOS gene may contribute to meningococcal disease severity. Further (on-going) work is required to establish if the increased Nox levels (and thus NO production) are associated with the intron 4 variations or other polymorphisms in the eNOS gene, leading to the increased severity. Also being investigated is the contribution that iNOS gene promoter polymorphisms may make to Nox concentrations in meningococcal disease.

The role of CD46 as a cellular receptor for pathogenic *Neisseria.*

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The cellular receptor CD46 (also called membrane cofactor protein, MCP) is an abundant glycoprotein expressed on all human cells except erythrocytes. It acts as a receptor for several pathogens including pathogenic *Neisseria* species, measles virus and *Streptococcus pyogenes*. CD46 interacts with type IV pili of *N. gonorrhoeae* and *N. meningitidis*. In order to analyze and examine the interaction between pathogenic *Neisseria* and CD46 in greater detail, we used RNA interference mediated by small inhibitory RNA molecules (siRNA) to silence CD46 expression in human cells. siRNA molecules are small duplexes of 21-nucleotide RNAs that knock down the expression of specific proteins (1).

The human epithelial cell line (ME180) showed greatly diminished levels of CD46 after transfection with *cd46*-specific siRNA. Adherence to transfected ME180 by piliated *N. gonorrhoeae* was accordingly reduced to 35% compared to non-transfected cells. This suggests that CD46 expression is very important for efficient attachment of piliated gonococci.

In contrast, invasion of ME180 cells by gonococci was strongly enhanced in transfected cells, reaching levels of almost 200% compared to non-transfected cells. These results accentuates the complexity of the interaction between pathogenic *Neisseria* and CD46. Further elucidation of the role of CD46 in adherence and invasion by pathogenic *Neisseria,* with respect to cellular signaling events and surface accessibility, is needed. We are currently performing corresponding adherence and invasion studies with *N. meningitidis*.

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Neisseria meningitidis protects HeLa cells from apoptosis

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Introduction: The pathogenic bacteria *Neisseria meningitidis* are the causative agents of meningococcal meningitis and sepsis. We have previously shown that purified meningococcal porin PorB associates with mitochondria and prevents apoptosis of B cells, Jurkat cells and HeLa cells induced by the protein kinase C inhibitor, staurosporine (STS). This work examines if intact meningococci have a similar effect and if this effect is mediated by the porins.
Results and Discussion: We first determined that incubation of HeLa cells with meningococci does not induce apoptosis, in and of itself, shown by direct visualization and measurement of DNA degradation by several methods (FACS analysis, fluorescent microscopy, electrophoresis). Moreover, intact meningococci did not elicit mitochondria-related apoptotic events, such as mitochondrial depolarization and cytochrome c release, or caspase 9 and 3 cleavage. We then examined if incubation of HeLa cells with meningococci could prevent apoptosis induced by staurosporine. We found that upon incubation with live meningococci, STS-induced apoptosis of HeLa cells was greatly reduced, as measured by the methods described above. We detected the presence of PorB in association with the mitochondria of cells incubate with intact meningococci, while another outer membrane protein, RMP, was not found associated with the mitochondria. Thus suggesting that a specific mechanism of translocation of porins from the bacteria to the intracellular environment maybe involved in this phenomena. Furthermore, we found that this anti-apoptotic effect was not related to changes in the levels of members of the Bcl-2 family.
Conclusions: Upon incubation of HeLa cells with *N. meningitidis* followed by induction of apoptosis, cell death is greatly reduced as compared to cells not incubated with the organism. The data suggest that the meningococcal porin, PorB, translocates from the bacterial outer membrane into the cytosol of the infected cells, where it has been shown to interact with the mitochondria. As a consequence of this interaction, mitochondria are protected from entering into the apoptotic cascade induced by STS treatment. These data are consistent with our previous findings demonstrating that purified meningococcal PorB can modulate STS induced apoptosis. During an acute infection, pathogenic Neisseria can invade and penetrate the mucosal tissue. Therefore, neisserial anti-apoptotic activity may have evolved for suppressing the host apoptotic response to the initial intracellular invasion.

Investigations of 17-β estradiol on *Neisseria gonorrhoeae* and HEC-1-B cell culture

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Humans are the sole natural host for *N. gonorrhoeae,* the etiological agent responsible for gonorrhoeae. It is imaginable that the gonococcus has developed mechanisms that respond to varying conditions of epithelial cell environment of the urogenital tract. The absence of a natural animal host has resulted in the use of *in vitro* studies for gonococcal pathogenesis using immortalized cell lines. Investigations that explore possible affects of female reproductive hormones on infection have been limited. Such studies could potentially have great impact on women. Clinical observations note susceptibility to gonococcal infection and PID presentation can depend on the stage of menstrual cycle, hormonal status or on oral contraceptive use. Previously our laboratory reported that higher numbers of gonococci were bound to HEC1B cells in the presence of physiological concentrations of 17-β Estradiol compared to the no hormone control. In addition, examination by SDS-PAGE of outer membrane proteins isolated from estradiol induced adherent bacteria revealed alterations in expression of several proteins when compared to controls. In this study, liquid chromatography mass spectroscopy/mass spectroscopy(LC-MS/MS) was used to sequence and identify proteins showing alterations in expression levels. Four proteins were analyzed by LC-MS/MS. Sequencing revealed the following: Catalase, Hsp60(GroEL), DNAK, and a putative neisserial lipoprotein. Currently our laboratory is constructing reporters for each identified sequence to quantify transcription levels in the absence and presence of hormone. In addition, higher percentage Tris-Glycine gels (16%) and silver staining, revealed several lower molecular weight OMP's (14-35 kDa) that also had altered expression levels in estradiol influenced adherence assays. LC-MS/MS is again being employed to determine sequence of tryptic peptides for these proteins. Our laboratory has conducted studies to determine if the estradiol influences the HEC1B cell culture system. MTT assays used to measure HEC1B cell proliferation showed that cells were viable and had a reproducible increase in cell number in the presence of physiologic concentrations of estradiol. Membranes isolated from HEC1B cells incubated with estradiol for at least 24 hours showed two proteins at 80 kDa and 180 kDa that differed in expression compared to control. These proteins will also be analyzed by LC-MS/MS. Data obtained in the above studies indicate that estradiol effects adherence of GC to HEC1B cells and alters expression of membrane proteins for both the bacteria and the HEC1B cells. Further study of the identified proteins may provide information regarding the mechanism of increased adherence. These findings suggest that hormones are a possible factor in gonococcal infection and indicate that use of reproductive hormones in laboratory studies may more closely mimic GC infection in women.

Identification of human antibodies against *Neisseria meningitidis* serogroup B from a VH family with intrinsic autoantibody potential

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Introduction: Antibody phage display libraries consist of antibody variable (V) domains expressed on the surface of M13 bacteriophage (phage antibodies). The genes encoding these antibody V domains are contained within each phage antibody, thus linking gene to protein and allowing selection of antigen specific antibodies.
Materials and Methods: A diverse (>1.3x 10^{10} recombinants), naïve library of human single chain Fv (scFv) fragments containing V genes from multiple tissue sources from a large number of human donors was panned against outer membrane vesicles (OMVs) produced from *Neisseria meningitidis* strain 44/76 (B:15:P1.7,16). The library contains both germline and mutated genes and would be expected to contain antibodies which have specificity for meningococci and which may have undergone affinity maturation through exposure to meningococci via nasopharyngeal carriage.
Results: Following 3 successive rounds of selection 70 clones were isolated which bound to OMVs in ELISA. Sequencing revealed that 10 clones had been selected, all of which contained a VH 4 family germline gene V4-34 (DP63). Further study of three of the clones demonstrated that they bind to the surface of intact meningococci of strains with the B15:P1.7,16 phenotype using flow cytometry. This binding was partially inhibited by 9G4, a rat monoclonal that recognizes the V4-34 framework. In order to identify the antigen recognized by these antibodies we have assessed their binding to mutant meningococcal strains and their inhibition by antibodies of known specificity.
Discussion: Antibodies belonging to the VH gene family V4-34 and with the ability to bind to surface antigens of *N. meningitidis* have been preferentially selected from a naïve human phage antibody library. V4-34 antibodies are frequently found to be autoantibodies, particularly targeting the i/I antigens. V4-34 producing B-cells are tightly regulated to prevent autoimmune disease and are concentrated in the non-immune repertoire. It has been postulated that V4-34 encoded antibodies may provide early or non-specific immunity against micro-organisms, thus explaining why they are maintained in the human B-cell repertoire. The discovery of a number of human V4-34 encoded antibodies with specificity for *N. meningitidis* may provide further information regarding the early immune response to *Neisseria*.

MCP-1 and MIP-1α in patients with systemic meningococcal disease

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Background: Recruitment of leukocytes from the circulation to sites of inflammation is essential for host defense against infectious agents. Regulation of the cellular trafficking involves adhesion molecules such as integrins and selectins and chemoattractant proteins and their receptors. The inflammatory chemokines, monocytes chemoattractant protein-1 (MCP-1) and macrophages inflammatory protein-1α (MIP-1α), induce migration of monocytes, basophils, T-and B-lymphocytes and dendritic cells. Proinflammatory cytokines and LPS (from *E. Coli*) induce synthesis of these chemokines. In patients with systemic meningococcal disease there is a complex interaction between cytokines and different celltypes. Very little is known about the level of chemokines in these patients, only levels of IL-8 and RANTES have been described.
Aim: To analyse plasma samples from patients with meningococcal disease for MCP-1 and MIP-1α and to correlate the levels with the endotoxin-level (LPS).
Metods: Plasma (heparin) collected from patients with fulminant septicaemia (n=25), meningitis (n=31) or mild disease (n=12) were analysed for MCP-1 and MIP-1α according to the manufacturers instructions (RandD systems Inc., MN, USA and Biosource International Inc., CA, USA). LPS quantification was by the Limulus Amebocyte Lysate (LAL)-assay.
Results: Patients with fulminant septicaemia had significantly higher levels of both MCP-1 og MIP-1α compared with patients with meningitis and mild disease. The concentration of MCP-1 was about 100 fold the concentration of MIP-1α in the patients with fulminant septicamaemia. The chemokine concentration was rapidly downregulated after treatment with antibiotics (after 2h, 85 % reduction). The correlation coefficient between the chemokines and LPS were: r= 0.88 (MIP-1α), r=0.90 (MCP-1).
Conclusions: Our data shown that patients with fulminant meningococcal septicaemia have very high leves of the chemokines (MCP-1 og MIP-1α) that attract monocytes/macrophages. These chemokines are important for the cells to migrate to the site of inflammation where an immunological response is required.

The separate signal pathways of the death of DT40 B cells were initiated by interaction of CEACAM1 (CD66a) and CEACAM3 (CD66d) with *Neisseria gonorrhoeae*

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CEACAM1 and CEACAM3 surface antigens serve as receptors for some of the opacity (Opa) proteins of *Neisseria gonorrhoeae* (gonococci, GC), promoting adherence and phagocytosis of this microorganism (4, 5, 7, 10) in epithelial cells and neutrophils. We and others found that GC may exploit a well-characterized intracellular signaling pathway, namely the immunoreceptor tyrosine-based activation motif (ITAM) signal transduction pathway, to mediate phagocytosis by interaction with CEACAM3 (2, 3). We further showed that CEACAM3-mediated phagocytic pathway of GC is also the pathway to cell death, which is involved in Syk kinase and phospholipase C activity (3). On the other hand, CEACAM1 was identified as an inhibitory receptor able to mediate negative signals (6), delivered through its immunoreceptor tyrosine-based inhibition motif (ITIM), which recruits SHP-1 and SHP-2, but not SHIP phosphatases (1, 6, 8). Unexpectedly, the interaction of CEACAM1 with GC results in induction of cell death, which does not involve SHP-1, SHP-2, Syk kinase or phospholipase C activity, but partially depend on SHIP and BTK kinases in DT40 cells. Thus, the cellular responses initiated through CEACAM1 resemble the events following activation of FcγRIIB, a well-documented inhibitory receptors also able to stimulate the death of DT40 B cells (9). These results support the notion that, in order to exert their inhibitory effects, inhibitory receptors should be co-ligated and co-stimulated with activated receptors. Otherwise, the so-called inhibitory receptors may deliver an activation signaling if they are induced directly.

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Role of surface components of *Neisseria meningitidis* in proinflammatory activation via Toll-like receptors

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The severity of disease caused by *Neisseria meningitidis* is correlated with the release of proinflammatory cytokines. The lipopolysaccharide (LPS) of *N. meningitidis* is a potent stimulator of the proinflammatory response, and plasma LPS levels also correlate with the severity of disease. Using a transfected HeLa cell method in which an IL8-promoter reporter construct is used to measure proinflammatory cell signalling, we have shown that *N. meningitidis* signals through both Toll-like receptor (TLR)2 and TLR4/MD2; and that LPS is necessary for the response of TLR4. Other bacterial surface components also play a role in meningococcal virulence, including sialylated terminal sugars of LPS and the polysialic acid capsule. We present data showing that mutants deficient in capsule and/or LPS outer core (preventing terminal sialylation) do not modify proinflammatory signalling though TLR2 or TLR4/MD2. However, purified lipopolysaccharides derived from diverse strains of *Neisseria* (including *N. meningitidis* and *N. gonorrhoeae*) cause widely variant levels of signalling through TLR4/MD2. These differences in signalling in HeLa cells correlate with the production of proinflammatory cytokines from THP-1 cells (a human monocyte derived cell line). The data suggests that while gross modification of the polysaccharide chain of LPS does not affect signalling through TLR4/MD2, fine structural differences in the lipid A portion of LPS do influence signalling through TLR4/MD2; and through this pathway influence the proinflammatory response elicited by the bacteria.

The human immune response to meningococcal carriage and disease

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Neisseria meningitidis colonises the human nasopharynx, from where invasion of underlying tissues may result in severe clinical syndromes. Immunity to meningococcal disease is dependent upon serum IgG with bactericidal activity but little is known about the cellular response, which regulates the humoral response and is important for the generation of immunological memory.

In order to study cellular and humoral immunity during carriage, pharyngeal swabs and samples of peripheral blood mononuclear cells (PBMCs) and serum were collected from a cohort of 200 undergraduate students at the University of Nottingham over the first 21 weeks of the academic year (1). The students proved to be subject to increasing rates of acquisition and carriage of meningococci. Progressively elevated anti-meningococcal serum IgG levels were detected in almost all cases, and this was accompanied by significantly increased serum bactericidal activity.

In order to investigate T cell responses, PBMCs taken on three occasions during the 21-week study were stimulated with a whole cell lysate of the H44/76 meningococcal strain (B:15:P1.7,16). The cells were stained to detect cell-surface markers and intra-cellular cytokines, and examined by flow cytometry. The cells were analysed for expression of CD69 (to indicate activation), IFNγ (a representative T-helper 1 subset (Th1) -associated cytokine) and IL-5 (a Th2-associated cytokine). Following meningococcal stimulation both IFNγ+ and IL-5+ events were detected amongst the CD69+, CD4+ population. The ratios of mean IFNγ+ to IL-5+ events amongst CD69+ CD4+ cells in cultures were between 0.81 and 1.83, and a positive correlation of IFNγ and IL-5 events was detected at all of the time-points ($p<0.005$). These data led to the conclusion that an unbiased T-helper subset response was elicited by meningococcal carriage.

PBMCs from patients proliferated in response to meningococcal antigens with significantly higher stimulation indices than those of carriers. Higher Th1 and Th2 responses were detected, although the frequencies of IFNg and IL-5-positive CD4 cells were again similar.

These data indicated that similar unbiased T-helper subset responses were elicited by both meningococcal carriage and disease.

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Meningococci influence inflammatory and apoptotic gene expression in human meningeal-derived cells.

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Introduction: During invasive disease, meningococci are thought to penetrate the nasopharyngeal mucosa and disseminate through the body via the bloodstream. High concentrations of inflammatory factors, e.g. IL-1β, TNFα can be detected in serum and CSF, but the cellular source of these cytokines remains unknown. Using expression arrays, we sought to determine

i) the effect of viable meningococci and their secreted proteins (SP) upon human gene expression in meningeal-derived, and
ii) whether meningeal cells could be a source of the cytokines detected in CSF during infection.

Materials and Methods: Human meningothelial cells, obtained from surgically removed tumors, were isolated and cultured to confluence. Viable meningococci or SP (LOS-depleted) were added to test monolayers whilst medium alone was added to control cells. After incubation, RNA was extracted from the human cells, radio-labelled probes were synthesized and used to probe human cDNA expression arrays. RT-PCR was used to confirm array data. Expression of human cytokines and other gene products were quantified by ELISA or immunocytochemistry.

Results and Discussion: In response to either viable meningococci or SP, up-regulated expression of pro-inflammatory factors including TNFα, IL-6, IL-8, MIP-2α, MCP-1, and VEGF, and adhesion molecules ICAM-1 and VCAM-1 was detected. Early effects of meningococci upon meningeal cells therefore include the production of pro-inflammatory cytokines, chemokines and adhesion molecules. Macrophages, polymorphonucleocytes and lymphocytes would thus be recruited to the area of infection, promoting the removal of invading pathogens.

Additionally, changes in gene expression that would lead to the inhibition of apoptosis were observed. Up-regulated expression of anti-apoptotic genes, including IEX-1L anti-death protein, FLIP, survivin and IAP-1, was detected. Observation of such changes in expression of apoptosis-related genes by cells of the meninges is an important novel finding.

Conclusion: These studies represent evidence that cells of the meninges take measures to resist the damaging effects of pathogenic bacteria and secreted virulence factors. Meningeal cells could indeed be a source of the cytokines detected in CSF during infection. Such responses may ultimately have a role in maintaining the integrity of the blood-brain barrier during meningitis.

Antithrombin, protein C and protein S in patients with meningococcal disease

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Introduction: Disseminated intravascular coagulation (DIC) presents important complication of meningococcal disease. Antithrombin, protein C and protein S are natural inhibitors of coagulation and play important role in the development of DIC.
Methods: Antithrombin, protein C and protein S were investigated in 64 patients (23 females, 41 males) with invasive meningococcal disease, who were treated at the University hospital Ostrava in the Czech Republic from January 1996 to November 2001. Natural anticoagulants were determined by using standard commercial kits, levels below 70% were considered as pathological.

Meningococcal meningitis was proved in 11 patients, septicaemia in 20 patients and meningitis/septicaemia in 33 patients. Four patients died, 3 of them suffered from septicaemia and 1 of them from meningitis/septicaemia. The highest morbidity was in the age groups 0-4 years (26 children, 1 death) and 15-19 years (16 patients, 1 death). The serogroups C and B were absolutely predominant, new emerging clone *Neisseria meningitidis* C:2a:P1.2,P1.5, ET-15/37 was the most frequent strain.
Results: Antithrombin was determined in 64 patients, at least twice in 56 patients. Initiative antithrombin levels below 70% or 50% were observed in 13 patients (2 deaths) or in 4 patients (0 death). Decrease of antithrombin level below 70% or next depression of pathological level during the course of disease were observed in 13 patients (2 deaths).

Protein C was investigated in 50 patients, at least 2 investigation were performed in 44 patients. Initiative levels of protein C below 70%, 50% or 30% were observed in 36 patients (1 death), in 25 patients (1 death) or in 10 patients (0 death). Decrease of protein C level below 70% or next depression of pathological level during disease were observed in 12 patients (1 death).

Protein S was determined in 38 patients, at least twice in 31 patients. Initiative levels of protein S below 70%, 50% or 30% were observed in 29 patients (0 deaths), in 20 patients or in 3 patients. Decrease of protein S level below 70% or next depression of pathological level during disease were observed in 14 patients (0 death).
Conclusion: Decrease of protein C and protein S in approximately two-third of patients and decrease of antithrombin level in fifth of patients with meningococcal disease were observed. These changes occurred nearly equally in patients with all clinical forms of meningococcal disease. Levels of protein C and S did not correlate with severity of disease and are not suitable for evaluation of meningococcal disease prognosis.

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The failure of protective immunity against genital infection with *Neisseria gonorrhoeae* is twofold

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Introduction: We have previously demonstrated that genital tract as well as circulating IgG and IgA antibody responses in both males and females infected with uncomplicated gonorrhea are weak at best, and show little evidence for the generation of effective immunological memory. Two nonexclusive hypotheses can be postulated to account for these findings: that the genital tract is poorly responsive to immune stimuli; and that gonococci are poorly immunogenic or possibly immunosuppressive.
Materials and Methods: To compare the relative immune responsiveness of the genital tract to another mucosal site, female mice were immunized intravaginally (i.vag.) or intranasally (i.n.) with a bacterial protein test antigen coadministered with cholera toxin (CT) or with *E. coli* labile enterotoxin (LT) type IIa or IIb, and specific antibody responses to both components were evaluated in serum, saliva, and vaginal wash. To investigate whether *Neisseria gonorrhoeae* can affect immune responses to another concurrently administered antigen, mice were immunized intraperitoneally (i.p.) with a potent bacterial protein antigen alone or mixed with live or killed *N. gonorrhoeae*, and serum antibody responses were determined.
Results and Discussion: i.vag. immunization with CT as an adjuvant induced genital and circulating but not salivary antibody responses against itself and the coadministered antigen, but the antibody levels were markedly less than the strong systemic and disseminated mucosal responses induced by similar i.n. immunization. LT-IIa and LT-IIb were ineffective as i.vag. adjuvants or immunogens,; however, they served as i.n. adjuvants as effectively as CT, but without inducing significant responses to themselves. The coadministration of live, but not killed, gonococci i.p. with another potent sentinel antigen suppressed the response to that antigen. Moreover, live gonococci induced a lower level of response against themselves than killed gonococci, and they shifted the serum antibody response from IgG1 towards IgG2a, indicating an effect on the balance of type 1 vs. type 2 T cell help. Conclusion: The dependence of i.vag. immunization on the exceptional adjuvant properties of CT, and the lack of dissemination of responses to remote mucosal sites, in contrast to the strong and widely disseminated responses induced by i.n. immunization, indicate that the genital tract is poorly responsive relative to other mucosal sites. This finding may be related to the lack of organized lymphoid follicles in the genital tract, and is consistent with its reproductive functions. The diminished response to a potent immunogen given with live gonococci suggests that *N. gonorrhoeae* interferes with the normal course of an immune response, and is supported by the recent demonstration of immunosuppressive effects exerted through gonococcal Opa interactions with CEACAM1 on T cells (Boulton and Gray-Owen, Nature Immunol. 3:229-236, 2002). These combined effects may account for the usual failure of gonorrhea to elicit protective immunity.

Investigation of host responses to water-soluble antigens from five strains of serogroup B *Neisseria meningitidis* using a novel competitive inhibition flow cytometric assay

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Objective: To determine whether individuals produce antibodies that bind to water-soluble extracts obtained from different serogroup B *N. meningitidis* strains.
Methods: Water-soluble antigens were obtained from 5 serogroup B *N. meningitidis* strains– MC58 (B:15:P1.7,16); the Cuban B vaccine strain (B:4:P1.15); and clinical isolates NM48 (B:4:P1.4), NM76 (B:2b:P1.10) and NM83 (B:NT:P1.13)– by repeated freeze-thawing. Lysates obtained from *H. influenzae* and *S. pneumoniae* were used as controls to assess non-specific binding of donor antibodies to bacterial proteins. Plasma was obtained from 2 adult donors who killed all 5 strains of bacteria in the whole blood assay (WBA) (Ison et al., 1999). After heating to inactivate complement, plasma was pre-incubated for 30 minutes with different concentrations of lysate, and then added to either homologous (parent) or heterologous meningococcal strains and incubated for 30 minutes at 37°C. After washing, the bacteria were incubated with FITC-rabbit anti-human IgG, A, M for 30 minutes at 37°C. After an additional wash the bacteria were processed in a flow cytometer and the level of inhibition of antibody binding to the meningococcal bacteria calculated.
Results: Antigens in meningococcal lysates were able to inhibit the binding of antibodies to the surface of the homologous strain in a dose dependent manner. Using a lysate concentration of 4000µg ml^{-1} we observed inhibition of 86%, 76%, 93%, 86% and 94% of antibody binding with antigens obtained from MC58, Cuban B, NM48, NM76 and NM83 respectively. When the lysates were used to inhibit binding to the MC58 strain to determine the presence of cross-reacting antigens, the greatest level of inhibition was observed using the homologous (parent) strain lysate. Greater than 75% inhibition of antibody binding was observed using the other lysates. Similar levels of inhibition were observed using antibody from each of the donors. The results were less consistent when using NM83 as the target strain. Plasma from donor 1 had levels of inhibition of 39 – 57% after incubation with the heterologous lysates, in contrast to plasma from donor 2 which had levels of inhibition of 64 – 70%. Significantly lower inhibition of binding was observed after incubation of plasma with lysates obtained from *H. influenzae* and *S. pneumoniae*.
Conclusion: A competitive inhibition flow cytometric assay has demonstrated that individuals who have bactericidal activity in the WBA have antibodies directed at surface expressed epitopes present on a diverse set of serogroup B *N. meningitidis* strains.

As the donors used in this study had not been infected with *N. meningitidis*, it is possible that exposure to the bacteria by carriage stimulated the production of cross-reacting antibodies. These antibodies may also have been induced by exposure to commensal neisserial spp. such as *N. lactamica,* which may be expressing antigens that cross-react with meningococcal structures.

Modification of a whole blood assay to detect antigens involved in bactericidal activity against serogroup B *Neisseria meningitidis*

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Objective: To determine the ability of water-soluble lysates obtained from serogroup B *N. meningitidis* to inhibit bactericidal activity in an *ex-vivo* model of whole blood killing.
Method: Adult donors were selected for their ability to kill five different serogroup B *N. meningitidis* strains in the whole blood assay. 15mls of fresh blood was obtained from adult donors and 1ml incubated without diluent, with diluent or various concentrations of lysates obtained from *N. meningitidis*, *H. influenzae* and *S. pneumoniae* and 400µg of Soybean Trypsin Inhibitor (STI) for 30 minutes at 37°C. The whole blood assay was conducted as previously described (Ison et al 1995). Briefly 10^7 cfu of MC58 were added to each ml of blood, mixed gently and incubated at 37°C. The blood was sampled at 0 and 90 minutes and 10µl aliquots plated onto dried GC plates. The numbers of bacterial colonies present at 0 and 90 minutes were counted the next day and the results expressed as percentage survival of the numbers of bacteria present at 0 minutes. Data was obtained from triplicate experiments.

The water-soluble antigens were obtained from serogroup B *N. meningitidis* strain MC58 (B:15:P1.7,16), *H. influenzae* and *S. pneumoniae* by repeated freeze thawing followed by centrifugation and freeze-drying before being at stored at –20°C. They were resuspended in water to a protein concentration of 10 mg ml^{-1} prior to use.
Results: Using final concentrations of 400, 200, 100 and 50µg ml^{-1} in blood, the lysate obtained from MC58 was able to inhibit the killing of MC58 bacteria. In donor 1 bacterial survival occurred at the highest concentration only with 40% of the bacteria surviving at 90 minutes. In donors 2 and 3 a dose-response effect was seen with bacterial survival decreasing with decreasing concentration of MC58 lysate with 200 and 150% of the initial bacterial inoculums surviving at 90 minutes. The lysates from non-neisserial bacteria and STI caused significantly reduced bacterial survival of *N. meningitidis* at 90 minutes compared to that observed using the MC58 lysate.
Conclusion: We have modified an *ex-vivo* model of whole blood bacteraemia and used it to investigate the effect of a water-soluble *N. meningitidis* lysate on bacterial growth. The data we have obtained indicates that the meningococcal lysate produced is able to inhibit the killing of the parent *N. meningitidis* strain in whole blood. This suggests that the lysate contains antigens, which are recognised by antibodies involved in the killing of MC58. Further investigation will allow us to determine whether the lysates we have produced contain antigens that are cross-reactive in nature and are able to inhibit the killing of different strains of serogroup B *N. meningitidis*.

Vaccination responses to *Neisseria meningitidis* serogroups W-135 and Y in adults with homozygous C2 deficiency

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Homozygous C2 deficiency (C2D) has an estimated prevalence of 1:40,000 in Western populations, and is known to be associated with increased susceptibility to invasive infections caused by meningococci and other encapsulated bacteria. C2 is necessary for activation of C3 and C5-C9 through the classical pathway of complement, and also supports complement activation through the lectin pathway. The alternative pathway is functionally intact in C2 deficiency. Previous studies indicate that anticapsular antibodies are capable of supporting serum bactericidal activity against *Neisseria (N.) meningitidis* through activation of the alternative pathway and that vaccination might be protective in classical pathway deficiencies. In the present study, adults with C2D (n=20) and a group of complement-sufficient controls (n=29) were immunized with tetravalent meningococcal vaccine containing capsular polysaccharides of the serogroups A, C, W-135 and Y (Menomune®). The protocol also involved immunization with polyvalent pneumococcal vaccine (Pneumovax®), and a *Haemophilus influenzae* type b vaccine with protein-conjugated capsular polysaccharide (ActHib®). Specific IgM and IgG antibodies to the *N. meningitidis* serogroups W-135 and Y were measured by ELISA in sera obtained before and 4-6 weeks after vaccination. Prevaccination antibody levels were low or undetectable in the C2D group as well as in the control group. With few exceptions, vaccination resulted in marked antibody responses. Postvaccination antibody levels in the C2D group did not differ significantly from the levels found in the control group. In bactericidal assays performed with C2-deficient sera at a final concentration of 25 %, killing of serogroups W-135 and Y appeared to require fairly high IgG antibody levels, i.e. in the range between 5 and 10 mg/L. Control sera demonstrated bactericidal activity both before and after vaccination. In addition to their bactericidal function it seems likely that anticapsular antibodies in C2 deficiency also contribute to defense by supporting opsonization through the alternative pathway and by complement-independent mechanisms.

Neisseria gonorrhoeae survive and proliferate within polymorphonuclear leukocytes

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Infection with *Neisseria gonorrhoeae* in males results in an acute urethritis with a massive infiltration of polymorphonuclear leukocytes (PMN). Despite the impressive inflammatory response, gonococcal urethritis often persisted for weeks to months in the pre-antibiotic era suggesting that this organism could resist PMN killing mechanisms. Many studies have examined the interactions of gonococci with PMN and have identified virulence factors that may be important for survival in phagocytes. However, the extent to which gonococci survive PMN killing and the ability to proliferate within these cells still remains debatable. Many of these studies examined PMN-GC interactions in suspensions, but in the host PMN function on surfaces. To simulate the natural environment, we have developed a phagocytosis assay that mimics the interactions between gonococci and PMN during *in vivo* infection. PMN are adhered to a glass surface coated with a combination of serum and extracellular matrix proteins and cooled to 10^{o}C to prevent phagocytosis. Serum opsonized gonococci are added to PMN in media containing amino acids and centrifuged to induce contact with the cell surface. The media is warmed to 37^{o}C and viable bacteria are quantified over time. We have demonstrated that gonococci are rapidly internalized in this assay and that the intracellular bacteria are capable of surviving for long periods of time within PMN. Real time RT-PCR analysis of the essential genes ftsZ and tsf revealed that intracellular gonococci express these genes at levels similar to log phase bacteria grown in culture. Finally, pulse-labeling experiments with ^{35}S-Met/Cys demonstrated that intracellular gonococci are metabolically active and synthesize protein. Taken together, these results support previous findings that *N. gonorrhoeae* can survive PMN killing, but also suggest that gonococci are capable of replicating within these cells. We are currently addressing the extent of gonococcal proliferation in PMN and how the bacteria may manipulate the host cell environment to its advantage.

Effect of Neisserial porins on dendritic cell function

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Neisseria meningitidis PorA (class 1 protein) and PorB (class 2 or 3 proteins) and *Neisseria gonorrhoeae* PIA (protein IA) and PIB (protein IB) are the major outer membrane proteins of the pathogenic Neisseria. It has been shown that Neisserial porins act as B cell mitogens and immune adjuvants. The mechanism of the immunopotentiating ability of porin is mediated predominately by its up-regulation of the T cell co-stimulatory ligand B7-2 on the surface of B cells. Because of Neisserial porin's ability to activate B cells and potentiate immune responses, we hypothesized that porin also employs the potent immune stimulatory function of dendritic cells (DC). In this work, we examined the ability of purified *N. meningitidis* PorB to induce maturation of splenic derived C3H/HeJ dendritic cells. C3H/HeJ mice have a point mutation in the TLR4 gene, thereby rendering cells hyporesponsive to LPS, which allows for the study of the effect of PorB on DC in the absence of LPS stimulation. We demonstrated that 24-hour PorB incubation induces the up-regulation of B7-2, class I MHC and class II MHC molecules on the surface of dendritic cells (characteristic of DC maturation), similar to levels induced by 24-hour treatment with the pro-inflammatory cytokine TNFα, as evidenced by flow cytometric analysis. PorB-induced up-regulation of class II MHC molecules was also evident by confocal microscopy, where class II molecules in medium treated DC localized within vesicles, while class II molecules in PorB treated DC were present at the cell surface. We also demonstrated that PorB treatment enhances the allostimulatory activity of DC, as evidenced by their increased activity in the mixed lymphocyte reaction (MLR), as compared with medium treated DC. The ability of Neisserial porin to induce dendritic cell maturation is significant for understanding the mechanism of its immune stimulatory activity.

Differential signalling events during *Neisseria meningitidis* and *Haemophilus influenzae* transcytosis across human epithelial cells

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Neisseria meningitidis (Nm) and *Haemophilus influenzae* (Hi), both colonisers of the human respiratory tract, target the human carcinoembryonic antigen family of cell adhesion molecules (CEACAMs). These events *in vitro* result in association of actin cytoskeleton with the receptors and the normally detergent soluble receptors become insoluble on ligation of bacteria. F-actin-capping under bacterially induced receptor caps occurred with both bacteria. However, after adhesion, Nm translocated through epithelial cells significantly less efficiently than Hi. Moreover, no discernible decrease in transepithelial electrical resistance was observed during translocation of either bacterium. This suggests that binding to CEACAM by specific bacterial adhesins might trigger differing signal transduction events in host cells allowing passage across target barriers without gross disruption and by distinct mechanisms. By confocal microscopy we have observed that haemophili were frequently associated with intercellular junctions and co-localised with occludin and cadherin, whereas meningococci were observed less frequently but were found largely intracellularly. Although, little is known about signalling events in target cells induced by Hi, it has been reported that binding of *Neisseria* to CEACAMs results in signal transduction involving tyrosine phosphatase SHP-1, tyrosine kinases such as Src and small GTPases like Rac1 and Cdc42. To investigate the involvement of such pathways during Nm vs. Hi invasion of target monolayers, we used a series of inhibitors and studied their effects on bacterial translocation across fully differentiated human epithelial monolayers. Interestingly, genistein an inhibitor of tyrosine phosphorylation reduced the rate of transcytosis of Hi across epithelial cells, whereas passage of Nm was unaffected. On the contrary, AG1478, an inhibitor of ErbB2, and Herbimycin A, a Src inhibitor, increased translocation of both Nm and Hi. Since SHP-1 can associate with CEACAMs in some cellular systems including epithelial cells, we used anti-SHP-1 antibodies in co-precipitation experiments. After Nm and Hi infection of target cells, two members of the CEA family of apparent MWs of *c.* 100kD and 140kD, could be co-precipitated together with SHP-1. In particular, the 100kD moiety was tyrosine phosphorylated and the level of its phosphorylation was higher during Hi infection compared with Nm infection. In addition, sodium orthovanadate, an inhibitor of phosphatase activity, blocked the passage of Nm and Hi through the monolayers, suggesting that association of CEACAMs with phosphatases might play a key role during bacterial translocation. Thus several early events such as actin polymerisation and SHP-1 recruitment are shared by both bacteria, but later signalling events diverge resulting in transcytosis of Nm but paracytosis of Hi across epithelial barriers *in vitro* where CEACAMs are the major target receptors.

Denitrification mechanisms of *Neisseria meningitidis* confers resistance to nitric oxide-related killing by human macrophages

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Neisseria meningitidis invades following a period of colonisation of the nasopharynx, during which it must evade innate immune responses mediated by resident macrophages. Activated macrophages kill bacteria by a number of mechanisms, including the oxidative burst, which is associated with release of reactive oxygen (ROS) and nitrogen (RNS) species. The genome of *N. meningitidis* includes the genes *aniA* and *norB,* predicted to encode nitrite reductase and nitric oxide reductase, respectively, and *cycP*, which encodes cytochrome *c'*. We hypothesised that these gene products allow the bacterium to denitrify nitrite to nitrous oxide, or consume NO, thereby conferring resistance to nitric oxide-mediated killing within human macrophages. We conducted deletion mutagenesis of the *cycP* and *norB* genes and found that *N. meningitidis* can grow microaerobically in the presence of nitrite via nitric oxide (NO), and that *norB* is required for microaerobic growth with nitrite. Continuous accumulation of NO at an initial rate of approximately 2.9 $nmol.min^{-1}.mg\ protein^{-1}$, in the presence of 1mM nitrite, was observed in suspensions of *norB* mutant strain but not wild-type *N. meningitidis*, as measured using an NO electrode, suggesting that wild-type *N.meningitidis* can consume NO. Expression of the *norB* gene and, to a lesser extent *cycP,* were able to counteract toxicity due to exogenously added NO and conferred protection against macrophage killing, presumably due to resistance to nitrosative stress. These data suggest a mechanism whereby *N. meningitidis* may survive nitric oxide-related host immune responses.

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Enhanced serological diagnosis of invasive meningococcal disease (IMD)

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Introduction: Serodiagnosis retains a significant role in confirmation of IMD. A test developed in the UK based on measurement of antibody to outer membrane protein [OMP] was sensitive in adults but not children with well developed features of IMD but also lacked specificity when used in less well defined clinical settings. This study sought to increase the specificity of this existing OMP IgM test and to increase its sensitivity in children.
Methods: To increase specificity, an LPS absorption step was introduced into the UK assay protocols and the test was then re-calibrated and re-evaluated using sera from controls, culture-positive throat carriers and culture positive IMD cases. Specificity was re-examined using sera from patients with immuno-proliferative disorders, arthralgias, infectious diseases accompanied by a rash and other infectious diseases. To increase the sensitivity of the test in infants, the OMP IgM test parameters were re-calibrated in children by using age specific sera from well infants.
Results: Significant improvements in both the sensitivity [in infants] and the specificity of the OMP IgM test were observed. Age-specific cutoff values were determined for infants and revised test parameters for the test in young children were then developed. In an initial study with sera from the only three culture positive cases available in children aged 1 year or less, the revised test parameters, but not the original test, gave a positive result. The specificity of the OMP IgM test in all age groups was significantly enhanced by the introduction of an LPS absorption step and the sensitivity of the original test was maintained. The revised OMP test eliminated false positive tests due to EB virus infection and demonstrated an absence of cross reactivity in infections due to *Mycoplasma pneumoniae, Yersinia*, and enteroviruses. Lack of cross reactivity with many other agents was previously demonstrated in our earlier (published) study. IMD was demonstrated as a cause of acute arthralgia and arthropathy of recent origin in a small number of RF and ANA negative patients. However low level false positive reactions occur in some RF and ANA positive sera.
Conclusions: The role of serological diagnosis in the laboratory confirmation of IMD has been confirmed and enhanced. Tests currently used internationally have been refined in terms of both specificity and sensitivity. The increased specificity of the revised test allows confirmation of the disease in atypical IMD presentations such as post IMD arthralgia syndromes. The increased sensitivity of a single-sample assay in infants, accompanied by the higher specificity, provides improved case ascertainment and confirmation of IMD in this age group. Continued assessment of possible causes of other non-specific and/or cross-reactions in the Nm OMP IgM test is appropriate.

Neisseria meningitidis activated human dendritic cells: role of bacterial structure and phagocytosis

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Introduction: Infections caused by *Neisseria meningitidis* are an important cause of mortality and morbidity worldwide. Effective sub-unit vaccines have been developed against serogroups A and C, but a safe vaccine has not yet been developed against serogroup B. Dendritic cells (DCs) phagocytose and process particulate antigens, such as bacteria and are therefore important in initiating an immune response to bacterial infections. In this study, the importance of phagocytosis in DC activation was investigated.
Materials and Methods: Human monocyte derived DCs were stimulated with purified LPS, serogroup B H44/76 wild type (WT) or LPS deficient, *lpxA*-, *N. meningitidis*. Phagocytosis and intracellular cytokine responses by DCs were studied.
Results: We show that LPS expressed on the surface of *N. meningitidis* is critical for a rapid internalisation of *N. meningitidis*. WT *N. meningitidis* were rapidly (after 1h) bound and internalised by the DCs, whereas LPS deficient bacteria, *lpxA*-, were internalised only after a significant delay compared to the WT (after 6h). Addition of exogenous purified meningococcal LPS did not enhance the internalisation of the *lpxA*-. WT bacteria were rapidly phagocytosed and induced high levels of TNF, IL6 and IL12 production, whereas the *lpxA*- bacteria were poorly phagocytosed and induced reduced levels of cytokines. We show that the majority of the cells that produced cytokines in response to the WT were those that were also in contact with the bacteria. These results suggested a possible link between phagocytosis and cytokine production. Role of phagocytosis in the DC activation and cytokine production was investigated by inhibiting phagocytosis with Cytochalasin D. A significant reduction in the TNF-α, IL6 and IL12 production in response to the WT bacteria was found. TNF-α and IL6 production in response to *lpxA*- organisms or LPS were not significantly reduced after cytochalasin D treatment suggesting that this was not dependent on phagocytosis. In contrast, IL12 production in response to LPS and *lpxA*- bacteria was inhibited by Cytochalasin D.
Conclusion: In conclusion, we show that LPS expressed on the surface of *N. meningitidis* is critical for a rapid internalisation of these bacteria by DCs. These data suggest that the reduced production of cytokines in response to LPS deficient *N. meningitidis* is due to reduced phagocytosis because of the absence of LPS on the outer membrane of the mutant bacteria. We show that cytokine production by DCs can be induced by different components of *N. meningitidis*, but it is only after phagocytosis of the intact, whole *N. meningitidis* that maximal activation for the cytokine production is obtained.

The lack of anti-gonococcal immunity in infected asymptomatic female contacts to men with gonorrhea

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We have previously demonstrated that symptomatic men and women with gonococcal disease develop a significant anti-gonococcal immune response as measured by anti-porin IgG and T cell responses and cytokine production (Simpson et al., 1999, *JID*, 189:762-773). In order to investigate the potential role of anti-gonococcal immunity in prevention of gonococcal sexual transmission the following study was initiated. Using extensive contact tracing and questioning to determine the direction of transmission, we began to enroll female contacts to males with culture documented gonococcal disease. At the time of their visit to our clinic, the patients had not been treated prior to the obtaining of peripheral blood mononuclear cells for porin specific T cell stimulation studies and the obtaining of sera to determine the level of anti-PIA and PIB specific IgG. In addition, if any of these women were symptomatic, as evidenced by an purulent exudate and inflammation, at presentation to our clinic, they were excluded from the study, as we have previously determined that the presence of significant inflammation can will induce a significant anti-Por IgG and T cell response (Simpson et al., IBID). Therefore, the anti-Por response measured in these symptomatic contacts would not be indicative of a potential pre-existing anti-gonococcal immune response, but would be more indicative of the immune response induced by this co-existing acute infection. Of the 25 cases obtained at Boston University that met inclusion criteria, 6 were uninfected and 16 were infected, and 3 were excluded as they were symptomatic at presentation. This ratio of infected to uninfected female contacts is similar to previous epidemiologic studies regarding gonococcal transmission. The similarity of the gonococcal strains infected the men and their female partners was determined by porin genotypic analysis performed by Dr. Margaret Bash at the Food and Drug Administration in Bethesda MD. T cell proliferation towards either Protein IA or Protein IB was not demonstrated in any of the infected contacts (stimulation index [SI] < 2). The lack of T cell reactivity to the porins in these infected female contacts is in stark contrast to our previous data demonstrating that patients with symptomatic inflammatory gonococcal disease (25% female) have increased T cell responsiveness to Protein I (average SI >7,5 for both PIA and PIB). Thus far we have also enrolled 6 uninfected female contacts to men with gonococcal infection and interestingly the average T cell SI to PIA and PIB is 13.9 and 12.1 respectively, but due to the small number of patients enrolled so far, statistical analysis cannot be performed as of yet. This data suggests that pre-existing immunity to gonococcal surface antigens might be associated with protection from gonococcal disease, or at least, women who do become infected with the gonococcus lack an immune response towards gonococcal surface antigens prior to infection.

Immune response to meningococcal carriage in a student population

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Introduction: University students are at an increased risk of meningococcal disease compared to non-students of a similar age. The risks of contracting meningococcal disease are increased when many young adults, a group with a high nasopharyngeal meningococcal carriage rate, are brought into close proximity within the confines of the university environment. First year students are most at risk when they first enter the university environment and are exposed to new meningococcal strains not encountered previously. The student's immunological status at the time of exposure to new strains will likely determine whether the organism is carried or causes disease. To investigate the role of carriage in inducing protective immunity we undertook a longitudinal study into the dynamics of meningococcal carriage and the immune response to meningococci in first year students attending an English university in 1999.
Materials and Methods: Mouth washings, throat swabs, and blood samples were collected from a cohort of 42 university students at four time points during the academic year. Meningococci were isolated from throat swabs using standard techniques and were serologically characterised by the PHLS Meningococcal Reference Laboratory (Manchester, UK). The sensitivity of carriage detection was enhanced by the addition of gargle-culture and PCR of gargles and throat swabs. Immunity to the group B strains detected during the study was determined by the serum bactericidal assay (SBA). Fifty percent bactericidal activity at a serum titre of ≥1/4 in the SBA was considered protective.
Results and Discussion: Since there is currently no effective vaccine for group B meningococci, this study focused on group B carriage and host immunity**.** Group B meningococci were carried by six (14%) out of a total of 42 students, four of whom acquired their strains during the study period. Two of the group B strains were found in more than one student, and one student harboured two different group B strains simultaneously. Sera both from carriers and non-carriers were investigated for the presence of protective antibodies, as measured by SBA against the carriage group B strains. Carriage stimulated an immune response against homologous group B strains. This protective immunity was not solely confined to the homologous strains but showed cross-reactivity to some of the other group B strains isolated in the study. Levels of protective immunity showed variation between the group B strains; for example 13% of students were susceptible to one particular strain while 73% were susceptible to another. Only one individual was susceptible to infection by all of the group B strains. Two individuals who lacked bactericidal antibodies at the beginning of the study were subsequently colonised by group B strains and developed protective immunity.
Conclusions: Natural immunity to group B strains was both strain and individual dependent. Many students were not protected against the circulating group B strains, however a lack of protective immunity can lead to carriage instead of disease.

Serological correlates of protection in non-infected 'at-risk' students prior to an outbreak of meningococcal infection at an English university

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Introduction: In October 1997 an outbreak of serogroup C meningococcal disease occurred amongst first year students at an English university. Six cases were reported, with three fatalities. We were able to obtain serum, taken from one of the cases and 89 asymptomatic classmates one month prior to the outbreak. This provided a unique opportunity to investigate levels of immunity to meningococcal infection in students, prior to and during an outbreak. We present here, a detailed analysis of humoral immunity to meningococci in the 'at risk' student population. Specifically, the relationship between antibody reactivity to individual meningococcal surface antigens and serum bactericidal activity (SBA) was explored.
Materials and Methods: Bactericidal activity against the outbreak group C strain, and a reference group B strain, was investigated in the 89 'at risk' students. Antibodies to group C and B polysaccharide and LPS were determined by specific ELISA assays. Serum antibody levels to outer membrane protein antigens were also investigated by SDS PAGE and western blotting. Correlations between antibody reactivity to individual meningococcal surface antigens and SBA against the homologous strain were determined with the Mann-Whitney *U* and the Pearson Chi-Square tests.
Results and Discussion: Most of our knowledge of events prior to an outbreak of meningococcal infection is based on the classic studies of Goldschneider *et al.* (1969). These investigators demonstrated a correlation between *in vitro* SBA and protection against invasive meningococcal disease *in vivo*. These studies demonstrated that the majority of individuals were immune to infection by the outbreak group C strain and high levels of carriage of this strain were noted. In contrast, our studies showed that carriage of group C strains was rare, and that 90% of the students were susceptible to infection by group C meningococci. This change over the last 30 years has important implications for vaccine policy. In our study, antibody to group C polysaccharide was the only individual meningococcal surface component consistently associated with SBA against group C strains. In contrast, no correlation was detected between the presence of antibodies to group B capsule and SBA. However, a strong relationship was noted between reactivity to PorA and PorB proteins and SBA of the group B strain. These results are in accord with the protection afforded against group C meningococcal disease by anti-C capsular vaccines and demonstrate that both the PorA and PorB proteins are potential candidates for inclusion in a group B vaccine.

A type I secretion machine in *Neisseria meningitidis*

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Introduction: To determine whether *Neisseria meningitidis* is able to secrete proteins via the Type I secretion pathway we searched for homologues of the *hlyB* and *hlyD* genes, which encode components of the *E. coli* hemolysin secretion system. Type I secretion systems consist of a hydrophilic inner membrane-associated ATP-binding protein, a hydrophobic membrane fusion protein, which spans the periplasm and interacts with components on both inner and outer membranes, and the outer membrane TolC protein, which is involved in the transport of a number of molecules including proteins and drugs. Typically the ATP-binding protein and the membrane fusion protein are genetically linked to each other and to the cytotoxin gene. The *E. coli* hemolysin operon, for example, comprises the genes *hlyABCD* which encode the cytotoxin, the ATP-binding protein, a toxin activating protein and the membrane fusion protein respectively. The *tolC* gene, by contrast, is usually genetically unlinked. Meningococcal homologues of *hlyB* and *hlyD* were identified but they were not linked to each other or to any putative cytotoxin genes. An *hlyB* homologue (NMB1400 in strain MC58) is flanked by two insertion sequences while a homologue of *hlyD* (NMB1737) is immediately upstream of a *tolC* homologue and downstream of a putative transposase.

Results and Discussion: We have cloned and mutated the *hlyB* homologue of *Neisseria meningitidis* strain MC58. Two high molecular weight proteins detected in concentrated culture supernatants of wild type meningococci were absent in supernatants of the isogenic *hlyB* mutant. Using antisera to the iron-regulated cytotoxins FrpA and FrpC we showed that these proteins were the substrates of the Type I secretion system. In a *frpC* mutant FrpA was secreted at higher levels compared to the wild type. These data indicate that NMB1400 is the ATP-binding component of a Type I secretion system specific for the secretion of FrpA and FrpC and that, when FrpA and FrpC are both expressed, the capacity of the secretion system rather than toxin gene expression is the limiting factor in export of FrpA. We are currently constructing a mutant in the *hlyD* homologue to determine whether the product of this gene is the membrane fusion protein component of this secretion machine. We will also probe concentrated culture supernatants of the mutants with antisera raised against meningococcal secreted proteins to determine if any other proteins are secreted via this pathway. The unusual genetic arrangement of the *hlyB*, *hlyD* and *tolC* gene homologues in the meningococcus suggests that these genes have been recruited from other organisms allowing the meningococcus to secrete the FrpA and FrpC cytotoxins.

Sialylation of gonococcal LOS occurs during experimental murine gonococcal genital tract infection

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Introduction: Gonococci in urethral exudates or blood demonstrate increased resistance to killing by normal human serum (NHS), which is lost by treatment with neuraminidase (NANase) or upon subculture. Serum resistance can be restored by incubation with cytidine monophosphate neuraminic acid (CMP-NANA). This unstable type of serum resistance results from the addition of sialic acid to the terminal N-lactose-N-tetraose molecule present on some gonococcal LOS species. Host sialic acid is used as the substrate in vivo, and the reaction is catalyzed by gonococcal sialyltransferase. In addition to conferring increased serum resistance, LOS sialylation increases resistance to opsonophagocytosis and to bactericidal activity of porin-specific antibodies. Collectively, these observations suggest that LOS sialylation confers a survival advantage. Sialylation also reduces gonococcal invasion of epithelial cells, however, a finding that suggests a more complex role for LOS sialylation in infection. Here we report the construction of a genetically defined sialyltransferase mutant, and evidence that LOS sialylation occurs during experimental murine infection in an effort to define the potential of the murine model for studying this aspect of gonococcal pathogenesis.
Methods and Results: The sialyltransferase gene of *N. gonorrhoeae* strain MS11 was cloned and mutated via the insertion of a nonpolar kanamycin resistance cassette. A sialyltransferase-deficient mutant (GP300) was constructed by allelic exchange. As shown by others, wild type MS11 was more resistant to NHS after culturing in the presence of CMP-NANA, and serum resistance was reduced after incubation with NANase (bactericidal$_{50}$ titers: wild type, 3%; wild type + CMP-NANA, >16%; wild type + CMP-NANA + NANase, 4%). In contrast, mutant GP300 was equally sensitive to NHS regardless of growth conditions or NANase treatment (bactericidal$_{50}$ titers: 3-4% for all conditions). To determine if LOS sialylation occurs during experimental murine infection, estradiol-treated BALB/c mice were inoculated intravaginally with wild type MS11 or mutant GP300, and bactericidal assays were performed directly on gonococci in vaginal swab suspensions collected at selected time points. Gonococci from mice infected with wild type MS11 demonstrated increased resistance to normal human serum. Incubation of vaginal swab suspensions with NANase resulted in a serum sensitive phenotype (bactericidal$_{50}$ titers: >16% and 2-4%, respectively). Serum resistance was detected as early as 2 days following intravaginal inoculation with wild type MS11, and was lost upon subculture to artificial media. In contrast, gonococci in vaginal swab suspensions from mice infected with mutant GP300 were sensitive to NHS (bactericidal$_{50}$ titer: 2-4%), regardless of the time point at which they were collected.
Conclusions: These results are indirect evidence that sialylation of gonococcal LOS occurs in the lower genital tract of mice as early as two days after inoculation. The estradiol-treated mouse model may therefore be useful as a tool for studying LOS sialylation in vivo.

Unglycosylated lipid A of *Neisseria meningitidis* attenuates, via the TLR-4 pathway, cytokine expression by human macrophages

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Background: *Neisseria meningitidis* (NM) is responsible for severe, often fatal septicemia and meningitis. Lipopoly(oligo)saccharide (LOS) of NM [NeuNAc-Galβ-GlcNAc-Galβ-Glcβ-Hep2(GlcNAc, Galα) PEA-KDO2-lipid A] is a major inflammatory mediator and is believed to activate cytokine production in human macrophages via specific stimulation of the Toll-like receptor 4 (TLR-4). Lipid A of LOS is believed to be the biologically important ligand, but the precise structural components of LOS required for TLR-4 activation are not completely defined.
Methods: We used purified meningococcal LOS of defined structure to study TLR-4 activity. LOS was quantitated and standardized based on lipid A content per sample and not on dry weight bases. We stimulated 106 human monocytes (THP-1 and U937 cells) with 0.56 pmoles of purified meningococcal LOS. Biological activity was measured with TNF-α, IL-8, and nitric oxide assays. Antibodies to TLR-4 and CD14 confirmed the role of TLR-4 in this pathway. LOS was purified from genetically defined meningococci of the serogroup B strain (NMB) that contained specific mutations in LOS biosynthesis genes. LOS structures of these mutants were characterized by NMR and mass spectrometry.
Results: Unglycosylated lipid A from mutants with defects in Kdo biosynthesis or transfer produced significantly less TNF-α ($p<0.01$) and nitric oxide ($p<0.05$) than the parent strain and a truncated KDO2-lipid A structure. Oligosaccharide inner core and α-chain structure alterations did not significantly influence TLR-4 activation.
Conclusion: These results suggest a structural requirement for Kdo glycosylation of lipid A to stimulate the inflammatory cascade of human monocytes via TLR-4.

Quantitative association between plasma LPS and load of bacterial DNA in systemic meningococcal disease as evaluated by robotized quantitative PCR

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Background: In systemic meningococcal disease (SMD) the clinical severity and outcome is closely associated with plasma levels of LPS. The association between the real bacterial load (the sum of live and dead bacteria) and levels of LPS has previously not been known in in SMD.
Aim: To quantitate the amount of *Neisseria meningitidis* (Nm)-DNA in plasmas and cerebrospinal fluids (CSF) from patients (n=62) with SMD and relate the amount to the levels of LPS, clinical presentation and outcome.
Materials and Methods: DNA from plasma (100 ml), collected from patients with fulminant septicemia (n= 20, 10 dead), septicemia and meningitis (n=1, 0 dead), mild SMD (n=16, 0 dead) and distinct meningitis (n=25, 1 dead) was extracted by magnetic beads in a robotized system (GenoM48, GenoVision AS, Oslo, Norway). Realtime PCR (LightCycler ™, Roche) was performed using specific PCR primers for *N. meningitidis* capsular transport gene (ctrA) and SYBR Green fluorescense detection. Standard curves were constructed by diluting known amounts of heath killed meningococci in plasma (*numbers based on living bacteria counts). The amount of Nm-DNA in each patient sample was extrapolated by comparing the amplified PCR values with the standard curve. The method was evaluated by running negative and positive controls for each run. Nm-DNA isolated from serum is currently under investigation. LPS quantification was made by the Limulus amebocyte lysate (LAL) assay and the results given as endotoxin unit (EU)/mL.
Results: The standard curves were reproducible ($CV_{slopes\ n=11}$= 4,3). Within run variation was 6 % (n=6). The overall variation coefficient between runs of the longitudinal control was 22% (n= 15). Median Nm-DNA and LPS amount in plasmas from patients with fulminant septicemia were 4635*/40.5 EU/mL, septicemia and meningitis: 928*/25 EU/mL, mild SMD: 1.2*/0.25 EU/mL and distinct meningitis: 0.7*/<0.25 EU/mL, respectively. Non-survivors of fulminant septicemia had more Nm-DNA/LPS 9322*/135 EU/mL than survivors 1342*/24 EU/mL ($p<0.03$). Nm-DNA in CSF in fulminant septicemia was 3*/0.3 EU/mL (n=4) and distinct meningitis 10140*/277 EU/mL (n=9) The correlation coefficients between levels of Nm-DNA and LPS in plasma were: 0.86 ($p=0.0001$, n = 62) and 0.87 ($p=0,006$ n = 13) for CSF (Spearman rank).
Conclusions: Nm-DNA levels, indicating the real bacterial load, may be specifically quantitated in plasma and CSF from patients with SMD using robotized DNA isolation and realtime PCR. There is a close quantitative association between Nm-DNA and LPS in plasma underscoring the usefulness of the LAL assay in meningococcal infections.

SESSION VI

Vaccines and methods for vaccine evaluation

Functional antibody activities in sera after intranasal and intramuscular immunisations with outer membrane vesicle vaccines against group B meningococci

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A serogroup B meningococcal outer membrane vesicle (OMV) vaccine was delivered either intranasally or intramuscularly to 12 and 10 volunteers, respectively. The mucosal vaccine was given once weekly for four weeks followed by a fifth dose five months later; each dose consisted of OMVs equivalent to 250 μg of protein. The intramuscular (i.m.) vaccine, consisting of the same OMVs but adsorbed to $Al(OH)_3$, was administered twice as doses of 25 μg of protein given at 6 weeks' interval, followed by a third dose after 10 months. Both groups of vaccinees demonstrated significant immune responses when measured as specific IgG antibodies against live meningococci, as serum bactericidal activity (SBA) and as opsonophagocytic activity. However, there were several low- and non-responders in the intranasal group, particularly when measured as SBA. Two weeks after receiving the last dose, the anti-meningococcal IgG concentrations were significantly higher in the i.m. group (median IgG concentration: 43.1 μg/ml) than in the intranasal group (10.6 μg/ml) (P = 0.001). The corresponding opsonophagocytic activity was 7.0 and 3.0 (median $\log_2$ titre) (P = 0.001), and the SBA was 5.0 and 2.0 (median $\log_2$ titre) (P = 0.005), for the i.m. and intranasal groups, respectively. The last immunisation induced an enhanced immune response in the i.m. group, whereas the intranasal group showed no significant booster-response. Accordingly, affinity maturation of anti-OMV-specific IgG antibodies was seen only after i.m. vaccination. The IgG1 subclass dominated the responses in both groups, whereas the significant IgG3 responses observed in the i.m. group were absent in the intranasal group. We observed higher correlations between the level of specific IgG antibodies and functional activities in the i.m. group than in the intranasal group. Although the intranasal OMV vaccination schedule used here induced functional immune responses relevant to protection, an improved mucosal immunisation regimen must be established to achieve a systemic effect comparable to that seen after three doses of intramuscular vaccination.

Development of candidate glycoconjugate vaccines against *Neisseria meningitidis* serogroup A

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Introduction: The meningococcal polysaccharide vaccines are not effective in infants where the incidence of meningococcal meningitis is higher. In order to improve their immunogenicity, meningococcal polysaccharides or derived oligosaccharides need to be conjugated to protein carriers. The aim of the present pre-clinical study was to evaluate the immunogenicity of two different models of meningococcal group A (MenA) conjugates.We prepared two classes of MenA conjugates, one using the native polysaccharide and the other using derived oligosaccharides; in both cases the protein CRM197 was used as carrier. The Men A polysaccharide-CRM197 conjugates were prepared following two procedures: one involving a carbodiimide mediated coupling reaction, the other using reductive amination. The MenA oligosaccharide conjugates were instead prepared according to the following steps: polysaccharide hydrolysis, oligosaccharide sizing by chromatography, oligosaccharide derivatisation to active ester and conjugation to CRM197. The different conjugates were tested for their immunogenicity in animals.
Results: MenA conjugates prepared with the oligosaccharide technology showed a very good immunogenicity. The immunogenicity of the conjugates prepared using the native polysaccharide resulted to be influenced by the conjugation chemistry.
Conclusions: Polysaccharide and oligosaccharide based molecular models of MenA conjugates have been produced, characterized and compared in animal models. The oligosaccharide conjugates resulted immunogenic and this model, basing on our previous experience with *Haemophilus influenzae* type b and Meningococcus group C, offers advantages in term of molecular characterization and manufacturing consistency.

Immune response to meningococcal serogroup C conjugate vaccination in asplenic individuals

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Introduction: The absence of a functional spleen predisposes individuals to an increased risk of overwhelming infection, especially due to encapsulated bacteria such as *Neisseria meningitidis*. Formerly, administration of unconjugated meningococcal polysaccharide vaccine was recommended for asplenic individuals. However, the limitations of the plain polysaccharide vaccine are well documented and administration of meningococcal polysaccharide-protein conjugate (MCC) vaccines to asplenic individuals was advised by the DoH from March 2001. As no data currently exists on responses to MCC vaccine, we have been determining antibody status to ascertain whether subjects require further doses of MCC or not.
Methods: Eighty-two subjects who had undergone splenectomy were followed up from the South Cheshire area. Historical data from thirteen age-matched individuals (with spleens) were included as a control population. Splenectomised subjects and control individuals received a MCC vaccine and were bled prior to and one month post-vaccination. Functional antibody was determined by serum bactericidal assay (target strain C11: C:16:P1.7-1,1) and serogroup C-specific IgG were quantified by ELISA. For the purpose of this investigation a SBA titre ≥ 16 was considered to be protective.
Results: The geometric mean titre (GMT) at one month post-vaccination for the asplenic individuals was 135.8 (95% CI, 67.7-272.6) compared to 2160.2 (493.9-9447.7) for the control population ($p < 0.001$). Thirty percent of asplenic individuals had a one month post-vaccination titre < 16 whereas no individuals in the control population had a GMT < 16. The GMT of asplenic individuals with a chronic illness was observed to be less than those asplenic individuals without chronic illness, 50.8 (8.8-292.6) and 222.2 (99.6-495.6) respectively, however this difference was not significant ($p = 0.06$). No other variable analysed such as age, previous unconjugated polysaccharide vaccination, reason for splenectomy and time since splenectomy influenced the SBA titre. Similar geometric mean concentrations of serogroup C-specific IgG were observed for asplenic and control individuals.
Discussion: A significant number (30%) of asplenic individuals did not respond to a first dose of MCC vaccine and the response to a second dose of MCC vaccine is currently being determined. Currently, due to the number of non-responders, serological testing of subjects' sera by the SBA assay is recommended. This is to advise on whether revaccination is necessary or not, however, to date, the benefit of this is yet to be determined. The reduced response to MCC vaccination observed in asplenic individuals indicates the importance of a functional spleen in the generation of the T cell dependent immune response to polysaccharide-conjugate vaccines. However, it is currently unclear why a significant proportion of asplenic individuals fail to generate a protective response.
Conclusion: Vaccination of asplenic individuals with MCC vaccine successfully induces protective levels of bactericidal antibody in 70% of vaccine recipients. However, significantly lower SBA GMTs are observed as compared to persons with fully functional spleens, which may have implications for the duration of immunity.

Adaptation of the Whole Blood Model of meningococcal bacteraemia for the assessment of the total bactericidal activity of antibodies raised against target vaccine antigens

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Objective: To modify the Whole Blood Model of bacteraemia to assess exogenous antibodies for their total bactericidal activity against meningococci.
Introduction: The identification of antigens for use in meningococcal vaccines commonly involves the production of antisera or monoclonal antibodies to the purified target protein. The antibodies raised need to be tested for their functional ability to kill meningococci. By depleting whole blood of endogenous antibody, target antibodies can then be added to the otherwise intact system and assessed for serum bactericidal and opsonophagocytic activities in a single assay.
Methods: Freshly isolated, heparinised blood from healthy adult volunteers was centrifuged to separate the plasma and cellular fractions. Aliquots of un-separated blood were retained as controls. Plasma was incubated with either Protein G sepharose or control beads, for 1 hour at 37°C with mixing. The cellular fraction was washed twice in RPMI, and recombined with the bead-treated plasma. In some experiments, Octagam (pooled human immunoglobulin) was added to the antibody-depleted samples to assess whether killing could be restored by the addition of human immunoglobulin. The whole blood assay was carried out as described previously, using an inoculum of 10^7 meningococci (strain MC58) per ml of blood (Ison *et al* 1995).
Results: Incubation of plasma with Protein G beads reduced the percentage of meningococci killed in the Whole Blood Assay from nearly 100% to 39% in one donor and 0% in a second donor (in the latter donor, growth of MC58 was recorded). Control beads were tested with blood from donor 1 and were found to have no effect on the percentage of meningococci killed. The reduction in killing resulting from Protein G treatment corresponded to a 98 and 99% reduction in MC58-specific antibody respectively, as measured by binding to whole, intact MC58 in a flow-cytometric assay. The addition of Octagam to antibody-depleted blood restored killing to its original level, indicating that only the immunoglobulin level had been reduced by the Protein G treatment and that complement was still functional. Octagam alone had no effect on bacterial viability. Both bead treatments caused activation of neutrophils (measured by staining for CD11b and L-selectin and flow cytometry), but it is not yet clear whether this led to an increase in opsonophagocytosis in samples inoculated with meningococci. Neither bead treatment significantly increased either background TNFa or its production in response to MC58.
Conclusion: The depletion of immunoglobulin from whole blood using Protein G sepharose may provide a simple method for testing target antibodies for their total bactericidal activity against meningococci.

Antibody persistence and immunological memory 4 years following meningococcal C conjugate vaccination in UK children

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Introduction: Meningococcal serogroup C conjugate (MCC) vaccines were introduced in the UK from November 1999. Although the immediate post-vaccination antibody levels that predict short-term protection are now established in the UK, these levels rapidly decline within one year of immunisation. Long-term protection following vaccination will therefore be reliant on the presence of immune memory. The ability to demonstrate the persistence of immune memory for following MCC vaccination is thus of particular importance.
Methods: Antibody persistence and immunological priming of 2 formulations of a meningococcal C conjugate (MCC) vaccine (containing 2 or 10μg of meningococcal C {men C} capsular polysaccharide) administered at 2/3/4 months of age was investigated by boosting at either 13-16 months or 4 years of age with a 10μg dose of unconjugated meningococcal C polysaccharide.
Results: At 4 years of age geometric mean concentrations (GMCs) and titres (GMT) of menC-specific IgG and serum bactericidal antibody (SBA) had declined to pre-vaccination levels. For both dose formulations only 8% of subjects had SBA titres ≥ 8 at this time point. In contrast geometric mean avidity indices (GMAI) increased post-primary to 13-16 months of age and then remained constant to 4 years of age. One month following the booster dose administered at 4 years of age, menC IgG and SBA increased significantly. For those primed with the 2μg MCC, the post booster SBA GMT (2181.2; 95% CI 975.9-4875.1) was 2 fold higher than that achieved in those primed with the 10μg MCC (931.6; 95% CI 338.0-2568.1). GMAI increased pre- to post-booster with no significant differences between the two formulations. The GMAI attained after boosting at 13-16 months was similar to that seen following boosting at 4 years.
Discussion: At 4 years of age, UK children immunised with MCC vaccine at 2/3/4 months have both group C-specific IgG GMCs and SBA GMTs similar to those pre-vaccination at 2 months of age. Despite the low titres of antibody, immunological memory was demonstrated by high SBA and menC levels achieved 1 month following a booster dose of plain polysaccharide and by the demonstration of a sustained increase in avidity. True validation of these measurements as correlates of immunity will emerge as the ongoing monitoring of the efficacy of the MCC vaccine introduced into the UK in 1999 continues.
Conclusion: This is the first demonstration of immunological memory at 4 years of age in UK children immunised with the routine 2/3/4 month schedule.

New recombinant human group B meningococcal antibodies from *in vitro* antibody libraries

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Introduction: Human recombinant antibodies can be developed from antibody libraries originating from the isolation of Variable genes from human B-lymphocytes. Human meningococcal antibodies can be cloned by this method and studied for antibacterial activities and ultimately designed for medical use.
Materials and Methods: Most approaches are based on naive libraries generated from the IgM and IgD V-gene repertoires of several «healthy» donors. Since these libraries are extremely complex, isolating specific antibodies requires iterative selection strategies. However, when humans are immunologically stimulated by infection or vaccination, there is an *in vivo* enrichment of functional high affinity IgG antibodies. These repertoires contain the ideal antibodies that have biologically evolved to combat the particular disease in question.
Results and Discussion: We have utilized the power of in vitro screening of such antibody libraries generated from peripheral blood B-lymphocytes from volunteers receiving the Norwegian group B-meningococcal outer membrane vesicle (OMV) vaccine. A very high yield of functional antibody fragments were cloned and analyzed for binding activity to OMV shown by ELISA-method and to intact meningococci shown by flow cytometry.
Conclusion: We have generated several meningococcal specific human antibodies by efficient screening of human antibody libraries derived from peripheral blood B-lymphocytes from vaccinees receiving the Norwegian group B OMV vaccine.

Excellent thermo-stability of meningococcal serogroup C conjugate vaccine, lyophilised formulation

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Introduction: New meningococcal group C conjugate (Men C) vaccines have been licensed and offer distinct advantages over the plain polysaccharide vaccines. A long shelf-life and stability at high temperatures are important attributes to ensure product effectiveness during conditions of routine use which may inadvertently include increased storage temperature. Therefore, we have analysed the thermo-stability of Menjugate®, a Men C vaccine available in a lyophilised formulation.
Material and Methods: Three clinical and three commercial product lots of Menjugate were stored at 8°C, 25 °C or 30 °C for a period of 18 months after they had already been stored at 2-8 °C for 18 months (real time stability, 36 months in total). The vaccine was stored under raised temperatures up to 40 °C or 50 °C up to 24 months and the physicochemical integrity of the vaccine was analysed: pH, free saccharide, percent moisture, sialic acid content, aggregation (by size exclusion chromatography), free CRM (the carrier protein), photostability. In addition, the immunogenicity was analysed in mice by a specific ELISA.
Results and Discussion: The appearance of the product remained acceptable under all storage conditions. The pH of the product, the free saccharide content, the percent moisture, the sialic acid content and the percent aggregation (measured by HPGPC and/or SEC HPLC) remained within the specification limits. In addition, stability studies at 30 °C for 24 months with one week excursions to –20 °C and +40 °C were performed and demonstrated that all physicochemical parameters remained within the specifications. Results of mouse-immunogenicity studies with vaccine stored at 8 °C for 24 months (validation lots) and for 36 months (clinical lots) confirm the stability of the vaccine after extended storage. Even upon incubation of vaccine at 55 °C for 6 and 9 weeks, no reduction in immunogenicity in mice could be determined compared to storage at 2-8 °C.
Conclusion: The lyophilised Men C vaccine is extremely stable with respect to hydrolysis and release of free saccharides, if stored for a long time or at high temperatures. Based upon the physicochemical and immunogenicity data, a shelf-life of 24 months has been approved by the authorities for Menjugate®. Data from lots stored at 8 °C with temperature excursions to –20 °C and +40 °C for up to 1 week did not show any change in the vaccine release specifications. Due to the outstanding stability, this conjugate vaccine should be favoured in regions with warm climate and if interruptions in the transport and storage are a possibility.

Optimization of preclinical immunization with outer membrane vesicles vaccine

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The Cuban outer membrane vesicles (OMV) vaccine against *Neisseria meningitidis*, VA-MENGOC-BC™, induces a preferential cellular (Th1) pattern in mice and human. It contains OMV and polysaccharide C (PsC) from B and C serogroups at equal concentrations, respectively. Traditionally, relative high concentrations of OMV and PsC and several weeks between each two doses for mice immunisation were used. Nevertheless, the optimal time and interval for each antigen dose were never been addressed. Therefore, we compared doses of: 25, 12.5, 6.25, 3.125, 1.56, and 0.7 μg *per* mouse and intervals of: 7, 14, 21, 28, 35, 42, 56, and 63 days between each dose. The anti-OMV and anti-PsC IgG class and IgG_2a and IgG_1 subclasses were determined by ELISA. The production of γIFN from spleen cells restimulated *in vitro* with OMV and the delayed-type hypersensitivity (DTH) were measured. All schedules (doses and intervals) showed high anti-OMV IgG responses although the anti-PsC IgG was more dependent of the schedule used. DTH was higher when lower doses and intervals times were applied although higher doses require longer intervals period for good DTH induction. The anti-OMV and PsC IgG_2a subclass were well induced in all schedules. The γIFN production was demonstrated in all schedules, but it was higher with concentration of 3.125 or 6.25 μg. In summary, in preclinical studies we can used short intervals between doses and preferentially low concentrations, but the response induced by PsC seems to need more interval between doses in agreement with the reported more permanence of this antigen in inductive sites.

Assessment of immune response against C11 (ATCC) strain induced by Cuban meningococcal vaccine (VA-MENGOC-BC®) in adolescents of Ciego de Avila, Cuba

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Introduction: Immunological memory lasts for a long time in humans, even when the serum antibody concentration diminishes the detection threshold. The antibody response induced by a vaccine depends, among other factors, on the quality and dose of the inoculated antigen. On the other hand, the booster with vaccines of polysacchaide antigens could induce an hyporesponse.
Materials and Methods: In this study, we assessed the immune response elicited by Cuban meningococcal vaccine *(VA-MENGOC-BC®)* against to C11(ATCC) strain, in 184 adolescents (ages 15 to 18 years) from a school in Ciego de Avila, Cuba. This vaccine is included in the Cuban National Immunization Program since 1991. These adolescents had been vaccinated with it 12 years before. The blood samples were taken immediately before of the first dose of vaccine (T0), four weeks after it (T1) and the last one, was taken four weeks after second dose (T2). Serum Bactericidal Assay (SBA) and ELISA were done
Results and Discussion: The percent of adolescents having serum bactericidal activity (titer≥8) against C11(ATCC) strain was 25 in T0. By ELISA, 78% of adolescents had an antibody concentration higher than the detection limit against polysaccharide of serogroup C meningococcus. The percentages of seroconversion (by SBA) after the first and second dose were 59 and 55 respectively. The percentages of seroconversion (by ELISA) after the first and second dose were 82 and 82 respectively. There were no significant differences between the results after the first and second doses, by SBA and ELISA.
Conclusion: These data show that the adolescents that had been vaccinated 12 years before with *VA-MENGOC-BC®* had significant antibody response after the application of one dose. These results show that the booster did not induce an hyporesponse.

Peptide conformational mimics elicit type 1 and type 2 T-cell responses in mice to meningococcal LOS

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Introduction: In the search for vaccine components that elicit protection against meningococcal disease, particularly to group B, peptide conformational mimics of the outer membrane lipooligosaccharide (LOS) are being studied. Several immunotypes of LOS have been identified, but L3, L7, and L9 share the same structure (L3,7,9) that is frequently associated with disease. This maybe partly due to a distal region of L3,7,9 mimicking human neural cell surface antigens. However, LOS also has a region within the inner core that is immunogenic. Peptide conformational mimics were identified from a cyclic peptide phage display library by interaction with an anti-LOS monoclonal antibody, 9-2-L379, that interacts with the immunogenic region (Brett *et al*, 2002, J. Biol. Chem, in press). Immunisation trials indicated that cross-reactive antibody responses to LOS were elicited by vaccination with a peptide mimic. We have extended these trials to include strains of in-bred mice with different haplotypes, and antigen stimulated cytokine responses to establish the T-cell responses mediated.
Material and Methods: Since standard methods to conjugate carrier proteins to conformational peptide mimics resulted in loss of antigenicity, we developed an alternative multiple antigen display method. Cyclic peptide C22, that binds tightly to antibody and competes with LOS binding, was synthesised with a lysine residue at its C-terminus, to which biotin-amide was reacted and then complexed with NeutrAvidin®. Avidin occurs naturally as a tetramer with four biotin binding sites with a biological affinity of K_D 10^{-15} M. The peptide-NeutrAvidin complex was tested for antigenicity and then used to vaccinate BALB/c and C3H/HeN mice with haplotypes H^{2d} and H^{2k}, respectively, in either Freund's or $Al(OH)_3$ adjuvant. Cross-reactive antibody responses to LOS were investigated in mouse anti-sera, and antigen stimulation cytokine secretion assays were performed on the same vaccinated mouse splenocytes.
Conclusion: Following a prime-boost regimen dose-dependent cross-reactive antibody responses to LOS were observed in both strains of mice with total IgG end-point dilution titres up to 3200 in the presence of wither Freund's or $Al(OH)_3$ adjuvants. Control animals vaccinated with either PBS or NeutrAvidin had higher background levels when Freund's was the adjuvant. The predominant antibody subclass was IgG1 and to a lesser extent IgG2b in both mouse strains. However, BALB/c mice also had low levels of IgG3 antibodies. Peptide-NeutrAvidin stimulated the secretion of IFNγ, a type 1 CD4+ T cell response, and Interleukins 4, 5, and 10, indicative of a type 2 T cell response. The relative amounts of cytokines indicated that the type 2 response was more dominant, in agreement with the IgG subclasses observed.

Identification of molecular mimics of MenB LOS as vaccine candidates

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The production of specific antibodies against *Neisseria meningitidis* LOS plays an important role in the host's defence against this organism. Unfortunately group B meningococcal LOS is poorly immunogenic, toxic and does not induce a sufficient secondary response. Therefore, we have investigated peptide mimics of the LOS epitopes as alternative vaccine candidates against group B meningococcal disease. The first stage of this work was to screen random phage display libraries with LOS specific monoclonal antibodies to identify peptide mimics. Two monoclonal antibodies were chosen for this purpose on the basis that they recognised epitopes that were relatively conserved, non-cross-reactive with human antigens and were protective. Consensus peptide sequences were identified with both antibodies, individually. The specificity of these enriched peptides sequences for the appropriate monoclonal antibodies were confirmed by phage capture assays. Real-time kinetic measurements on a resonant mirror biosensor were used to determine which sequence motifs had the greatest affinity for one of the monoclonal antibodies. The results suggested that peptides that were conformationally constrained had binding affinities to the monoclonal antibody that were comparable with native LOS, whereas this degree of binding was not observed with linear peptides. Preliminary immunogenicity studies have been carried out using a variety of immunising approaches. Results suggested that a strong anti-peptide response can be induced and that this response is cross-reactive with *N. meningitidis* LOS.

Evaluation of naturally acquired mucosal cellular immunity to *Neisseria meningitidis* serogroup B antigens

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Introduction: An understanding of the mechanisms behind the development of natural immunity to *Neisseria meningitidis* serogroup B (Men-B) will greatly benefit the development of future vaccines. The natural immune response to Men-B usually occurs following colonisation of the nasopharynx. Within the nasopharynx, immune cells are localised in secondary lymphoid tissues such as the palatine tonsil, which is consequently thought to play a key role in the immune surveillance and the induction of immunity to locally encountered antigen. We have therefore investigated the presence of immunity to Men-B in the tonsils of children and adults and sought to compare the findings with systemic immunity as defined by serum bactericidal activity (SBA).
Materials and Methods: Tonsil mononuclear cell (MNC) suspensions were generated and employed in a standard thymidine proliferation assay to determine the presence and kinetics of antigen-driven mucosal T cell proliferation. Meningococcal outer membrane vesicles (OMVs) from strain H44/76 and isogenic PorA derivatives TR52 (P1.5,2), TR4 (P1.7,4) and TR10 (P1.5,10) were employed as the source of meningococcal antigens. A spontaneous PorA deficient strain (B:15:-) was also examined. SBA was measured by standard assay.
Results and Discussion: We have shown that mucosal T cells from adult tonsils proliferate in response to both recall and primary antigens *in vitro*. In addition, responses were observed to all the meningococcal OMVs. LPS controls and CD19 depletion experiments demonstrated that the proliferation was T cell and not B cell dependent. The kinetics of the responses to meningococcal OMVs implicated a memory type response in some individuals. Responses were also seen with the PorA negative OMVs suggesting that alternative antigens were also involved. Both adults (age 17-31) and children (3-15) demonstrated a range of responses to the OMVs but the majority of high responses were in the older individuals. There was no clear correlation between mucosal T cell responses and SBA.
Conclusion: Tonsils from adults and children were found to contain meningococcal-specific T cells. These mucosal cellular immune responses were found to be independent of SBA levels and appeared to be acquired with increasing age. Whether these responses are primary or secondary in nature remains to be elucidated.

In silico screening of serogroup B *Neisseria meningitidis* genome for protein-based vaccine development

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Whole genome sequencing opens the possibility of identifying *in silico* new surface-exposed or secreted proteins that can be tested as new potential vaccine candidates. The genome of *N. meningitidis* strain ATCC13090 (serogroup B) was used for screening while the sequencing by Incyte Genomics was in progress. The sequence data of the last genome screening were assembled into 393 DNA fragments of a total length of 2.194 Mb, in which 2051 open reading frames (ORFs) were predicted. For comparison, the complete genome of 2.272 Mb of the serogroup B strain MC58 contains 2158 predicted ORFs (1).

The first step of the screening was based on a search by homology to identify surface-exposed proteins like porins, fimbrial and non-fimbrial adhesins, lipoproteins, other outer-membrane proteins, periplasmic and secreted proteins. Thus, surface-exposed or antigenic proteins previously described in other bacteria were used as queries for performing BLAST searches. To increase the sensitivity, multiple alignments were retrieved from the Prodom database. Hidden Markov Models derived from the multiple alignments (HMMER) were used to screen the ORFs.

The second step was based on specific identification of porins or porin-like proteins and lipoproteins. Porin was determined by identification of a signal sequence using SignalP and GCG SPScan, by prediction of a predominance of beta-strands in a region of at least 100 amino acids using DSC, and by finding a C-terminal aromatic amino acid. Lipoprotein determination was based on identification of a type II secretion signal (Prosite prokar lipoprotein pattern PS00013) using GCG FindPatterns.

The third step consisted in annotation and characterisation of the selected ORFs. Each ORF was annotated by homology using BLAST. They were also screened for regular expressions of Prosite using GCG FindPatterns, for Hidden Markov Models of Pfam using HMMSearch, for motifs of Blocks and fingerprints of Prints using Blocksplus, and for transmembrane regions using TopPred.

By using *in silico* genome screening techniques, 50 new surface-exposed or secreted proteins were identified. They included 4 autotransporter proteins, 7 porins involved in haeme acquisition, 1 porin-like protein that is part of type IV pili, 7 other outer membrane proteins, 27 lipoproteins of which 9 were predicted to be anchored in the outer membrane, and 4 potential proteases.

Even though many surface-exposed or secreted proteins of *Neisseria meningitidis* were identified before the genome sequencing era, many new proteins could be identified by *in silico* genome screening. This approach will facilitate and accelerate the antigen discovery process.

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Clinical development of the Norwegian meningococcal group B vaccine (MenBvac)

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Introduction: An epidemic of serogroup B meningococcal disease in Norway motivated the development of a vaccine based on group B meningococcal outer membrane proteins in the form of vesicles (OMVs).
Materials and Methods: MenBvac has been extensively tested in several large clinical trials. Approximately 250 000 doses have been given in clinical studies from 1987 until today.
Results and Discussion: Increasing doses from 14 up to 100 μg (measured as protein) were given in the phase I trial. The safety and immunogenicity of different doses and formulations were compared in phase II trials including approximately 450 adults. A 25 μg dose consisting of OMV adsorbed to aluminium hydroxide as an adjuvant was chosen for further phase II and phase III trials. More than 80 % obtained bactericidal antibodies after 2 doses.

From 1988 to 1991, two placebo-controlled, double blind protection studies including about 55 000 military recruits and 171 800 secondary school children were performed. In the school trial the efficacy of a two-dose schedule for prevention of serogroup B meningococcal disease was estimated to 57 % during an observation period of 29 months. Simultaneous safety and immunogenicity trials in the same populations were conducted to establish serological correlates to protection and lot to lot consistency. After the protective effect was established, 49 000 teenagers from the former placebo group were vaccinated and included in an open follow-up study. Results from both the blinded and open part of the efficacy trial suggested that the protective effect of the vaccine is very good during the first year after vaccination (87 %), but then declines.

Clinical studies in Norway and Iceland have shown that a boosterdose given after 6 to 10 months induced higher antibody levels of longer duration and more cross-reactivity. In a Chilean trial even infants and small children responded with marked increase in bactericidal activity against the vaccine type strain after three doses.

After upscaling and re-establishing the process in new facilities, the vaccine has recently been given in a regimen of three doses six weeks apart in two clinical trials in students and secondary school children. The teenagers also received a fourth dose one year after the primary vaccination.

MenBvac has been well tolerated, although most of the vaccinees have reported pain or tenderness at the injection site. Systemic adverse events, mainly mild, have been reported by 30 – 40 % in both the vaccine and placebo groups.
Conclusion: MenBvac has been shown to be safe, immunogenic and efficacious. An application for marketing authorisation will be submitted in 2003. Partly based on the clinical documentation for this parent vaccine, clinical trials of a tailor-made vaccine for New Zealand has recently started.

Production of recombinant *Neisseria meningitidis* B outer membrane vesicles enriched with potential vaccine antigens.

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Outer membrane vesicles (OMVs) derived from Serogroup B meningococcal wild-type strains of *Neisseria meningitidis* (menB) were found to be protective in teenagers of homologous settings, but were only poorly effective in young infants. There is a great need for a menB vaccine that would be safe and immunogenic in the pediatric population and elicit protection against a wide range of clinical strains.

Beside immuno-dominant and variable integral OMPs like PorA, Opas, and OpC, we postulated that minor and conserved OMV protein components might confer cross-protective immune responses (1, 2).

Conserved and surface-exposed antigens were selected by genome mining and produced in recombinant OMVs via up-regulation in the menB chromosome. Two different methods were applied, referred to as gene delivery and promoter replacement. Both allowed us to enrich recombinant OMVs with proteins that might have a potential as vaccine antigens.

Meningococci tend to excrete large amounts of OMVs into their environment. Intact vesicles were partially purified from the culture of these modified strains by mild deoxycholate extraction followed by several filtration steps. Data on OMV protein patterns will be presented. Pre-clinical evaluation of the antibodies elicited after immunisation with those recombinant OMVs is currently ongoing.

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Immunogenicity of 3 doses a group B meningococcal outer membrane vesicle (OMV) vaccine in adults

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Introduction: A phase II trial has been undertaken to evaluate the safety and immunogenicity of the candidate group B meningococcal OMV vaccine, "MenBvac" in UK adults. Residual sera are being distributed for international efforts for the standardisation of assays. The vaccine was produced by the NIPH in Norway, and is based on the strain 44/76 (B:15:P1.7,16). Blood samples were taken prior to and six weeks after each immunisation. The Whole Blood Assay (WBA) which has recently been suggested as an alternative and more sensitive measure of bactericidal activity than the Serum Bactericidal Assay (SBA) was evaluated to determine its suitability for use in any future vaccine trial.
Methods: UK laboratory staff (n=21) were recruited and vaccinated using a three-dose 0-6-12 week schedule. Functional and non-functional meningococcal assays were used incorporating the vaccine strain (H44/76) and two other heterologous strains (NZ 98/254 {B:4:P1.7-2,4} and M01-240013{B:NT:P1.22,9}). The WBA was performed on fresh whole blood with percentage killing of each target strain calculated. Bactericidal activity against the target strains was determined using the SBA using human complement as an exogenous complement source. IgG and IgM antibody titres to OMVs from the target strains were determined by ELISA.
Results: Twenty (95.2%) subjects received all three doses of the vaccine with 19 (90.5 %) giving all four bleeds. High percentage of killing was seen in the WBA prior to the first dose (n=9), therefore fold rises following vaccination could not be determined for these subjects. Subjects (n=13) with low initial percentage killing showed an increase in killing following each dose against the vaccine strain. IgG ELISA results using the vaccine strain have also shown an increasing immune response, rising from a pre bleed GMT (95% CI) of 2507.1 (1545-4066) to 34067.1 (23519.5-49344.8) six weeks following the final dose. An increase in SBA antibody from a pre bleed GMT (95% CI) of 2 (1.3-3), to 4.4 (2.4-8.1) post one dose, to 4.1 (2.5-6.7) post 2 doses, to 6.3 (3.3-12.4) post three doses was observed, with 14 (73.7 %) having obtained a titre $\geq$4 against the homologous strain following three doses. Complete ELISA and SBA data using heterologous target strains are to be presented.
Discussion: The WBA was extremely labour intensive and coupled with the inability to standardise, control or repeat the assay makes it unsuitable for use in any future vaccine trials. Responses to heterologous strains will determine any cross-protective response induced by the vaccine.
Conclusion: This OMV vaccine was immunogenic as measured by OMV ELISA and elicited an SBA response $\geq$4 following three doses in 73.7% of subjects against the homologous strain.

A genomic approach to identify vaccine candidates against gonococcus

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Introduction: *Neisseria gonorrhoeae* (GC) is the causative agent of gonorrhoeae, one of the most prevalent sexually-transmitted diseases. The incidence rate of the disease is approximately 100/100,000. Since antimicrobial resistance is increasing and there are no methods available for an active prevention against gonorrhoeae, the development of an effective vaccine is a priority.

A genome analysis of *Neisseria meningitidis* serogroup B strain (MC58) allowed the identification of 600 novel surface-exposed antigens, 380 of which were expressed as recombinant proteins in *Escherichia coli*, purified and used to immunize mice. Analysis of the immune sera showed that 85 of them are surface-exposed in meningococcus and 28 of them are able to induce antibodies with bactericidal activity. Many of the genes encoding for the 85 novel surface-exposed antigens are present also in gonococcus and highly conserved in sequence. Therefore, these antigens represent potential vaccine candidates against *N. gonorrhoeae*.

Materials and Methods: Fluorescence-Activated cell sorting (FACS) was used to verify the presence of the antigen on the surface of gonococcus GC strain. Bactericidal assay was performed using F62 strain and human complement as exogenous source of complement. Complement binding was also evaluated by FACS analysis using an anti human complement C3c FITC conjugate as secondary antibody.

Results and Discussion: Many of the antisera tested were positive in FACS analysis indicating that the corresponding proteins are expressed and surface-exposed also in gonococcus. To evaluate whether these antibodies were also able to bind the complement and induce a complement-mediated bacterial killing, we set up bactericidal and C3 binding assays using human complement as complement source. Preliminary data show that at least two of the 10 sera analyzed up to now have bactericidal activity. These data correlate with the ability of the antisera to recognize the protein on the surface of gonococcus and to bind the complement.

Conclusions: The antigens identified could represent novel candidates for a vaccine against gonococcus.

Immunological response to the Norwegian meningococcal group B OMV vaccine in teen-agers with a condensed three-dose regimen

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Introduction: The Norwegian outer membrane vesicle vaccine against group B meningococcal disease is based on a representative epidemic *Neisseria meningitidis* B:15:P1.7,16 strain from 1976. In a double blind, placebo-controlled protection trial among 172,000 teenagers in 1988-91, two vaccine doses, administered 6 weeks apart, gave 57% protection against serogroup B disease during an observation period of 29 months. In a later immunogenicity study the effect of a third vaccine dose given 10 months after the second dose was examined. A considerable increase in responder rate was achieved. The vaccine is now being produced in new facilities, with minor modifications of the procedure. The newly produced vaccine was used in a 3-dose regimen with a condensed schedule, to investigate if an increase in response rate could be achieved as soon as 6-weeks after the second dose.
Materials and Methods: An observer blind placebo controlled immunogenicity and safety study of the present vaccine was performed. A total of 374 teenagers volunteered for the study. They received either the Men B vaccine or a placebo in a 3-dose regimen plus an additional dose 10-months later. Antibody levels, in blood samples drawn several times during the first 6-months of the study period, were determined by serum bactericidal assay with the vaccine strain. A subject is regarded as a responder to the vaccine if there is either a conversion from not having detectable antibodies to having such, or a four-fold increase in antibody levels.
Results and Discussion: Preliminary results after three doses are as follows: In the group not having detectable antibody levels before vaccination the responder rate by bactericidal antibody assay was 88%. Among those having pre-vaccination antibodies, the responder rate was 64%. The overall responder rate was 75% after three doses, a moderate but significant increase from 66% after two doses. In the previous three-dose immunogenicity study (see introduction) the overall responder rate increased from 73% after two doses to 96% after three doses. In that study however the third dose was given ten months after the previous dose. No serious adverse events due to the vaccine were observed, but a majority of the subjects experienced local pain.
Conclusion: The present study demonstrates that the current vaccine is safe and immunogenic, and confirms that a three-dose regimen renders a higher response rate than the two-dose regimen. This indicates that a condensed regimen may be valuable in an outbreak situation. Results from the fourth dose in the present study are not available yet. In addition, cross-reactive bactericidal antibodies will be examined.

Assessment of the potential of TbpA+TbpB vaccines to protect against experimental meningococcal infection

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Introduction: *Neisseria meningitidis* transferrin binding proteins (TbpA and TbpB) form a complex responsible for the acquisition of host iron from the human iron transport protein transferrin. A number of studies have demonstrated that TbpA and TbpB are both promising vaccine candidiates. TbpB has been shown to protect against meningococcal challenge and elicit a bactericidal antibody response in laboratory animals[1], whilst TbpA provides protection in the absence of detectable bactericidal antibodies[2]. We have further assessed the vaccine potential of combinations of recombinant TbpA and TbpB compared with TbpA+B complex isolated from the meningococcus.
Methods: Recombinant TbpA (strain K454), recombinant TbpB (strains M982, isotype II and B16B6, isotype I) and the native TbpA+B complex (strain M982) were prepared as described previously[1,2]. Antibody responses to these antigens were assessed in mice and rabbits following immunisation with Freund's adjuvant by ELISA, serum bactericidal assay (SBA) and a flow cytometric antibody surface labelling assay. Protection against meningococcal challenge was assessed following intraperitoneal challenge in adult mice following the standard protocols at AvP[1] and CAMR[2].
Results: Vaccination with TbpB elicited higher IgG ELISA titres than TbpA and TbpB gave high SBA titres against the homologous strains. TbpA did not induce clear SBA activity, except against strain BZ83 and 8680 in mice. Some differences in antibody surface labelling were observed between mouse and rabbit sera. Rabbit responses to TbpB were specific to strains with a homologous isotype, whilst cross-reactive surface labelling was observed with TbpA serum. In both species of animal, surface labelling did not reflect the large differences between homologous and heterologous responses against TbpB seen in ELISA assays. This may be indicative of the surface accessibility of the epitopes recognised by the sera. Protection against meningococcal challenge was assessed in both laboratories with either strain M982 or K454 and comparable results were obtained. The greatest number of survivors was seen in groups immunised with TbpA+TbpB. This combination provided greater protection than either individual protein but without an increased SBA response. TbpA +TbpB provided a level of protection close to that conferred by the native TbpA+B complex isolated from *N. meningitidis* strain M982.
Conclusion: The combination of TbpA+TbpB shows promise for providing enhanced protection against meningococcal disease compared with either individual TbpA or TbpB.

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Development of meningococcal whole cell antibody surface labelling and opsonophagocytic assays for assessment of vaccines containing proteins

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Introduction: It is considered that opsonisation and phagocytic killing, in addition to complement-dependent bactericidal killing, are important host defence mechanisms against meningococcal disease. The evaluation of protein-based serogroup B vaccines requires that assays are developed to assess responses against a panel of diverse meningococcal strains. It is also important that these target bacteria are carefully preserved and labelled to maintain the structure of surface epitopes. We have developed an antibody surface-labelling assay against a panel of meningococcal strains and an opsonophagocytosis assay utilising fluorescently labelled bacteria as the target.
Methods: *Bacterial preservation:* Meningococci were killed by various methods then assessed for surface protein functionality using labelled human transferrin.

Antibody surface labelling: Bacteria killed using azide and PMSF in combination were washed in PBS+1%BSA and incubated with serum dilutions for 2h at 4°C. After washing, the bacteria were incubated with anti-Ig-FITC conjugate for 1h at RT then analysed using a flow cytometer.

Opsonophagocytosis (Op): Meningococci labelled with BCECF/AM were killed as described above and opsonised with serum for 30min at 37°C. After 15min further incubation with complement, bacteria were then incubated with phagocytic cells and analysed by flow cytometry.
Results: The preservation of bacterial surface proteins was found to be critically dependent on the agent/s utilised to kill meningococci. Surface protein was abolished after killing with ethanol and aldehydes but preserved using azide and PMSF in combination. Using meningococci killed in this manner, the antibody surface labelling assay was found to be reproducible with a CV of <10% for a panel of sera assessed by different operators. Binding of antibody raised against *N. meningitidis* and *N. lactamica* outer membrane vesicles was assessed against a panel of strains representing the major hypervirulent lineages and a high level of cross reactivity was detected.

Using fluorescently labelled meningococci, non-opsonic and opsonic uptake by HL60 and U937 cells was assessed using flow cytometry. Cell differentiation factors and complement sources have been compared and optimal conditions selected. Clearer Op antibody titrations were obtained with U937 cells than with HL60 cells.
Conclusion: Antibody surface labelling of meningococci detected by flow cytometry is an important new assay for assessing meningococcal vaccines. The bacteria are not dried onto a surface as with whole cell ELISA, which may expose inappropriate epitopes. However, it is vital that the bacteria are not killed using denaturing conditions. Op assays are also important for assessing serogroup B meningococcal vaccines, as bactericidal antibody may not be the only mechanism of protection. As for surface labelling, conditions for fixing and fluorescent labelling must be carefully chosen to maintain surface epitopes. Human cell lines offer the opportunity for reproducibility and standardisation but the cell line and differentiation conditions can greatly affect results obtained.

Murine anti-polysaccharide antibodies: responses to meningococcal group C conjugate vaccines

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Introduction: Different meningococcal conjugate vaccines containing polysaccharides (PS) that are O-acetylated (OAc+); or lack O-acetyl groups (OAc-) have been approved for routine immunization in the Europe. In nature 90% of meningococcal group C strains contain PS that are OAc+, while 10% are OAc-. We compared the specificity and real time binding avidity of the murine anti-PS sera generated by commercial OAc+ and OAc- vaccines as well as different meningococcal group C strains using BIAcore technology.
Materials: Serum pools were prepared from groups of mice immunized with three UK licensed meningococcal conjugate vaccines or OAc+ or OAc- group C bacterial strains. Mice immunized with PBS were used as controls. Four group C murine anti-PS monoclonal antibodies (mAbs), 1 that reacts preferentially with OAc+ and 3 that react preferentially with OAc- epitopes, were used to validate the BIAcore chip. Total IgG and IgM anti-PS antibody (Ab) titers and relative specificity for OAc+ and OAc- were determined by fluorescent ELISA (FELISA). Specificity and relative avidity were determined by BIAcore.
Results: Sera from mice immunized with bacteria had more IgM than IgG and showed approximately equal reactivity on OAc+ and OAc- antigen by FELISA. In contrast, sera from conjugate immunized mice had more IgG than IgM and the two vaccines containing OAc+ PS (Chiron, Wyeth-Lederle) showed approximately equal reactivity on OAc+ and OAc- antigens by FELISA. The OAc- vaccine (Baxter) stimulated higher titer IgG Abs for OAc- than OAc+ antigen by FELISA.

Studies with mAbs established the ability of the BIAcore to discriminate between Abs of low and high affinity and specificity for either OAc+ or OAc- antigens. Sera from immunized mice studied by BIAcore showed similar results to FELISA in specificity and relative avidity.
Conclusions: The O-acetylation status of the PS moiety of conjugate vaccines determines the relative specificity of anti-PS Abs. Compared to bacteria, conjugates induce high affinity IgG Abs of either equal reactivity on OAc+ or OAc- or higher OAc- reactivity. The BIAcore technology can be used to measure both relative specificity and avidity of Abs in serum. BIAcore results correlate with FELISA.

Investigation of IgG antibody responses to meningococcal serogroups A, C, Y and W135 polysaccharide vaccination in human volunteers, for clinical trial serology reagent production

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Background: Meningococcal capsular polysaccharides (MCP) have been and remain a primary target for vaccine development. These structures are the basis for serogroup identification, and among them, four serogroups are currently used in vaccine formulations. Serogroups A [ManNAc-(α1P-6)] and C [NeuNAc-(α2-9)] MCPs are homopolymeric glycans, while Serogroups Y [6-D-Glc(α1-4)-NeuNAc(α2-6)] and W135 [6-D-Gal(α1-4)-NeuNAc(α2-6)] MCPs are polymers of disaccharide repeating units. Bactericidal antibodies specific for MCP confer protection against systemic meningococcal illness. Only antibodies against serogroups A and C MCPs currently have been shown to have good correlation between total serum IgG antibody concentrations and bactericidal titers (Sikkema, et al., 2000; Maslanka, et al., 1997). In this study, pre-and post-vaccination sera from human volunteers were analyzed for the purpose of generating standards, controls and test panels to be used in assay development and validation. The results of these experiments have contributed to our understanding of humoral immune responses to these carbohydrate structures.
Methods: A total of 54 adult volunteers, ages 21 to 57, were pre-screened for anti-MCP serum antibodies (serogroups A, C, Y and W135) by IgG enzyme-linked immunosorbant assay (IgG-ELISA) and serum bactericidal assay (SBA). Twenty-eight individuals with little or no anti-MCP IgG antibodies and low bactericidal titers were selected from this group to undergo pre-vaccination apheresis followed by a single subcutaneous injection of Menomune, (A, C, Y, W135 MCP). After 4 weeks, all volunteers underwent post-vaccination apheresis and the resulting sera were tested for the presence of anti-MCP antibodies by various methods including IgG-ELISA and SBA.
Results: The optimal antigen-coating concentrations for the serogroups A and C ELISAs were the same for all of the serum and antigen lots tested. The range of serogroup C pre-immunization IgG concentration was from 0.05 μg/ml to 1.33 μg/ml (mean = 0.228 μg/ml ±0.33) and from 2.78 μg/ml to 54.38 μg/ml (mean = 22.3 μg/ml ±18.66) for post-immunization samples. IgG values were based on the pooled standard serum, CDC1992 (Elie et al., 2002). Serogroup A IgG concentrations fell within the range of 0.05 μg/ml to 4.49 μg/ml (mean = 0.647 μg/ml ±1.16) for pre-immunization samples and from 1.55 μg/ml to 356.44 μg/ml (mean = 33.36 μg/ml ±67.55) for post-immunization samples. Various pooled sera were generated for use in these assays, as well as SBAs. Optimization of antigen coating concentrations revealed that serum antibodies to serogroups Y and W135 were selectively sensitive to specific epitopes. Availability of different epitopes in the ELISA appears to be sensitive to the coating concentration. Therefore, serum samples display different IgG values based on the coating conditions.
Conclusion: The poor correlation between ELISA data and SBA titers for serogroups Y and W135 may be due to the lack of consensus among serum samples for a single optimal ELISA coating concentration.

Comparison of the immunogenicity of synthetic linear and multiple antigen peptides containing a protective epitope derived from PorA (subtype P1.15)

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Introduction: Peptide vaccines can provide an effective vaccine by focusing the host's immune response on epitopes known to play a role in protective immunity. Among the major outer membrane proteins of *Neisseria meningitidis*, the porin PorA has been considered as an important vaccine candidate (1). In this work, Linear and Multiple Antigen Peptide (MAP), comprising B cell epitopes from PorA and T cell epitopes derived either from TT or from the P64k meningococcal protein were evaluated, in terms of immunogenicity in mice.
Materials and Methods: Peptides and Multiple Antigen Peptides (MAPs) contained a B cell epitope derived from the surface loop 4 of PorA from the *Neisseria meningitidis* strain CU385 and refered T-helper epitopes. They were synthesized manually using the Boc/Bzl chemistry as described previously (2). Peptides and MAPs were purified by reversed-phase HPLC and characterized by mass spectrometry or amino acid analysis. The immune response was examined in mice immunized with three doses (two weeks apart) of peptide or MAPs, emulsified in Freund´s Adjuvant. Sera were studied by ELISA, immunoblot and whole cell ELISA.
Results and Discussion: Immunization with all MAPs evoked potent anti-peptide titers, even in the absence of a T cell epitope. The subclasses of anti-peptide antibodies elicited by these immunogens were characterized. Moreover, in all groups that received MAPs, the anti-peptide sera reacted with meningococcal outer membrane vesicles in immunoblot, but only sera produced against MAPs containing a T-cell epitope significantly reacted with native meningococcal outer membrane vesicles in ELISA. There were no statistically significant differences between the reactivity with whole meningococci exhibited by sera from mice immunized with the MAP containing a P64k-derived T cell epitope and the one produced in response to a MAP containing a TT-derived T cell epitope.
Conclusion: Even when the anti-peptide titers can be significantly increased using a MAPs system without T cell epitopes, the presence of such epitopes is highly recommended not only for the quantity but for the quality of the antibody response directed to conformational epitopes.

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Characterization of recombinant P64k as a carrier protein

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Introduction: The P64k meningococcal protein, an antigen of 64 kDa expressed in *Escherichia coli,* has been extensively characterized. Its availability, molecular weight and immunogenicity encouraged its previous use as a carrier molecule in a candidate vaccine against cancer (1). However, due to the existing homology between the P64k lipoyl-binding domain and the one present in the dihydrolipoamide acetyltransferase (E2) subunit of the pyruvate dehydrogenase complex, the lipoyl-binding site of P64k was genetically modified to allow the use of this recombinant protein in prophylactic vaccines. Here, we show some of the P64k properties that support its use as a carrier for conjugate vaccines.
Materials and Methods: First, we investigated the immunogenicity in mice of several hapten-P64k conjugates. Six synthetic peptides, derived from viral proteins were conjugated to P64k by the glutaraldehyde method (2). Besides, serogroup C capsular polysaccharide of *Neisseria meningitidis* was chemically coupled to this protein. All the conjugates were injected in BALB/c mice and the antibody titers were determined by ELISA. In addition, the effect of presensitization with P64k on its carrier ability was studied. Moreover, a Phase I clinical study, was designed and conducted in 26 healthy volunteers to determine the safety of two formulations of P64k, adsorbed onto aluminum hydroxide.
Results and Discussion: P64k increased the immune response against six out of seven peptides and against the serogroup C capsular polysaccharide of *Neisseria meningitidis* chemically coupled to this antigen. Moreover, it was demonstrated that P64k-induced epitope-specific suppression might occur, depending on the hapten and the pre-existing levels of immunity against the protein. This is in agreement with results found by others, who showed that epitope specific suppression exists for TT, KLH and other widely used carrier proteins. In the clinical trial, it was found that the P64k formulations were well tolerated and safe in healthy adults. It was expected having into account that this is a recombinant protein produced under GMP. Moreover, this antigen induced a specific humoral immune response in 15 out of 18 individuals inoculated with it and did not elicit anti-mitochondrial antibodies.
Conclusions: P64k is a ready available protein, safe and immunogenic in humans which can be used as a carrier not only for therapeutic but also for prophylactic conjugate vaccines.

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T-cell response against the P64k meningococcal protein in BALB/c mice

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Introduction: Our group has previously characterized the P64k protein of *Neisseria meningitidis*. Due to its relatively high molecular weight, demonstrated immunogenicity and availability, P64k was employed as a carrier protein for poorly immunogenic peptides and *Neisseria meningitidis* serogroup C polysaccharide with good results (1). In this study, we characterized the T-cell response developed by lymph node cells of mice sensitized with this antigen.
Materials and Methods: BALB/c mice were subcutaneously immunized at the base of tail with 20 µg/mouse of recombinant protein as an emulsion in Freund´s Adjuvant. Seven days later, mice were killed, and inguinal lymph nodes were removed. Lymph node cells (LNC) were cultured in triplicate in RPMI 1640 containing 10% FCS, gentamicin and 2-mercaptoethanol in wells of a 96-well plate. In addition, different concentrations of the homologous immunogen were included as the challenge antigen. Proliferative response was measured as published elsewhere (2). In similar experiments, the supernatants were collected 24 h and 72 h after stimulation with P64k. The presence of IL-4 and IL-12 in such supernatants was analyzed by ELISA. For the T-cell epitope mapping, 59 overlapping synthetic peptides that encompassed the full-length 596 amino acids of the protein were synthesized, using the Multipin cleavable peptide kit (Chiron Technologies), and tested for proliferation in P64k-sensitized mice. Then, selected peptides were synthesized using Fmoc chemistry, purified to at least 95% purity by reverse phase HPLC and used to confirm the results obtained with overlapping peptides.
Results: BALB/c mice subcutaneously immunized with P64k in CFA provided, after seven days, inguinal LNC that proliferated in the presence of P64k in a dose-dependent manner. Proliferating cells secreted IL-4 while the concentration of IL-12 remained unaltered in culture supernatant. Proliferation of LNC from P64k-sensitized BALB/c mice was also observed with two overlapping 20-amino-acid peptides (P48 and P49), from a series of 59 synthetic peptides. Consistently, lymph node cells obtained from either P48- or P64k-sensitized mice, produced a statistically significant proliferative response when challenged with the homologous peptide or the recombinant protein, respectively. Finally, three overlapping peptides spanning the P48 sequence were tested for proliferation in homologous peptide- and P48-sensitized mice. The highest proliferative response was obtained against a peptide that includes amino acids 470-485.
Conclusions: As expected, P64k induced a significant proliferative T cell response in mice, with secretion of IL-4. The peptide IPGVAYTSPEVAWVG (aa 470-485) contains an immunodominant T-cell epitope for BALB/c mice.

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A novel antigen of *Neisseria meningitidis* as target for bactericidal antibodies

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Introduction: NadA is a novel surface exposed antigen of *Neisseria meningitidis* identified by computer analysis of the MC58 genome (1). The protein has a predicted molecular structure similar to that of known adhesion-invasion proteins. The gene encoding for NadA is present in approximately 50% of strains analysed, but is overepresented in hypervirulent strains. The protein is encoded by three well-conserved alleles. Here we show that NadA is able to induce bactericidal antibodies against all strains in which the gene is present. The bactericidal titers induced are different against the various strains. These differences are not due to diversities in protein sequences but to the expression levels, which are different in the various strains and vary during growth.
Materials and Methods: Immunizations: Groups of four to eight CD1mice per group were injected intraperitoneally with 20 μg of recombinant antigens in alum hydroxide. Each animal received three doses. Western immunoblot was performed on whole cell lysates of *N.meningitidis*. Samples were taken at different times during growth and equal amounts of proteins were subjected to SDS-PAGE on polyacrilamide gels and electrotransferred onto nitrocellulose membranes. Serum bactericidal assay (SBA) against Neisseria strains was assessed for the pooled sera serially two-fold diluted using baby rabbit complement. Bactericidal titers were determined as the reciprocal dilution of test serum yielding >= 50% bacterial killing
Results and Discussion: We performed Western blot analysis with anti-NadA serum of samples collected at different time of the bacterial growth. An high molecular weight reactive band, corresponding to an oligomeric form of NadA was detected in all strains carrying the gene. The size of the protein correlates with the length of the encoding allele. Furthermore, the Western blot showed also that the levels of expression varied during the growth curve, reaching the maximum at stationary phase.

We tested whether the anti NadA serum had a bactericidal activity against a panel of strains carrying the gene. The bactericidal titers varied between strains and correlate with the expression levels. Moreover, the antiNadA immune sera obtained from the different alleles were tested in a cross bactericidal assay against the different strains. The results support the hypothesis that the difference in the bactericidal titers were due to a different level of expression of the antigens in the various strains, rather than to a sequence specificity in the immune response.
Conclusion: NadA is a novel meningococcal antigen that is differentially expressed in a subset of hypervirulent strains, forms oligomers anchored to the outer membrane, and induces bactericidal antibodies with different titers related to the various expression levels.

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Improved formulation and immunization regime may be the key to effective nasal vaccines against meningococcal disease

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Introduction: Nasal vaccines consisting of outer membrane vesicles (OMVs) from group B meningococci can induce serum antibodies with strong bactericidal activity, as well as local mucosal antibodies, in both mice and humans. Since some individuals will not respond to these vaccines, even when given repeatedly, we have evaluated the effect in mice of different doses and immunization regimes.
Materials and Methods: Groups of mice were immunized intranasally with OMVs prepared from group B meningococci, in 25 μg or 100 μg doses without adjuvant, at intervals from one hour to six weeks. In some, immunizations were repeated for up to one year. Separate groups were immunized during general intravenous anesthesia, and some were immunized subcutaneously in 2.5 μg doses with aluminum hydroxide as adjuvant. IgG and IgA antibodies in respectively serum and saliva were analyzed by ELISA, and serum bactericidal activity (SBA) was measured by an agar overlay method. Vaccine-specific *in vitro* spleen cell proliferation was measured by thymidine incorporation.
Results and Discussion: Repeated intranasal (i.n.) immunizations boosted serum IgG antibody concentrations to the same level as were induced by subcutaneous (s.c.) immunizations. Measurements of SBA correlated with the corresponding serum IgG concentrations. I.n. immunizations could prime for later boosting of serum IgG by s.c. immunizations, and vice versa. I.n. immunizations were necessary for induction of secretory IgA antibody responses, but s.c. immunizations could prime for later boosting of IgA in saliva by i.n. immunizations. After repeated i.n. immunizations for up to one year, stimulation of antibody production was still possible. Moreover, priming by i.n. immunizations did not reduce proliferation of spleen cells in response to later s.c. immunizations, indicating that the nasal vaccine had not induced immunological tolerance. IgA in saliva were boosted within a four-week-period by repeated i.n. immunizations, whereas more than four weeks were needed for boosting of IgG antibodies in serum. A six-week-interval regime with three doses induced maximal antibody responses in both serum and saliva. The antibody responses following two 100 μg doses intranasally were equal to those after two series of four 25 μg doses administered at one-hour-intervals. Increased dose, and not time of contact with mucosal surfaces, might thus improve the vaccine-induced antibody production. High serum antibody concentrations were obtained in animals that had been anesthetized during i.n. immunizations, indicating that vaccines reaching the lungs will increase systemic antibody responses.
Conclusion: Studies in mice indicate that the responses to nasal OMV vaccines can be improved by adjusting the dose or increasing their availability, and by allowing sufficient time for induction of immunological memory. Such vaccines may be favorably combined with similar vaccines for injection. Improved systemic responses may be obtained with vaccines administered as nasal spray that can be inhaled.

Antibody avidity and O-acetylation of meningococcal group C capsular polysaccharide in relation to protective activity of murine and human group C *Neisseria meningitidis* anticapsular antibodies

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Introduction: In 90% of group C strains the polysaccharide is O-acetylated (OAc+); and in 10%, it is not O-acetylated (OAc-). Little is know about the role of O-acetylation on the protective activity of anticapsular antibodies.
Materials: Pre- and post- serum pools were prepared from 2 groups of children immunized with meningococcal polysaccharide vaccine. Six group C murine anticapsular mAbs, 3 that preferably reacted with OAc+ and 3 with OAc- epitopes, were tested. Total anticapsular antibody (Ab) concentrations and avidity were measured by a radioantigen binding assay and IgG by ELISA. Passive protection was measured in infant rats challenged with an OAc+ or an OAc- strain (N=4 to 7 pups/Ab dose).
Results: The three OAc+ mAbs were bactericidal against the OAc+ strain (BC_{50}<0.05 µg/ml) but were much less bactericidal (BC_{50} up to 14 µg/ml) against the OAc- strain. At an antibody dose of 0.5 to 1µg/rat, the OAc+ mAbs completely protected infant rats (i.e. sterile blood cultures) challenged with the OAc+ strain but 2 of the 3 mAbs failed to protect against the OAc- strain (geometric mean CFU/ml of blood [GMB] >500,000). The three mAbs that preferentially reacted with OAc- epitopes were bactericidal (BC_{50}<0.05 µg/ml) and completely protected against both the OAc+ and OAc- strains. Their activity against the OAc+ strain is consistent with non-stoichiometric O-acetylation. None of the pediatric serum pools was bactericidal against the OAc+ strain and only post-pool 3 was bactericidal against the OAc- strain. None of the pre-pools tested protected infant rats (GMB>500,000 for both strains). At a dose/rat of 0.2 µg of anticapsular antibody, post-pool 1 failed to protect (GMB >500,000 with either strain) but post-pools 2 and 3 protected against the OAc+ strain (GMB 400 and <1) despite lack of SBA. Against the OAc- strain, pool 3 at a dose of 0.2 µg/rat was more protective than pool 2 (GMB 45 Vs. 21,000 vs 45). A representative adult post-immunization serum (Ka = 28.3 nM^{-1}) protected rats at this dose against both strains (GMB 2 and <1), a result suggesting that the adult antibody may be skewed towards different capsular epitopes than children.

Study	Mean Age, years	Number subjects in pool	Pre-pools	Post-Pools			
			Tot. Ab, µg/ml	Tot. Ab, µg/ml	IgG Ab, µg/ml	K avidity, nM^{-1}	SBA OAc+,OAc-
1	2.6	5	<0.1	2.0	2.2	6.2	<4,<4
2	4.8	12	<0.1	12.0	9.7	12.6	<4,<4
3	4.5	15	<0.1	11.4	16.6	22.8	<4,12

Conclusions: Both antibody avidity and O-acetylation of the strain affect protective activity of anticapsular antibody. Some vaccine-induced anticapsular antibodies that lack bactericidal activity can confer protection against bacteremia.

The use of non-human complement sources in the serogroup B *Neisseria meningitidis* serum bactericidal assay facilitated by a colominic acid absorbent

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Background: The preferred complement source for serogroup B *Neisseria meningitidis* (NmB) serum bactericidal assays (SBA) is human serum that is found to lack SBA activity and is non-toxic for the target NmB strains. Non-human complement sources, with those same characteristics, have been evaluated in the NmB SBA. These sources have been shown to be associated with elevated SBA titers that seem to be connected to the presence of anti-NmB capsular polysaccharide (CpsB) antibodies in test sera. The process of obtaining the quantity and quality of human complement necessary to investigate non-CpsB based vaccine candidates and support clinical trials is quite tedious making the use of an alternative, commercially-available source of complement attractive. Colominic acid is a capsular homopolymer from *E.coli* K1 that shares the same alpha (2-8)-linked N-acetyl-D-neuraminic acid (NeuAc) residue linkage as the NmB capsular polysaccharide. Since anti-CpsB antibodies seem to be a major hindrance in the use of non-human complement in the NmB SBA when measuring non-capsular functional antibodies, we have examined the impact of the addition of colominic acid, as an absorbent in the bacterial buffer, on SBA titers generated by various complement sources.
Methods: Using H44/76 as the NmB target strain, normal human serum, hyper-immunized (PorA) animal serum, and Nm acute convalescence human serum, were evaluated for SBA titers using guinea pig, bovine, baby rabbit, and human complements. The SBAs were performed in the presence of bacterial buffer containing colominic acid as an absorbent.
Results: The addition of colominic acid to the bacterial buffer decreased SBA titers obtained with non-human sources of complement when compared to titers obtained without colominic acid. Overall, the most dramatic decrease was observed in the presence of rabbit complement giving SBA titers that were equal or $\pm$ 1 dilution to those generated by human complement in ~80% of the samples tested. To ensure that this decrease in SBA titer was not a result of complement exhaustion or antibody masking by colominic acid, a SBA positive serum sample was diluted with a colominic acid-absorbed negative serum sample. The SBA titers of the positive serum sample obtained in the presence of increased amounts of colominic acid-absorbed negative serum sample were $\pm$ 1 dilution from the mean SBA titer suggesting no exhaustion or masking by colominic acid.
Conclusion: The inclusion of colominic acid in the NmB SBA enables the use of non-human sources of complement and results in titers more in agreement with those generated with human complement. This impact is most pronounced using baby rabbit complement and promotes the possibility of using commercially available sources of complement in the NmB SBA targeted for measuring non-capsular functional antibodies.

Serum bactericidal activity as surrogate for efficacy in evaluation of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease

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Introduction: Formulations of outer membrane vesicle (OMV) vaccines against meningococcal serogroup B disease have shown to be protective through large clinical trials involving thousands of participants. A reliable and convenient *in vitro* assay is particularly important for evaluation of various modified and improved formulations of a vaccine. We have evaluated serum bactericidal activity (SBA) as an alternative to efficacy studies, using the unique set of data and sera from an efficacy trial performed in Norway.
Materials and Methods: A randomized, double blind, placebo-controlled clinical trial involving 172,000 secondary school students (aged 13-14 years) was performed in order to evaluate the protective potential of a meningococcal OMV vaccine in Norway (Bjune *et al.* Lancet 1991; 338: 1093). The vaccine was manufactured from the epidemic strain 44/76 (B:15:P1.7,16) and given twice intramuscularly six weeks apart, each dose consisting of 25 μg protein with aluminum hydroxide as adjuvant. A cohort of 880 individuals, representative of the larger study population, was selected for immunogenicity studies. The SBA test was performed with the vaccine strain, using 25% human complement and 60 min incubation. Titer was defined as the reciprocal of the serum dilution with >50% kill of the inoculum. Vaccine responders were defined as those individuals with equal or more than four-fold increase in SBA titer.
Results and Discussion: After 29 months, the point estimate of protection against group B meningococcal disease induced by vaccination was 57%. However, an estimated efficacy of 87% was calculated after a 10-months observation period. The immunogenicity study showed that 30% of the vaccinees had SBA titers ≥4 before vaccination and 97% reached this SBA level six weeks after the second vaccine dose. SBA titers ≥4 persisted in 42% of the vaccinees two years after vaccination. The cohort showed 80% responders six weeks after the second dose. The proportion of seroresponders decreased to 26% two years after vaccination.
Conclusion: The OMV vaccine protected against systemic disease and induced bactericidal activity in the same proportion of individuals. Good correlation between observed protection and mean SBA titers was found. Using the strain 44/76 as target in the SBA assay a tentative protective SBA level for the population was found to be a titer ≥4. Measurements of SBA may thus be used to evaluate various vaccine formulations and to optimize immunization regimes for OMV vaccines against group B meningococcal disease.

Vaccine strategies for recombinant meningococcal PorA protein

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Introduction: The Class 1 protein (PorA) of *Neisseria meningitidis* has been shown to induce a bactericidal immune response following natural infection and is under investigation as a potential antigen for inclusion in new meningococcal vaccines. Experimental vaccines containing recombinant PorA incorporated into monovalent liposomes have been found to induce bactericidal antibodies in animals. However, antigenic variation of PorA between strains of *N. meningitidis*, and the generation of subtype specific immune responses, suggests that multivalent PorA vaccines are required to provide broad protection. In this study, recombinant PorA (rPorA) of four different subtypes have been purified and used for immunisation as multivalent preparations. In addition, a number of different expression vehicles for the production of rPorA were also investigated.

Methods and Materials: Recombinant PorA of subtypes P1.7,16, P1.7b,4, P1.19,15 and P1.5a,10d were expressed in *E.coli* using the high expression vector pQE-30 and purified using 6-histidine tag affinity chromatography. For immunisation studies the purified recombinant proteins were incorporated into monovalent and tetravalent liposomes: in addition a mixture containing the four monovalent liposomes was prepared.

Recombinant PorA protein of subtype P1.7,16 was also expressed from three expression vectors, pRSETA, pQE-30 and pTWIN1, which introduce N-terminal leader sequences of 29 kDa, 11 amino acids and 3 amino acids respectively. These three rPorA were also incorporated into monovalent liposomes and detergent micelles and used for immunisations.

Results and Discussion: The three rPorA with varying length N-terminal leader sequences were all immunogenic when incorporated into liposomes and detergent micelles. They produced high titres of antibody against the homologous PorA within the outer membrane in ELISA and recognised the homologous native PorA on whole meningococcal cells as determined by immunofluorescence. The antisera raised against liposomes containing these three different proteins also had similar high levels of bactericidal activity against the homologous meningococcal strain.

The liposomes containing the four different subtype rPorA were immunogenic, inducing high titres of antibody against the respective homologous recombinant proteins and against the homologous PorA within outer membranes in ELISA. The antibodies induced also showed high reactivity to the homologous native PorA on whole meningococcal cells as determined by immunofluorescence. In serum bactericidal assays the antisera induced against the monovalent PorA liposomes were found to induce complement-mediated killing of the homologous meningococcal strains. However, following immunisation with multivalent preparations the antisera raised had bactericidal activity against strains of certain subtypes but not against others.

Conclusion: The differences in bactericidal response to the different subtypes when incorporated into multivalent preparations demonstrate that certain subtypes are immunodominant and it is therefore necessary to investigate alternate strategies for enhancing the immune response to these recombinant PorA proteins.

Characterization of NOMV prepared from *lpxL1* and *lpxL2* mutants of *Neisseria meningitidis* with L3,7 and L8 lipooligosaccharide

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Introduction: We have been investigating two approaches to using native outer membrane vesicles (NOMV) as a vaccine. The first approach is use of NOMV from a *syn*X(-) mutant as an intranasal vaccine. The second approach is use of NOMV from an *lpx*L2 mutant as a parenteral vaccine. Although highly immunogenic, NOMV retain up to 25% LOS resulting in high toxicity. Use of detergent to remove LOS may result in undesirable changes in OMP conformation and/or exposure of non-bactericidal antibody-inducing epitopes. We report here characterization of NOMV from *N. meningitidis* mutants, which contain deletions in the *lpx*L1 and/or *lpx*L2 genes and express full length LOS.
Materials and Methods: The *lpx*L1 and *lpx*L2 genes were amplified from strain 44/76, cloned into the pUC19 vector, and a 282-bp and a 260-bp deletion in the coding regions of *lpx*L1 and *lpx*L2, respectively, were introduced using reverse PCR. Either a tetracycline or a kanamycin resistance gene was inserted into the deleted region, and the resulting plasmids were used to transform the wild-type (WT) 44/76 and 9162 strains using the standard *Neisseria* transformation method. The transformants were screened by PCR and Southern blot analysis. NOMV were prepared as described previously. Endotoxicity of the mutant NOMV was determined by the rabbit pyrogen test and human monocyte cytokine release analysis. Human monocytes were incubated overnight with different concentrations of NOMV and the culture supernatants assayed for levels of TNF-α, interleukin (IL)-1β, IL-6, and IL-8 using the LINCOplex assay system (Linco Research, Inc.). *In vivo* immunogenicity was analyzed by administering NOMV to mice with or without alum and measuring levels of total anti-NOMV IgG and IgM, as well as bactericidal antibody response.
Results: LOS from the D*lpx*L1 and mutants showed slightly faster migration on gel electrophoresis as compared to WT LOS. LOS from D*lpx*L2 mutants was highly sialylated unless the mutant was also *syn*X negative. Growth of the D*lpx*L2 mutant was 2-fold slower than that of the WT and D*lpx*L1 strains. The D*lpx*L1 NOMV were marginally pyrogenic in rabbits at 0.1 μg/kg, whereas D*lpx*L2 showed no pyrogenicity at 1 μg/kg. Both mutant strains showed lower toxicity than the WT as measured by cytokine release and lower immunogenicity by IgG and IgM ELISA and by bactericidal assay. The immunogenicity was restored by co-administration of alum.
Conclusions: Mutant NOMV, especially from the D*lpx*L2 mutant, were less toxic than the WT, but were also less immunogenic. However, immune response was restored by use of an adjuvant. These data suggest that D*lpx*L2 mutant NOMV present a promising parenteral vaccine candidate.

The Whole Blood Assay is the most sensitive tool for evaluation of serogroup B meningococcal vaccines

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Introduction: The whole blood assay (WBA) has previously been shown to be more sensitive than the serum bactericidal assay (SBA) in measuring the bactericidal activity towards serogroup B meningococci[1], and in detecting a specific response to the Cuban serogroup B meningococcal vaccine[2]. However, evidence was still lacking regarding its ability to detect the acquisition of age-related bactericidal activity, as demonstrated by Goldschneider *et al.*[3] using the SBA, or to detect cross-reactive responses to more than one strain.

Materials and Methods: The bactericidal activity of whole blood was determined using samples from healthy children (82, aged <1 year to 15 years), adult volunteers (20), and children after serogroup B (56, aged <1 year to 18 years) and serogroup C (44, aged 1 year to 18 years) meningococcal disease. The WBA was performed as previously described using a panel of meningococci including strains: MC58 (B:15:P1.7,16, ET-5, MLST 74); the Cuban vaccine strain CU385/83 (B:4:P.19,15, ET-5, MLST type 34); the clinical isolate NM48 (B:4:P1,4, ET Lineage 3, MLST 41); and NCTC 8554 (C:NT:P1.15). Correlation coefficients were determined using Spearman's rank test.

Results and Discussion: Survival of MC58 in the blood of healthy children and adults was age-dependent (p=<0.001), with the greatest survival in infants less than 2 years of age resulting in growth up to 400% in some cases. There was a progressive increase in efficiency of whole blood killing with age with the exception of a dip between 12 to 16 years of age. There was a significant correlation between survival of MC58 and the two other serogroup B and the serogroup C strain. There was an acceleration in the acquisition of bactericidal activity in the blood of children convalescent from serogroup B disease, where even in samples from young children the survival of strain MC58 was reduced compared to healthy children (<2 years of age, median 68% compared to 194.5%). Surprisingly this was also evident in children convalescent from serogroup C infection.

Conclusion: Bactericidal activity as measured in whole blood is inversely related to the incidence of disease, a similar finding to that of Goldschneider *et al.*[3] but differs in that the SBA detected a significant titre in only 70-80% of adults whereas whole blood killing was demonstrated in almost 100% of adults. The WBA detects bactericidal activity elicited by carriage or infection that is directed at multiple strains of meningococci. It could therefore be used as a sensitive tool for the selection of candidate antigens and in Phase II studies of meningococcal vaccines.

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Immunogenicity and immunological priming of the serogroup A portion of a bivalent meningococcal A/C conjugate vaccine in two year old children

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Introduction: Meningococcal A/C polysaccharide (MACP) vaccines have proven to be effective in the short-term control of MenA disease and have been used extensively in the control of outbreaks and epidemics. MACP vaccines fail to induce immunological memory and antibody levels progressively decline to pre-immunisation levels in the three years following vaccination in both adults and children. Meningococcal serogroup C conjugate (MCC) vaccines have been shown to induce immunological memory in toddlers and meningococcal A/C conjugate vaccines studies have demonstrated these vaccines to be safe and immunogenic in children. The development of immunological memory may be demonstrated by the measurement of antibody avidity, the strength with which a bivalent antibody binds to complex antigens. Recently, avidity indices have been utilised to study meningococcal conjugate vaccines for both serogroup A and C. In this study we investigated the immune response and induction of immunological memory to the serogroup A portion of MACP and a meningococcal A/C conjugate (MACC) vaccines in subjects aged two years old.
Methods: Children aged 2-3 years were randomised to receive a single dose of either MACP or MACC. Blood was taken before, 1 month and 1 year after vaccination. Sera were tested for serum bactericidal antibodies (SBA) against a serogroup A meningococcal strain and IgG, IgM and IgA antibodies to serogroup A-specific capsular polysaccharide. Serogroup A-specific IgG avidity was measured by an elution ELISA using the chaotroph ammonium thiocyanate.
Results: Geometric mean SBA titres and serogroup A-specific IgG increased one month post primary for either MACC or MACP vaccination. SBA GMTs increased from <4 to 558.34 and 886.31 for the MACP and MACC respectively whilst serogroup A-specific IgG increased from 3.16 to 11.1 and from 4.86 to 14.32 for the MACP and MACC cohorts respectively. Both SBA GMTs and serogroup A-specific IgG declined to pre-vaccination levels in the following year. SBA GMTs were <4 for both MACP and MACC cohorts with serogroup A-specific IgG decreasing to 3.92 and 2.15 for the MACP and MACC cohorts, respectively. For the MACP cohort, serogroup A-specific IgG geometric mean avidity indices (GMAI) increased from pre-vaccination GMAI of 80 to 190 one month post-vaccination. One year post-vaccination, the GMAI of the MACP cohort then decreased to 130. In contrast, for the MACC cohort, the GMAI increased from a pre-vaccination GMAI of 112 to 210 one month following MACC and did not decline one year post-vaccination, remaining at a GMAI of 210.
Conclusions: In this study, MACC and MACP elicit high SBA titres one month post-vaccination but only the MACC was shown to induce immunological memory as measured by avidity indices. A single dose of MACC should be sufficient for the induction of both SBA titres in the short term and immunological memory in the long term for protection of two to three year olds children.

Meningococcal serogroup A avidity indices as a surrogate marker of priming for the induction of immunological memory following vaccination with a meningococcal A/C conjugate vaccine in infants

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Introduction: Meningococcal serogroup A (MenA) disease continues to cause major epidemics in the 'meningitis belt' of sub-Saharan Africa every few years. In 1996, 188,341 cases of meningococcal disease were reported to the WHO from African countries in the 'meningitis belt' highlighting the need for a programme of preventative vaccination. Meningococcal AC polysaccharide (MACP) vaccines have proven effective in the short-term control of both serogroups A and C. Concerns have now been raised about the induction of immunological hyporesponsiveness to serogroup A polysaccharide by MACP vaccines. Serogroup A conjugate vaccines can overcome this problem and are thought to elicit immunological memory.

In studies undertaken in Gambian infants with a meningococcal serogroup A/C conjugate vaccine, the serogroup A portion did not appear to induce immunological memory although the serogroup C portion did. A study in UK infants with the same vaccine appeared to induce immunological memory as measured by SBA titres following a booster dose of MACP. We have sought confirmation of this finding by measuring avidity indices during the primary immunisation course and before and after the MACP booster, since avidity maturation is also evidence of the induction of immunological memory.

Methods: Low avidity antibodies were eluted with the chaotroph isothiocyanate and the resulting high avidity antibodies were measured and expressed as a percentage of the total serogroup A-specific antibody termed the avidity index.

Results: Geometric mean SBA titres increased from the first dose (4.7) to the second (23.6) and third (26.4), fell in the following 9 months (2.2) and then increased upon challenge with serogroup A polysaccharide (394.8). The geometric mean avidity indices (GMAI) however continued to increase with GMAIs at the same time intervals of 129, 227, 254, 308, and 470 indicating an increase in the quality of the antibody even in the face of declining total antibody. The GMAI in a control group of age matched children given a single dose of MACP was 144.

Conclusion: Evidence of immunologic memory in this study was based on the response to MACP vaccine and maturation of antibody avidity. Avidity indices were shown to increase after each dose of conjugate and carried on increasing in the following nine months and increased further after challenge with polysaccharide suggesting an increase in the antibody quality.

Characterization of a genetically detoxified native outer membrane vesicle (NOMV) vaccine prepared for human use

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Introduction: Native outer membrane vesicles (NOMV) have excellent immunogenicity, but their high endotoxin content has prevented their use as a vaccine. We have identified two approaches to safely use NOMV as a vaccine. First, we have demonstrated in two human studies that NOMV may be safely used as an intranasal vaccine. The second approach is use of NOMV from a strain with genetically detoxified LOS (*lpx*L2 knockout) as a parenteral vaccine. We report here the initial characterization of a clinical lot of vaccine prepared from a mutant of strain 44/76 that has the *lpx*L2 deletion, stabilized Opc expression, and full length LOS.
Methods: The vaccine was prepared under U.S. current good manufacturing practice from pelleted cells of strain 44/76 MO grown in a 400 L fermenter on modified Catlin's medium with low iron. NOMV was extracted with tris-saline-EDTA buffer at pH 7.5 followed by differential centrifugation and ultrafiltration. The vaccine was analyzed for composition by chemical assays, SDS-PAGE and western blotting with a panel of monoclonal antibodies. Safety was evaluated in rabbit pyrogen, general safety, and human monocyte TNF-alpha release tests. Immunogenicity with and without aluminum hydroxide adjuvant was tested in mice and rabbits. Antibody response was evaluated by bactericidal assay and ELISA.
Results and Conclusions: Per milligram of protein the vaccine contained 50 μg LOS, 22 μg sialic acid, and 7.5 μg nucleic acid. Analysis by SDS-PAGE showed the presence of PorA, PorB, RmpM, Opa, Opc, and iron regulated proteins as major components. The LOS also contained several components including L3, L7, and L8. The most prominent band was L3 (the sialylated form of L3,7). The vaccine was less immunogenic in mice than NOMV prepared from the 44/76 wild type (WT) or an *lpx*L1$^-$ mutant. Geometric mean titers of bactericidal antibody were 696.72, 64.0, and 20.75 for mice immunized with 1 ug of WT, lpxL1$^-$, or *lpx*L2$^-$ NOMV, respectively. However, immunogenicity of the *lpx*L2$^-$ NOMV vaccine could largely be restored with an adjuvant such as aluminum hydroxide (GM SBA titer = 558). Safety and immunogenicity data obtained to date support the use of NOMV from a *lpx*L2$^-$ strain as a parenteral vaccine in human volunteers.

Tailor-made outer membrane vesicle (OMV) vaccine against the group B meningococcal epidemic in New Zealand – manufacturing and characterisation

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Introduction: Due to the epidemic situation in Norway between 1975 and 1995, NIPH developed an OMV vaccine against *Neisseria meningitidis* serogroup B, based on the Norwegian B:15:P1,7,16 strain 44/76. New Zealand has experienced a similar group B epidemic during the last ten years. In order to help in the combat of this epidemic, NIPH has now developed and manufactured a tailor-made vaccine based on the meningococcal B:4:P1.7b,4 strain NZ98/254 from New Zealand and the manufacturing process of the Norwegian vaccine.

Material and Methods: The New Zealand vaccine strain NZ98/254, characterised as B:4:P1.7b,4, originates from a clinical isolate in New Zealand, and was kindly supplied by Dr. Diana Martin, ESR, New Zealand.

The development and manufacturing was performed following Good Manufacturing Practice (GMP). The manufacturing process includes cultivation in a fermentor, concentration by ultrafiltration, inactivation of the bacteria and OMV formation by the addition of deoxycholate (DOC), removal of cell debris by centrifugation and further purification by ultracentrifugation. Purified OMV is subjected to sterile filtration and subsequently stabilised with sucrose and absorbed to aluminium hydroxide as an adjuvant.

Compared with the original Norwegian vaccine, the main improvement of the manufacturing process is the use of a chemically defined growth medium. In other respects, the process is essentially similar for the two vaccines.

Purified OMV is characterised by the concentration of proteins, lipopolysaccharides (LPS), DOC, DNA and endotoxins. Antigen pattern is studied with SDS-PAGE and immunoblotting. Vesicle formation is monitored with transmission electron microscopy. Potency test is performed by immunization of mice and measuring specific IgG antibodies against OMV by ELISA.

Results and Discussion: The final manufacturing process demonstrated consistency of the process parameters and product quality control. The New Zealand vaccine contains intact vesicles and vesicle fragments with the major outer membrane proteins PorA and PorB, minor amounts of TdfH, Omp 85, FrpB, FbpA, Class 4, Opc and NspA, and LPS of immunotype L3,7,9 and L1.

Conclusion: NIPH has successfully developed and manufactured consistent lots of a tailor-made OMV vaccine against the New Zealand meningococcal epidemic.

This robust manufacturing process can possibly be used for a rapid development of new OMV vaccines to combat future meningococcal outbreaks. NIPH has the required facilities and know-how for the manufacturing of such vaccines for clinical trials.

Antigen expression and immunogenicity of outer membrane vesicles from the Norwegian vaccine strain 44/76 cultivated in modified Catlin-6 and Frantz' medium

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Introduction: Strain 44/76 (B:15:P1.7,16; ET-5) was cultivated in a chemically defined (Modified Catlin 6; MC-6), and a complex medium (Frantz') to examine the effect of growth media on antigen expression and immunogenicity of extracted outer membrane vesicles (OMVs). The MC-6 vaccine was also compared with a corresponding vaccine from a recently isolated B:15:P1.7,16; ET-5 strain to look for changes in immunogenicity over time.
Materials and Methods: Bacteria were grown until stationary phase in a 2.5 litre fermentor and OMVs isolated by deoxycholate extraction. In total, we performed four cultivations in Frantz' and three cultivations in MC-6 medium. Based on antigen expression in SDS-PAGE, three of the four Frantz' batches and all three MC-6 batches were pooled into two vaccines, sterile-filtered and adsorbed to aluminium hydroxide. Doses of 0.5 and 2 μg OMVs were injected into mice (NMRI) on day 0 and 21 and sera collected two weeks after the last dose. Immune responses were examined with immunoblots, serum bactericidal assay (SBA) and ELISA. Fifteen patient isolates from year 2000, belonging to the ET-5 complex, were characterised with reference mouse monoclonals and human sera. From these results, OMVs were prepared from one strain (N24/00) with antigenic pattern similar to 44/76.
Results and Discussion: For the 44/76 OMV vaccines, PorA, PorB and class 4 proteins were equally expressed in both growth media. The MC-6 medium, however, induced increased levels of LPS and Opc as well as expression of two additional proteins with mol. wt. of 80 kDa and 100 kDa. The 100 kDa protein was identified as TonB-dependent protein H (TdfH). Both 44/76 vaccines induced similar total and specific IgG levels to the major outer membrane proteins and a significant dose-response in ELISA, SBA and immunoblotting. However, SBA titres were significantly higher in mice immunised with the MC-6 OMV vaccine. Six of the B:15:P1.7,16; ET-5 strains from year 2000 were used as target strains in SBA. They showed different titres with the human reference sera, indicating distinct antigen expression. The OMV vaccine from strain N24/00, grown in MC-6 medium, demonstrated reduced expression of Opc but increased expression of TdfH compared to 44/76. Results from immunogenicity studies in mice with this vaccine will be presented.
Conclusion: In comparison with Frantz' medium, cultivation of strain 44/76 in MC-6 demonstrated expression of TdfH and of an unknown protein with mol. wt. of 80 kDa, as well as increased levels of Opc and LPS. The two OMV vaccines induced similar total and specific IgG antibody levels in mice, but SBA titres were higher with the MC-6 vaccine.

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Cross-reacting and cross-protective antibodies against *Neisseria meningitidis* and *Neisseria lactamica* in human postvaccination and patient sera

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Introduction: The aim of the study was to compare antibody responses in patients infected with B:15:P1.7,16 strains and vaccinees who received a B:15:P1.7,16 outer membrane vesicle (OMV) vaccine. Cross-reacting antibodies in the postvaccination and convalescent sera were determined by immunoblotting with OMVs from three *Neisseria meningitidis* strains that represented major epidemic clones and one *N. lactamica* strain. The presence of potential cross-protective antibodies was tested in a serum bactericidal assay (SBA) and a mouse infection model.
Materials and Methods: Postvaccination sera were drawn 6 weeks after the third dose from 10 volunteers who received the Norwegian 44/76 (B:15:P1.7,16) OMV vaccine. Convalescent sera from 10 patients with B:15:P1.7,16 disease and 2 patients with C:15:P1.7,16 disease were collected 12 - 40 days after onset of disease. Sera were blotted against OMVs from the vaccine strain 44/76 (ET-5; ST32), C:2a:P1.5,2 (ET-37; ST11), B:4:P1.7-2,4 (lineage III; ST41) and Y92 1009 (*N. lactamica*). All sera were analysed in SBA with the ST32, ST11 and ST41 strains as targets. The passive protection afforded by pooled postvaccination and convalescent sera was assessed using a mouse intraperitoneal infection model. Following passive immunisation, mice were challenged with the same meningococcal strains as used for blotting except that the ST11 strain was exchanged with a corresponding group B strain.
Results and Discussion: The immunoblot patterns of the 12 convalescent sera varied, with IgG antibodies against different minor antigens in the four OMV preparations. We could not demonstrate common specific cross-reacting antigens. Corresponding experiments with the postvaccination sera will be presented. In SBA, the group C ST11 strain proved serosensitive with all sera. Postvaccination sera showed high geometric mean titres in SBA (GMT = 8.9) against the homologous ST32 strain but low titres (GMT = 1.3) against the ST41 strain. In contrast, patient sera demonstrated high bactericidal titres against both the ST32 (GMT = 6.9) and ST41 (GMT = 5.0) strains. In the mouse model, the pooled patient sera afforded 100% protection against ST32 as well as the ST11 and ST41 strains. Postvaccination sera were less protective, with the highest level of protection against challenge with the ST32 strain.
Conclusion: Convalescent sera from patients infected with 15:P1.7,16 (ST32) strains did not reveal common cross-reacting antigens in the four *Neisseria* strains on immunoblots. However, a high level of functional cross-reacting antibodies was found in SBA against both the ST32 and ST41 strains. In the mouse passive protection model, pooled convalescent sera demonstrated 100% protection against the ST32, ST11 and ST41 strains. Sera from vaccinees, receiving a B:15:P1.7,16 vaccine, only showed high SBA titres against the homologous ST32 strain. Such sera provided 100% protection against this strain in the mouse model but did not protect against the ST41 strain in agreement with the SBA results.

Expression of a recombinant bacterial luciferase in *Neisseria meningitidis* and investigation into its use as a viability marker

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Objective: We investigated whether artificially bioluminescent meningococcal strains could be used to provide a simple and rapid alternative to counting colony forming units (cfu) for the measurement of bacterial viability.
Background: Meningococcal killing assays such as the serum bactericidal assay and the whole blood assay measure bacterial viability by counting cfu after overnight growth. This can be a laborious process and introduces a delay into the conclusion of the experiment. We proposed that incorporating bioluminescent versions of meningococcal strains into such an assay would simplify the assay by allowing instantaneous measurement of bacterial killing via the light emitted from each sample. The *luxA* and *luxB* genes from *Photorhabdus luminescens* were selected for expression in *N. meningitidis*. These genes encode a bacterial luciferase enzyme, which catalyses the oxidation of long-chain fatty aldehyde substrates, resulting in the emission of a blue-green light. This reaction requires other cofactors present only in live bacteria and so should function as a viability reporter.
Methods: A vector was constructed using the *E. coli-N. meningitidis* shuttle plasmid pMIDG100 (derived from pCGLS1), in which expression of the *luxA* and *luxB* genes was driven by the strong constitutively active *ner* promoter. Subsequent bioluminescence produced by bacteria was measured in 1ml samples over 20 seconds following the addition of decanal (using a Labsystems Luminoscan TL Plus luminometer).
Results: When introduced into a number of *N. meningitidis* strains, the expression vector was stable and conferred the bioluminescent phenotype. When grown in Mueller-Hinton broth, bacteria generated in the order of 1 relative light unit (RLU) per cfu. RLU readings were proportional to cfu counts for samples containing between 10^2 and 10^9 cfu. (100 cfu was the lower limit of detection.) During an *in vitro* growth curve, changes in RLU correlated well with cfu during the exponential growth phase of growth ($R^2 > 0.9$). Expressing the luciferase enzyme had no noticeable affect on the growth kinetics.

In order to study the application of bioluminescent meningococci to a bactericidal assay, RLU were compared to cfu in a whole blood killing assay[1] using an inoculum size of 10^7 cfu/ml. In blood which showed no bactericidal activity, changes in RLU closely followed those of cfu, after an initial lag period. In blood which did exhibit bactericidal activity, RLU decreased as cfu decreased, but not proportionally in all cases. Sometimes RLU decreased much more slowly than cfu. We hypothesise that this is because damaged bacteria may not survive to produce colonies, but could still possess luciferase activity during the experiment.
Conclusion: Bioluminescent meningococcal reporter strains can be used to measure bacterial viability, but assay conditions will need to be adapted to enable comparison with existing bactericidal assays.

Reference

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O-acetylation status of the capsular polysaccharides of group Y and W135 meningococci isolated in England and Wales

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Introduction: Tetravalent conjugate vaccines for meningococcal groups A/C/Y/W135 are being formulated but the *O*-acetylation status of all their capsular polysaccharides have not been studied to date. *O*-acetylation status is known for group C, but not for groups Y and W135.
Methods: W135 (n=181) and Y (n=90) case isolates submitted to the PHLS MRU in 1996, 2000 and 2001 were investigated for *O*-acetylation capsular status by dot blot assay using monoclonal antibodies (mAbs) MnW78-59, MnW1-3, MnY38-49 and MnY4-1. De-*O*-acetylated (*O*ac-) W135 and Y isolates react with MnW1-3 and MnY4-1 respectively while *O*-acetylated (*O*ac+) isolates react with both mAbs directed against their respective serogroups. Sera were collected from healthy adults (n = 24) of a single dose of tetravalent polysaccharide vaccine. Serum bactericidal assay (SBA) titres were measured using *O*ac+ and *O*ac- W135 and Y strains. Isolates used were *O*ac+ W135 (NT: P1.18-1, P1.3)[M, 01.0240070], *O*ac+ Y (NT: P1.5-1, P1.10-22)[M, 01.0240539], *O*ac- W135 (NT: P1.18-1, P1.3)[M, 00.0242317] and *O*ac- Y (2a: P1.5, P1.2) [M, 00.0242975].
Results: The proportion of *O*ac+ isolates was 8% and 80% for groups W135 and Y respectively. The percentages for 1996, 2000 and 2001 were for W135 7%, 0% and 21% and for Y 83%, 90% and 71% respectively. Phenotypically the *O*ac+ W135 isolates were mostly serosubtype P1.3,6 (n=11, 73%). All of the P1.5, 2 isolates were *O*ac-. Both *O*ac+ and *O*ac- group Y isolates were phenotypically diverse. Pre and post immunisation sera show good SBA responses against all W135 and Y isolates used. Geometric mean titres (GMT) and 95% CIs were for W135 *O*ac+ 198, 75-516; *O*ac- 310, 137-699 and for Y *O*ac+ 535, 259-1104; *O*ac- 351 151-817.
Discussion: The phenotypic similarity of *O*-acetylated W135 isolates suggests that these may be clonally related and capsular *O*-acetylation status may be stable within clones.

Previous studies have demonstrated that tetravalent polysaccharide vaccines are immunogenic in children and adults but the *O*-acetylation status of the W135 and Y strains was not reported. We have shown that both *O*ac+ and *O*ac-W135 and Y isolates occur naturally in the UK with a predominance of *O*ac- W135 and *O*-ac+ Y strains. The exact biological relevance of *O*-acetylation status is as yet unclear but this information is useful at a time with a view to the conjugate vaccines being formulated to prevent disease due to serogroups A/C/W135/Y.
Conclusions: The minority of W135 (8%) and majority of Y (80%) isolates in the UK are *O*ac+. The tetravalent polysaccharide vaccine used in healthy adults evoked serum bactericidal activity against both *O*ac+ and *O*ac- W135 and Y isolates.

Avidity maturation following vaccination with a meningococcal recombinant hexavalent PorA outer membrane vesicle vaccine in UK infants

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Introduction: Serogroup B *Neisseria meningitidis* is the major cause of invasive meningococcal infections in most developed countries. The serogroup B polysaccharide capsule is poorly immunogenic, therefore subcapsular antigens are being considered for use in these vaccines. A recombinant hexavalent PorA outer membrane vesicle (OMV) vaccine containing six PorA outer membrane proteins was trialled in the UK. Serum bactericidal activity (SBA) titres ≥ 4 against all six isogenic strains present in the vaccine after four doses but against only two strains following three doses. As peak incidence of disease in the UK occurs by 6 months of age it was necessary to investigate evidence for immune priming. Measurement of IgG avidity indices was undertaken to investigate the maturation of antibody avidity against a single PorA (P1.7, 16).
Methods: Subjects received doses of the PorA OMV vaccine at 2, 3, 4 and 12 - 18 months of age. Controls received one dose at 12 – 18 months of age. OMVs were purified from strain H44/76 (B:15: P1.7,16). IgG avidity was measured by an elution ELISA using isothiocyanate. Sera were diluted to yield an optical density between 0.8 and 1.2, then incubated on antigen-coated plates for 2 hours at +37°C. Thiocyanate (2M – 18M) was added and the plates incubated at room temperature for 15 minutes. Antibody was detected using peroxidase-conjugated anti-human IgG and results were expressed as the log percentage reduction of absorbance in the presence of thiocyanate and plotted against thiocyanate concentration.
Results: The proportion of infants with SBA levels ≥4 increased following each of the first three doses. There was a significant increase following the fourth dose. ($p<0.001$). The geometric mean titre for the group post fourth dose was 28.7%. Between one and six months post third dose, a significant increase in geometric mean avidity indices (GMAI) was seen. This increased again following the fourth dose. The GMAI was significantly higher than the controls both pre and post booster vaccination.
Discussion: This is the first report to assess the use of avidity indices as a surrogate marker for inducing immunological memory following meningococcal OMV vaccination. After three doses, SBA GMT titres declined and GMAI had increased. These differences were significant. The increase in antibody avidity following the third dose implies immunological memory against strain H44/76. Antibody avidity indices are useful laboratory markers for the priming of immunological memory following vaccination with meningococcal serogroup B vaccines.
Conclusion: We demonstrated antibody avidity maturation between third and fourth doses of the PorA OMV vaccine. Further phase 2 trials are now needed to elucidate whether a fourth dose given between 5 and 12 months of age elicits protection against all components of the vaccine.

Reduced immunogenicity of neisserial porin A can be overcome by specific priming

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Introduction: The RIVM has developed a hexavalent outer membrane vesicle (OMV) vaccine (HexaMen) against meningococcal disease caused by *Neisseria meningitidis* serogroup B, containing six of the most common class I outer membrane proteins (PorA) in the Netherlands. In clinical studies and mouse models, this vaccine has been proven to be effective, although two of the six PorA serosubtypes (7-2,4 and 19,15) are less immunogenic. Since 7-2,4 is the most common serosubtype, accounting for up to half of the meningococcal serogroup B infections in recent years, it is important to improve the efficacy of the 7-2,4 response.
Material and Methods: Balb/c mice were immunised subcutaneously using 1/10 human dose of HexaMen and/or monovalent (7-2,4) OMV vaccine. IgG titres were measured in ELISA and in a bactericidal assay.
Results: When immunised with 7-2,4 alone or in combination with one or two other PorAs (5-2,10 and 12,13), 7-2,4 IgG titres are sufficient, even when the heterologous PorA is given in abundance (8x amount of 7-2,4). If mice are immunised with all six PorA's (HexaMen or 6x monovalent), IgG titres against 7-2,4 and 19,15 are significantly decreased as compared to the other PorA's. This weaker immunogenicity can be overcome in several ways: 1. by adding additional 7-2,4 to HexaMen; 2. by spreading the immune response over different draining lymph nodes by injecting 7-2,4 left and HexaMen right; 3. by specific priming with 7-2,4 and boosting with HexaMen.
Discussion and Conclusion: For broad coverage and applicability of HexaMen, it is important that all serosubtypes raise a sufficient IgG titre. Although it is not known what is the correlate of protection (mice and human), the decrease in immunogenicity of two of the PorA's should be overcome to guarantee a broad effective coverage of the vaccine.

These data indicate that there is competition when several highly (±90%) similar PorAs are given simultaneously, resulting in lower IgG levels against the weaker immunogenic PorAs. It is yet unclear at which level this competition occurs and what the role of APCs, B and T cells is. Although it is unknown which cells are involved in the competition, we here show that it is possible to circumvent this problem and raise sufficient IgG titres against all six PorA's. Further investigation will be needed to elucidate the role of the different cell types and to further improve the vaccine.

Selection of phage-displayed mimotope of *Neisseria meningitidis* serogroup B capsular polysaccharide

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Serum antibodies against capsular polysaccharide (CPS) from *Neisseria meningitidis* confer protection against disease. However, the immunogenicity of meningococcal group B CPS is poor. Further, a portion of the antibody elicited has autoantibody activity due to the similarity of B CPS with human polysialic acid. The bactericidal and protective monoclonal antibody (mAb) 13D9, raised against N-propionylated B CPS, reacts specifically with B CPS, but not with human polysialic acid (J Exp Med 185: 1929-38). This mAb was used to select mimotopes of B CPS from a phage-displayed peptide library. After four rounds of panning, a phage-displayed peptide able to bind mAb 13D9 was selected. The peptide competed with purified B CPS for mAb 13D9 binding. A linear peptide and a multi-antigen peptide containing four copies of the selected peptide were synthesized and conjugated to a carrier protein using different conjugation methods. Conjugated and unconjugated antigens were used to immunize BALB/c mice. High levels of specific anti-peptide antibodies were elicited after immunization with almost all immunogens, but the purified B CPS was poorly recognized by the murine sera and only low bactericidal activity was detected in the sera of few animals. At present, experiments are conducted to modify the peptide in order to increase its ability to generate protective immune response against *N. meningitidis* serogroup B.

Memory response generated in African Green Monkeys against polysaccharide C conjugate

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Objective: To study the memory response generated after immunization of African Green Monkeys (AGM) with 5μg of polysaccharide conjugated with the carrier protein P64k. To evaluate the correlation among the different ELISA procedures to measure the antibody titers and the functional activity of the antibodies generated.
Design: The polysaccharide conjugate was obtained by the selective periodate oxidation method. Three groups of AGM, three monkeys each, were immunized three times on days 0, 30 and 390. One group was immunized with 5 μg of polysaccharide conjugated to the P64k protein, the second with 25μg of polysaccharide C present in VAMENGOC BC and the other one with 5 μg of plain polysaccharide C. The antibody response was measured by ELISA using poly L Lysine plus PsC, human methylated albumin plus PsC, or modified polysaccharide C as coating antigen. The elicited antibodies were assayed in the bactericidal test using two complement sources. The sera obtained were also assayed in a model for passive protection in infant rats.
Results: For the three groups there was a good antibody response after the second dose with titers ranging from 50 to 550. After the last dose the boosting effect was observed in all groups, but the group immunized with the PsC-P64k conjugate showed the highest increment, generating a mean titer of 1800. When the sera obtained were assayed for the functional activity, the group immunized with the PsC-P64k conjugate showed the best results with bactericidal mean titer of 2517 for the human complement and mean titer of 4413 for the rabbit complement. Besides the sera of this group showed a high protective capacity when assayed in the infant rat model. On the other hand, when the three different ELISAs procedures were compared for its correlation with the functional assays, the ELISA using the modified polysaccharide as coating antigen showed the highest correlation (r^2= 0.650)
Conclusions: The PsC-P64k conjugate elicited the highest antibody response and the highest boosting effect when assayed in AGM. The PsC-P64k conjugate was able to generate a bactericidal and protective immune response. The ELISA using the modified polysaccharide as coating antigen showed the best correlation

Comparison of the immune response in mice against meningococcal group C polysaccharides conjugated to three carrier proteins

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Objective: To study the nature and kinetics of the serum antibody response in mice to meningococcal group C polysaccharide (C-Ps) conjugates to three carriers: Bovine serum albumin (BSA), Tetanus toxoid (TT) and recombinant P64k.
Design: Meningococcal group C polysaccharide was conjugated using carbodiimide as coupling reagent and adipic acid (ADH) as spacer between the C-Ps and the carrier. Carrier proteins, conjugates and free C-Ps were employed to immunize Balb/C mice. Aluminum hydroxide was used as adjuvant. The murine humoral immune responses were evaluated by ELISA after the third dose.
Result: The polysaccharide-protein ratios of the three conjugates were: TT-1.26, BSA-1.13 and P64k-0.4 mg/mL. The immune response against the three C-Ps conjugates was higher than against free C-Ps.

The levels of antibodies detected in the sera of mice immunized with C-Ps-P64k conjugate were higher than those detected against C-Ps-TT and C-Ps-BSA conjugates.
Conclusions: The carrier protein present in the C-Ps conjugates influenced the levels of the IgG antibodies elicited against to the Ps. In this regard P64k was superior to TT and BSA.

Primary and booster antibody response to *Neisseria meningitidis* B in vaccinated mice

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Neisseria meningitidis B is an important cause of bacterial meningitis worldwide. The Cuban produced serogroup B meningococcal vaccine is the only one available in Brazil. This vaccine is based on outer membrane proteins of a B:4:P1.19,15 meningococcal strain. Immunity to *N. meningitidis* has been associated with development of antibodies able to activate the complement system and induce bacterial lysis (bactericidal antibodies).

The goals of this study were (i), to evaluate the kinetics and duration of antibody response to *N. meningitidis* B:4:P1.15 induced by different vaccinating schedules (2, 3 or 4 doses of the Cuban vaccine) by quantification of IgG and bactericidal antibodies in Swiss mice (ii), to analyze the effect of a booster dose of vaccine given 7 months after the primary immunization schedules (iii), to investigate the IgG subclasses and IgG avidity index induced by vaccine.

The results showed that a significant antibody response (IgG levels and bactericidal activity) was induced by 2 doses of vaccine but not 1 dose. Four doses of vaccine induced a significantly higher antibody response than 2 doses**.** The duration of antibodies was longer in mice immunized with 4 doses compared with 2 or 3 doses of vaccine. After the booster dose of vaccine, the antibody levels were similar to the ones detected after the primary immunization schedules.

IgG1 and IgG2b were the main subclasses induced by vaccination. The IgG avidity index was significantly higher after 2 and 3 doses of vaccine and increased continually during 7 months following the primary immunization.

Taken together the results of antibody studies in the animal model used in this study showed that the vaccine induced persistent immunological memory. The duration of antibody response was associated with the number of doses of vaccine administered. However, immunological memory was not affected by the number of doses received during the primary immunization schedule. Bactericidal antibodies and the IgG avidity index were the serological parameters that more adequately indicated the immunogenic potential of the vaccine.

Complement regulatory proteins attenuate the functional effect of antibody elicited by a gonococcal vaccine candidate

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We have identified a conserved carbohydrate epitope on gonococcal lipooligosaccharide (LOS) that is recognized by a monoclonal antibody [mAb] 2C7. This structure is present in 95% of clinical gonococcal isolates [1] and may represent a potential candidate for an anti-gonococcal vaccine. In humans, the 2C7 epitope elicits a significant immune response after natural infection. Antibodies against this epitope mediate both complement-dependent bacterial killing and opsonophagocytosis. Because oligosaccharides are poor immunogens that usually result in a T-cell independent response, we developed a peptide mimic as a surrogate of the 2C7 epitope using a random peptide display library. Upon immunization, this peptide elicited cross-reactive anti-LOS antibodies in mice. We examined bactericidal activity of immune mouse sera against 2C7-positive gonococcal strains 15253 (highly resistant to nonimmune normal human serum [NHS]; binds factor H and C4b-binding protein [C4bp]) and F62Δ*lgtAlgtG*$^+$ (sensitive to NHS; weak factor H binder and non C4bp binder). Strains 15253 and F62Δ*lgtAlgtG*$^+$ bound similar amounts of mAb 2C7.

An inverse correlation between level of IgG anti-LOS versus percent survival was demonstrated for 2C7 epitope bearing gonococci. Fifty percent killing by immune mouse sera of strains 15253 and F62Δ*lgtAlgtG*$^+$ was attained with 1.53 and 0.62 μg/ml of specific IgG anti-LOS antibody, respectively. Similarly, 2.6-fold less mAb 2C7 was required to kill F62Δ*lgtAlgtG*$^+$, compared to strain 15253. These data suggest that IgG anti-LOS antibody elicited by peptide immunization possessed complement mediated bactericidal activity against gonococcal strains that express the 2C7 epitope. The vaccine candidate can kill even serum resistant gonococci. Higher titers of antibody, however, are necessary, to kill serum resistant strains because they can attenuate complement activation by virtue of their C4bp and factor H binding ability.

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Peptide mimic elicits bactericidal antibody response against an oligosaccharide epitope of *Neisseria gonorrhoeae*

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Gonorrhea, a sexually transmitted disease, is a major public health problem worldwide; the development of an effective vaccine might serve to prevent the serious sequelae of gonococcal infection while also controlling transmission of HIV in persons who are coinfected with HIV and *Neisseria gonorrhoeae*. We have identified a carbohydrate epitope (called the 2C7 oligosaccharide [OS] epitope, defined by reactivity with monoclonal antibody [mAb] 2C7) on gonococcal lipooligosacchaide (LOS), which is present in 95% of gonococcal strains as they exist *in vivo* [1]. This structure may represent a potential candidate for an anti-gonococcal vaccine. In humans, the 2C7 OS epitope elicits a significant antibody response that mediates both killing and opsonophagocytosis either after natural infection (4.4-17-fold increase in IgG antibody) or following vaccination with gonococcal outer membrane preparations that contain the antigen (44.5-fold increase in IgG antibody). Because oligosaccharides are poor immunogens usually resulting in a T-cell independent response, we approached the design of a vaccine candidate by developing peptides that mimic the 2C7 epitope, and which we believed might elicit a T-cell dependent response when used as an immunogen.

Using a random peptide library expressed by *E. coli* flagella, we identified a consensus sequence that bound mAb 2C7. A multiple antigen peptide (MAP) containing this consensus sequence was constructed and it was shown to inhibit binding of mAb 2C7 to LOS in a dose-responsive manner, indicating the sharing of antigenic determinants with LOS.

To investigate the immunogenicity of this peptide, we immunized 30 mice with two doses of MAP (50 μg). Twelve of the 30 mice (40%) showed an IgG anti-LOS antibody responses above baseline. The mean IgG anti-LOS antibody concentration in responder mice was almost 10-fold greater (6.8 ± 3.3 μg/ml) than in the negative control group (0.717 ± 0.026 μg/ml) or in the non-responder mice (0.725 ± 0.026 μg/ml). IgG anti-LOS antibody elicited by MAP immunization possessed direct complement dependent bactericidal activity against numerous gonococcal strains that express the 2C7 epitope, even those that resist killing because of their ability to bind complement (down)regulatory proteins. These data suggest that a peptide can act as a molecular mimic of a carbohydrate epitope and may form the basis for the development of a vaccine candidate(s) for human immunization against *N. gonorrhoeae*.

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1. Gulati S *et al.* (1996) *J Infect Dis* 174: 1223-37.

Intranasal immunization with meningococcal outer membrane protein induces a protective immune response in mice

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Introduction: The mucosal surfaces of the respiratory tract represent one of the main portals for the entry of many human pathogens. Meningococci induce disease only after first colonizing the nasopharynx. Induction of an immunologic response at the mucosal surface is an attractive approach to interdict infection (1). The direct immunization by this route may induce local mucosal as well as systemic immune response. The OMVs from group B meningococci have been shown to be immunogenic in mice, monkey and human when give intranasally (2). In the present study we have focused in the dose-reduction of OMVs as immunogen by intranasal route and we have shown that immunization by this route can induce a protective response.

Materials and Methods:

Immunization: In order to study antibody response to OMVs, groups of ten mice were immunized three times at two weeks intervals with 1, 2.5, 5, 10, 50, and 100µg of OMVs i.n. A control group was immunized s.c. with 5µg or 50µg of OMVs, respectively, absorbed onto aluminium hydroxide.

Collection of samples: Fifteen days after the last immunization, mice were bled and lungs were extracted and macerated with 1ml of PBS.

Detection of the immune response: Sera and samples from washed lungs were analyzed by ELISA. Bactericidal assays (3) were performed against B:4:P1.19,15 strain using pooled sera from individual mice previously positive by ELISA. The protective activity of pooled sera was evaluated *in vivo* in the infant rat model (4).

Results and Discussion: After three doses of OMVs vaccine, significant IgG antibody responses, measured by ELISA, were reached in serum for all groups. The concentration of IgG antibody was significantly augmented when the quantity of OMVs was increased up to 100 µg. For concentrations of OMVs lower than 10µg, we did not detect IgA antibody in serum, while very low levels were detected in washed lungs. Although mice immunized with 10µg of OMVs exhibited IgA and IgG antibody responses in serum and lung lavages; 50µg was the minimal dose required to elicit a functional antibody response, in terms of bactericidal activity and protective ability in infant rats, tested against the Cuban strain CU385 (B:4: P1.19,15).

Conclusions: The nasal immunization with OMVs is an effective approach to induce systemic and local immunity against the group B meningococcus, and doses as lower as 50µg or 100µg should be explored in the preclinical testing of formulations intended for human use.

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Development of an outer membrane vesicle vaccine against serogroup A meningococcal disease

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Meningococcal disease caused by serogroup A meningococci is a significant health problem in many countries, in particular in those situated in the area traditionally known as the meningitis belt of Africa. There is an urgent need for a serogroup A vaccine capable of inducing long-term immunological memory in all age groups. WHO and GAVI (Global Alliance for Vaccine and Immunisation) have now intensified the work on development of new polysaccharide (PS) conjugate vaccines, focusing on serogroup A disease. Whereas the serogroup C conjugate vaccines have proved safe and efficacious against group C disease, and are now used for large-scale population immunisation, the development of a serogroup A conjugate vaccine is still in progress. The possibility of developing vaccines against serogroup A meningococcal disease, based on other principles than the PS conjugate vaccine, should also be explored.

One approach is to use proteins of outer membranes from the meningococci as vaccine. NIPH has extensive experiences in this area and has established general methods for GMP production of outer membrane vesicle (OMV) vaccines from serogroup B meningococci. The Norwegian serogroup B OMV vaccine has been shown to be safe and efficacious , and it is highly immunogenic in all age groups. The production method for an OMV vaccine is relatively simple, compared to conjugation. In order to explore the possibility of producing an affordable meningococcal vaccine for Africa, we have therefore started research and pilot production on a serogroup A OMV vaccine, using the same technology as for the serogroup B vaccine. In addition, a non-covalent (1:1) complex of serogroup A OMVs and serogroup A PS was produced.

A panel of serogroup A strains representative of the epidemics from various African countries since 1988 has been collected and thoroughly characterised. An important feature of the recent epidemics of serogroup A disease in Africa was that all the bacteria belonged to the same clonal group (subgroup III) and showed nearly no variation in their antigenic properties, e.g. they all expressed the same PorA (serosubtype P1.9,20) and PorB (serotype 4/21) major outer membrane proteins. A strain from Mali was selected as a production strain for an experimental OMV vaccine. Characterisation of the serogroup A OMV vaccine showed quite similar protein patterns and lipopolysaccharide content as the group B OMV vaccine. Preliminary results in mice showed that the A-OMV vaccine induced high levels of bactericidal antibodies against three serogroup A strains from different African countries. These antibodies were mainly directed against the PorA outer membrane protein. Compared to the pure serogroup A-PS, a non-covalent complex of serogroup A OMV and serogroup A-PS strongly enhanced the immune response to the serogroup A-PS. Thus, an OMV vaccine might be an alternative to the conjugate vaccine principle, that can be included in the routine vaccination program in Africa at an acceptable cost.

Pathogenicity island a new target for gonococcal vaccine

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Introduction: The hypothesis that genomic island, which was recently discovered, has characteristics of the pathogenicity island (PAI) was tested. A laboratory strain F62 was transformed with SC-4 cosmid representing PAI in pelvic inflammatory disease (PID) strain.
Materials and Methods: To investigate our hypothesis, we sequenced and analyzed SC-4. Western blot analysis of purified membranes were used for identification of novel gene product. Animal model for gonococcal infections was used to evaluate preventive effect of antibodies against new membrane protein. PCR and RT-PCR was used for DNA and RNA detection, respectively. Alanine mutagenesis and stop codon mutation was used. *In vivo* transformation of PAI was performed. Transformants and mutants were evaluated in bactericidal assay.
Results: The transformant became both virulent to the rat pups and resistant to human serum. This data suggests that SC-4 of PID isolate harbors a virulence-encoding horizontally transferable variant of the genomic island, a property differentiating PAIs from genomic islands. Antibodies against novel membrane protein coded by PAI gene, significantly reduced the number of gonococcal colony forming units isolated from animals.
Conclusion: Possible contribution of novel protein, the cryptic open reading frames, type IV secretion system and long tandem repeats of PAI to gonococcal virulence is discussed. This investigation provides new strategies for understanding and prevention of gonococcal disease.

Immunogenicity of a combination of two different outer membrane protein based meningococcal group B vaccines

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Introduction: We wanted to investigate the immunogenicity of two group B outer membrane vesicle (OMV) vaccines given in combination in comparison to the immunogenicity of the vaccines given alone.

The vaccines consist of partially purified outer membrane proteins from strains 44/76, B:15:P1.7,16 (MenBvac) and NZ98/254, B:4:P1.7b,4 (NZ MenB), respectively, presented as proteoliposomic vesicles adsorbed to aluminum hydroxide. Both vaccines were developed and produced at NIPH. The MenBvac vaccine has been extensively tested in clinical trials and has been found to elicit strain-specific immunity and is efficacious in preventing group B meningococcal disease in adolescents in Norway. The NZ MenB vaccine is 'tailor-made' for the New Zealand epidemic and is being tested in an on-going clinical trial in New Zealand. The combination of these two vaccines cover the most prevalent serotype B strains circulating in Europe today.

Materials and Methods: Groups of outbred Bom:NMRI mice (n=12) were assigned to receive either three doses (s.c., 6 weeks apart) of MenBvac (0.5 or 2 mcg), NZ MenB (0.5 or 2 mcg) or a combination of the two (0.5 + 0.5 mcg or 2 + 2 mcg) mixed immediately before administration. Six control mice received no injection. IgG antibodies in serum were analyzed by ELISA with OMVs from the vaccine strains as antigen.

Results and Discussion:

IgG response against 44/76 OMV

After two doses, there was no significant difference in the IgG response between the group receiving the combined vaccine and the group receiving the corresponding dose of MenBvac alone. Interestingly, after 3 doses with vaccine there was a 3-fold increase in specific IgG antibodies in the group receiving the combined vaccine with 2 mcg + 2 mcg compared to the mice receiving 2 mcg MenBvac. A small IgG response was observed for the mice immunized with 2 mcg NZ MenB vaccine indicating that cross-reactive antibodies had been induced.

IgG response against NZ98/254 OMV

There was no significant difference between the groups receiving the combined vaccine and the groups receiving the corresponding dose of NZ MenB vaccine alone after 2 and 3 doses with vaccine. A small IgG response against the heterologous strain was observed for the mice immunized with 2 mcg MenBvac indicating cross-reactive antibodies.

Conclusion: The combination of the two outer membrane protein vaccines induced IgG antibodies against OMVs from both strains. The magnitude of the response was at least the amount of IgG antibodies induced by the single vaccines. The combination actually showed a 3-fold increase in IgG antibodies compared to the serotype 15 vaccine (MenBvac) given alone. This suggests a beneficial effect of combining different group B outer membrane protein vaccines in addition to the advantage of a vaccine that will cover different serotype B strains.

Meningococcal NspA expressed in commensal *Neisseria* – a vaccine paradigm

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Introduction: Infections caused by several clinically important capsular serogroups of *N. meningiditis* can be prevented with polysaccharide vaccines, but serogroup B strains, with capsular polysaccharide that is not immunogenic in humans, remain a serious problem. No single subcapsular antigen has been found that elicits an adequate, cross-protective, antibody response, but preparations of meningococcal outer membranes containing multiple antigens hold some promise. Several neisserial species (in particular *N. lactamica*) are notable for their significant carriage rate in humans, and antigen exposure in the course of such carriage has been proposed as the basis for the natural bactericidal activity against group B strains that develops during childhood[1]. These organisms, like meningococci, bleb to produce outer membrane vesicles (OMVs), and preparations of these OMVs therefore hold promise as cross-protective vaccines. We are seeking to enhance this potential by the strategic incorporation of meningococcal outer membrane proteins.

Results and Discussion: We examined different commensal *Neisseria* for their ability to take up the conjugative shuttle plasmid pMIDG100[2]. Attempts to transfer the plasmid into 52 diverse *N. lactamica* strains were unsuccessful, but 4/4 *N. cinerea* and 2/4 *N. flavescens* – as well as other commensals – were transformable. pMIDG100 was modified to allow the expression of antigens with vaccine potential under the control of a high level meningococcal promoter, and the gene encoding the vaccine candidate NspA[3] was inserted. Expression of NspA at high levels in different commensal *Neisseria* was demonstrated in an initial dot blot analysis. The localization and surface exposure of NspA in intact bacterial cells was studied using a panel of well-characterised monoclonal antibodies. NspA was demonstrated in apparently native conformation at the surface of recombinant strains of *N. flavescens* and *N. cinerea* by a flow cytometric assay and by immunogold electron microscopy (IEM). OMVs were isolated from these strains, and the presence of NspA again demonstrated by western blotting and by IEM. These recombinant OMVs containing NspA have been used to immunise mice and immune responses are currently being evaluated. This new approach to meningococcal vaccines allows expression of neisserial antigens in a neisserial background with the potential for correct conformation, and use of a non-pathogen has great advantages for large-scale production.

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How to harness the political will and implement an OMV vaccine solution to combat a devastating epidemic

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New Zealand is embarking on an ambitious programme to introduce a strain-specific group B meningococcal vaccine in the context of extraordinary levels of meningococcal disease.

New Zealand is in the 12th year of a large and widespread epidemic of group B meningococcal disease (2001: 17.4/100,000) increasingly dominated by a single subtype (P1.7b,4) causing 82 percent of all disease regardless of serogroup in 2001. Overall, New Zealand children under 5 have a 1 in 330 risk of contracting this disease.

It is expected that without a vaccine intervention this outbreak will continue for at least another ten years with potentially 4000 more cases, 200 deaths and perhaps 600 permanently disabled in some way mostly in the under 20 year olds.

It has been estimated that in societal costs the meningococcal epidemic to date has cost $500 million NZ dollars. Direct costs to the health sector have been estimated at $220 million NZ dollars.

A 'tailor-made' vaccine approach ie fitting the vaccine strain to match the outbreak strain has been recognised internationally and nationally as the correct approach, because of the highly clonal serogroup B outbreak in New Zealand.

The Government has committed significant funding to a mass vaccination campaign targeting all under 20 year olds in New Zealand. A cost benefit analysis provided justification for this intervention. A contract has now been signed with Chiron Corporation to supply for clinical trials and a mass vaccination campaign a New Zealand epidemic strain-specific meningococcal vaccine. Chiron Vaccines is working with Norway's National Institute of Public Health (NIPH).

New Zealand's approach has been to form a consortium of the Ministry of Health, Chiron Vaccines and a research team lead from the University of Auckland, to manage the clinical trials and roll out to all under 20 year olds.

Assessment of consistency and process reproducibility of the Cuban vaccine VA-MENGOC-BC by 2D PAGE and mass spectrophotometry

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Neisseria meningitidis (Nm) is the causative agent of epidemic bacterial meningitis, with serogroups A, B and C being responsible for 90% of the cases of meningococcal disease worldwide[1]. A tetravalent polysaccharide vaccine is available against serogroups A, C, Y and W135 [2], but an effective polysaccharide vaccine against all the organisms belonging to serogroup B is still lacking because of the poor immunogenicity of the group B capsular polysaccharide[3]. During the last decade some outer membrane protein (OMP)-based vaccines against serogroup B have been tested in humans. Cuban vaccine VA-MENGOC-BC is one of this vaccines, and so far is the only group B vaccine commercially available[4].

In this study the global protein content in the Cuban Vaccine VA-MENGOC-BC was investigated using current proteomics technology. Based on silver stained two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), up to 700 protein spots were detectable within the range of the immobilized pH gradient (IPG) system used and major protein components were identified by mass spectrometry.

Using this methodology, the consistency and reproducibility of the VA-MENGOC-BC vaccine were proved by studying four different production batches.

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Mouse monoclonal antibodies against outer membrane proteins of the Cuban vaccine strain of *Neisseria meningitidis* B:4:P1.19,15

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Introduction: Determining the profile of antigen expression among meningococci is important for epidemiologic surveillance and vaccine development. We report here the generation and characterization of mouse monoclonal antibodies against several relevant vacunal antigens of the Cu-385-83 strain**.**
Materials and Methods: BALB/c mice were immunized with outer membrane vesicle (OMV) protein and recombinant antigens TbpB and 5c, all from the CU-385-83 strain of *Neisseria meningitidis*. Western blot and whole cell ELISA, using strains with different serotype and subtype of *Neisseria meningitidis,* characterized the monoclonal antibodies that were obtained.
Results and Discussion: Using OMV as coating antigen six positive clones were obtained. Two monoclonal antibodies against PorA and three against PorB3 were obtained from OMV. One monoclonal antibody against the PorA proteins reacted in whole cell ELISA and immunoblotting only with subtype P1.15 strains. Three monoclonal antibodies against the PorB3 proteins reacted in whole cell ELISA and immunoblotting only with serotype 4 strains. One monoclonal antibody reacted strongly only in ELISA with OMV and whole cells with serotype 4 strains but not the immunoblotting assay. This result suggests that the monoclonal antibodies probably recognize a conformational epitope on the PorB3 protein[(1).] Three monoclonals against the 5c protein, three against TbpB and two against P64K were obtained using recombinant antigens. Monoclonal antibodies against the 5c protein were tested against different meningococcal strains and recognized a wide range of pathogenic strains of *Neisseria meningitidis*. All the monoclonal antibodies against the TbpB protein were specific to TbpB (85 kDa) Two antibodies were IgG1 and one IgG2b isotype. In addition, two monoclonal antibodies against the P64K protein were obtained, because the TbpB and 5c are recombinant proteins and were expressed fused to the first 23 and 46 aa from the P64k protein, respectively [(2-3).]
Conclusions: We have obtained and characterized mouse monoclonal antibodies against five proteins (PorA, PorB3, TbpB, 5c and P64K) in the outer membrane of *Neisseria meningitidis*.

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Immunoblot versus ELISA for the primary screening in the development of mouse monoclonal antibodies against outer membrane proteins of *Neisseria meningitidis* B:4:P1.(7b),4

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Introduction: Methods that employ a 96-well (ELISA and solid phase RIA) configuration are clearly the most practical for screening the large number of wells required for monoclonal antibody production. Here we used double-screening methods (immunoblot and ELISA) in the primary screening in order to identify positive clones.
Materials and Methods: The outer membrane vesicles (OMV) from B:4:P1.(7b),4 strain of *Neisseria meningitidis* were used in the immunization of mice and hybridoma supernatants were screened in ELISA and immunoblot. The monoclonal antibodies obtained were characterized by Western blot and whole cell ELISA, using reference strains with different serotype and subtype of *Neisseria meningitidis.*
Result and Discussion: The decision to use immunoblotting and ELISA as screening strategic clone hybridoma production of OMV proteins was based on several factors:
– the antigen preparation for immunization and screening was OMV proteins
– the inaccessible P1.7 subtype epitope from PorA protein is only available after denaturation of the proteins [(1)]
– finds previous used OMV from CU-385-83 strains only obtained monoclonal antibodies against PorA and PorB3 proteins [(2)]
– prior to the cell fusion, sera collected from mice immunized with OMV protein were examined by immunoblotting and recognized PorA, PorB3 and several other antigens.

As illustrated in Table 1, only monoclonal antibodies against to FrpB were obtained with immunoblotting assay as the selection method for primary screening. Monoclonal antibodies against PorA were analyzed in more detail and all reacted in whole cell ELISA and immunoblotting only with subtype P1.4 strains.

Table 1. Specificity of the monoclonal antibodies obtained using immunoblot and ELISA in the primary screening

Specific	Primary screening		Total
	Immunoblotting	ELISA	
FrpB (70 kDa)	6	–	6
PorA (P1.4)	2	3	5
PorB3	6	6	12
No. clones (+)	14	9	23
No. Clones assay	200	350	550

Conclusions: The use of this screening strategy contributed to monoclonal antibodies against FrpB, PorA subtype P1.4 and PorB3 proteins of the outer membrane of *Neisseria meningitidis*. The reason for the inaccessibility of the epitope from FprB in ELISA assay using OMV as coating antigen, is now being studied.

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Identity test of the OMV protein from the Cuba vaccine VA-MENGOC-BC® using monoclonal antibodies

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Introduction: Monoclonal antibodies have had a large impact on biological research due to their high specificity and reproducibility. They are used for the quality control in the vaccine production[1-2] and other biotechnological assays. The inherent specificity of the monoclonal antibodies broadens the utility of the test for identification of the OMV protein in vaccines from Meningococci B. Various authors used monoclonal antibodies to identify proteins in OMV vaccines[3-4].
Materials and Methods: Monoclonal antibodies against PorA, subtype P1.15, PorB3 serotype 4, 5c and TbpB from Finlay Institute [5] were used to identify OMV proteins from Cu-385-83 strains. The proteins content of three OMV batches was identified by Western blot using monoclonal antibodies with different specificity. Control monoclonal antibodies specific for P1.15 (NM3C5C), and serotype 4 (NM14G21) were supplied by Janet Suker and 5c (B306) provided by M. Achtman.
Results and Discussion: The profile antigens of the OMV were evaluated on immunoblot. The immunoreactive bands showed no distinct differences between batches. The results were similar using reference monoclonal antibodies. We have identified four proteins (PorA, PorB, TbpB and 5c) from Cu-385-83 OMV. All these proteins have had a promising approach for *Neisseria menigitidis* B vaccine.
Conclusions: The methods proposed here is useful for performing the identity test of the protein constituent of OMV from *Neisseria meningitidis* strains. At present, multi-component vaccines like this OMV are not fully identified. New work is in progress to identify other antigens present in the meningococcal vaccine VA-MENGOC-BC®.

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The anti *Neisseria meningitidis* B vaccine overcomes the thymus independence of polysaccharide C in toddlers

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VA-MENGOC-BC™ is an outer membrane protein based vaccine of serogroup B of *Neisseria meningitidis*. It also contains the serogroup C polysaccharide (PsC) which is non-covalently incorporated. We recently demonstrated the participation of other mechanisms possibly involved in protection which include, first a characteristic Th1 response induced by this vaccine in young adults and toddlers demonstrated at different levels: functional (presence of delayed-type hypersensitivity, opsonophagocytic and bactericidal activities and not immediate anaphylaxis nor passive cutaneous anaphylaxis); class and subclasses (IgG and IgG_1); molecular (induction of IL2 and γIFN mRNA and not IL4 nor IL5 mRNA); post-transcriptional (production of IL2 and γIFN and not IL4 nor IL5 in supernatant of re-stimulated peripheral cells); and the biological activity of γIFN produced[1]. Secondly, a typical anti-PsC IgG secondary response was induced in toddlers which demonstrated that this vaccine overcomes the absence of response of the PsC, a thymus independent (TI) antigen. As was expected, the unconjugated polysaccharide vaccine stimulated IgG_2 antibody subclass in agreement with previous reports. The subclass response is changed by VA-MENGOC-BC™ to IgG_4 and IgG_3. Thirdly, the priming induced by PsC did not induced hyporesponsiveness after a booster dose. In conclusion, we showed: the induction of Th1 pattern at different levels, the influence of this pattern over the response of a TI antigen in toddlers and the not induction of hyporesponsiveness by PsC antigens after a booster dose.

Reference

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Vaccine development and production for national domestic needs in developing countries. Experiences from the meningococcal group B vaccine field

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In public health circles the respective roles of public and private institutions in development, production, financing and delivery of pharmaceutical products in developing countries are intensely debated.

A survey of a sample of vaccine development projects, some of whom also known as Public-Private Partnerships, that taken together target a range of neglected diseases shows that a majority of the projects consider to have their vaccines, when developed, produced in countries in which the disease in question is endemic, or in a developing country. One of these projects is the Meningitis A Vaccine Project, a joint project that involves WHO and Program for Appropriate Technology in Health (PATH).

However, available literature paints a very mixed picture of the viability of vaccine development and production for national domestic needs, and poor vaccine quality, weak or absent national regulatory authorities, and unreliable supply are frequently mentioned problems.

The discrepancy between the ambitions of current vaccine development projects and the experiences documented in literature calls for research into successful attempts at vaccine development and production.

In the late 1970s and throughout the 1980s Cuba and Norway suffered from epidemics caused by two different serotypes of group B meningococci, and public national laboratories embarked on development of vaccines based on previous work by Carl Frasch at the FDA. The Cuban vaccine became widely applied, but in Norway the epidemic disappeared before the vaccine was ready for universal application.

In Cuba, one finds today an integrated vaccine industry, with development and production of viral and bacterial vaccines, and vaccines are now said to be a not insignificant export earner. In Norway, on the other hand, in the vaccine manufacturing facility that was created in the wake of the MenB vaccine project a vaccine intended for use in clinical trials in New Zealand has been produced only very recently.

This reasearch project aims at discussing the following questions: Is the Cuban vaccine industry an integrated part of an innovative and vital biomedical sector, or is it a cost-inefficient 'white elephant'? What factors – social, political and economic – contributed to the quite different outcomes of the vaccine development projects in the two countries? What are the conditions for successful transfer of vaccine technologies? How is a public health problem like an epidemic turned into a 'window of opportunity' for establishing a vaccine industry? To what extent are vaccine development and vaccine production interdependent activities? What are the roles of the different levels and agencies of the government, including the drug regulatory authorities? What is the scope for commercialization of technology and products that are developed to solve a 'neglected disease' problem? Or, in short: What are the social and political conditions for vaccine development and production for national domestic needs?

Data collection based on interviews, documentation and literature is taking place in Cuba, USA and Norway.

Development of a lipopolysaccharide based vaccine against invasive *Neisseria meningitidis* serogroup B disease

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Introduction: Our goal is to develop a vaccine to prevent *Neisseria meningitidis* (Nm) group B invasive disease. Our strategy is to use inner core lipopolysaccharide (LPS) as an immunogen to induce high affinity, protective serum antibodies. To this end, we have characterised the reactivity and functional activity of a murine monoclonal antibody (MAb), designated B5 (IgG_3) that binds to an inner core epitope for which phosphoethanolamine linked to the 3-position of heptose II is an essential structural requirement. This epitope is found in 76% of a representative global collection of NmB strains.

Materials and Methods: Western blotting (WB) and passive protection of infant rats were used to characterise a diverse collection of 32 NmB, MAb B5 positive strains (based on whole cell ELISA).

Results: On WB there was specific binding to 28 (88%) NmB strains that could be grouped into 3 patterns based on the relative prevalence of full length (sialylated lacto-N-neotetraose) and truncated (*galE*-like) alpha chain LPS glycoforms. In the first pattern (21% NmB), full length glycoforms predominated (G1); in the second pattern (57% NmB) truncated glycoforms were the most prevalent (G2); and in the remainder (21% NmB), the ratio of the glycoforms was about equal (G3). Three NmB strains (M986, 8047 and 2996) representative of G1, G2 and G3 respectively were investigated using a passive protection model.

Pre-treatment with 50µg/rat of MAb B5 completely protected 7 animals challenged 2h later with 8047 (0/7 bacteremic); rats were partially protected if pre-treated with 5µg/rat (2/5 bacteremic; Geometric Mean Bacteremia GMB $\log_{10}$ = 1.48 CFU/ml) or 1µg/rat (3/6 bacteremic; GMB $\log_{10}$ 1.76). An irrelevant MAb or PBS-albumin protected 0/12 animals (GMB $\log_{10}$ 5.4 CFU/ml), whereas rats pre-treated with anti-porin MAb were completely protected (0/4 bacteremic). Pre-treatment with 25µg/rat to 50µg/rat of MAb B5 failed to protect against strain 2996 (5/5 bacteremic; GMB $\log_{10}$ 4.86 CFU/ml) or strain M986 (5/5 bacteremic; GMB $\log_{10}$ 4.6 CFU/ml).

In further experiments using a mixture of MAb B5 and NmB, MAb B5 at 2µg/rat completely protected against 8047 with no detectable bacteremia compared to controls (GMB $\log_{10}$ 4.0 CFU/ml SE ± 3.9). MAb B5 at a dose of 10µg/rat partially protected against 2996 (GMB $\log_{10}$ 3.3 CFU/ml SE ± 1.6 vs 3 controls GMB $\log_{10}$ 4.6 CFU/ml SE ± 2.7 $p<0.1$) but at 7µg/rat did not protect against M986 (GMB $\log_{10}$ 4.3 CFU/ml SE ± 2.3, vs 6 controls with GMB $\log_{10}$ 4.7 CFU/ml SE ± 2.3).

Conclusions: Antibodies specific to the inner core LPS can protect against invasive infection caused by some but not all NmB strains; (ii) the affinity of MAb B5 for its cognate epitope may be insufficient to mediate protection against NmB strains possessing a predominance of fully extended alpha chain glycoforms.

Age-dependent incidence of meningococcal disease in British Columbia, Canada 1985-2000 in relation to meningococcal serum bactericidal titres

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Endemic meningococcal disease occurs at a rate of 1 per 100,000 of the population in Canada, a figure which has changed little in the past 50 years. Most cases are sporadic, but many clusters of disease in Canada since 1990 have been caused by serogroup C meningococci of the ET-15 complex leading to expensive public health measures for outbreak control. An outbreak of invasive serogroup C disease caused by this clone occurred in southwestern British Columbia during early 2001. We explored whether population immunity to this organism could explain the outbreak of disease that occurred.

We examined the age-dependent incidence of reported invasive meningococcal disease in the Province from 1985-2000 using surveillance data and related this to age-dependent serum bactericidal activity (SBA). SBA was measured in serum samples from 175 adults and children and the percentage of responders with a titre greater than 1 in 8 was calculated. In the assay baby rabbit complement was used and the prevalent outbreak strain of *N. meningitidis* (C:2a,P1.5) was the target organism.

Incidence of meningococcal disease ranged from 6.9 per 10^5 population in children under 2 years of age to 1.9, 0.5, 0.7, 1.7, 0.6 and 0.4 in individuals aged 2-4, 5-9, 10-14, 15-19, 20-29 and >30 years respectively. Serum bactericidal titres over 1 in 8 were present in only 3% of children under 18 years of age and 19% of adults, 18 years and over (median age 33).

Since SBA titres greater than 1 in 8 are believed to be required for protection against serogroup C meningococcal disease, these data indicate that a high proportion of adults, and almost all children remain susceptible to disease caused by this hyperinvasive serogroup C clone, supporting implementation of the new serogroup C meningococcal glyconjugate vaccines in British Columbia.

Validation of *Neisseria meningitidis* serogroups B and C colorimetric serum bactericidal assays

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Colorimetric serum bactericidal assay (cSBA) based on color change was recently developed[1]. Precision, specificity, and robustness were determined for cSBA validation. cSBA results were compared to those obtained for a traditional colony-counting microassay (mSBA). The precision measured as repeatability, intermediated precision, and reproductibility were determined assuming a percentage of coincidence between replicas ≥ 50. Specificity was measured by counting the number of CFU on each color (blue to green) at the titration moment. The robustness was measured changing independently four key parameters: bacterial suspension OD, decomplementation of sera, and conservation by freezing or freeze-dried of sera and complement. The results were the followings: presicion: titer ± 1 dilution, except for the highly positive serum against serogroup B, where it was titer ± 2 dilutions; percentage of coincidence: ≥ 50%; specificity: not growth in blue wells (bactericidal activity) and $5x10^7$ CFU/well (average) in green wells (not bactericidal activity), it demonstrated that the color change in the cSBA specifically depends on the bacterial growth; robustness: the change in density of the bacterial suspension and the freeze-dried of sera affected significantly the results. There was a good correlation between cSBA and mSBA ($r= 0.974$, $P<0.01$). Concluding, cSBA showed to be highly precise and specific, having an acceptable robustness and a good correlation with the mSBA.

Reference

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Immune memory induction to meningococcal B outer membrane vesicles through nasal immunization

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The outer membrane vesicle (OMV) vaccine used in this study derived from *Neisseria meningitidis* N.44/89 strain (B:4,7:P1.19,15:P5.5,7:L1,3,7,8, ET-5), recovered from spinal fluid of a patient from São Paulo, Brazil, in 1989 and it is representative of the most prevalent serosubtype in the country since 1980. This strain was also used in bactericidal tests and in the OMV production for immunoblotting and ELISA assays. Distinct mouse lines – 26 outbreed Swiss animals; 10 isogenic BALB/c mice and 6 genetically selected high responder line (H) – were immunized following two protocols: on day 0 and 28 with 2 μg of protein intramuscularly or 20 μg by the intranasal route. Seven months after the first dose both groups were boosted intramuscularly with 2 μg of protein in Al (OH_3) gel. Bleeds were done via the retro-orbital venous plexus 7, 14 and 27 days after the first dose, 15 days after the second and 8 months after the first immunization. The individual IgG and IgA anti-OMV antibody titers were determined by ELISA. The mean value of the observed optical density was transformed to U/ml by a sigmoid standard curve calculated from the value of a reference serum. The specificity of the IgG and IgA antibody secondary and tertiary responses were evaluated by immunoblotting of pooled sera. The quantification of the serum bactericidal activity was performed using the agar overlay technique in microtiter plates. The individual bactericidal titers were given as the reciprocal of the highest serum dilution yielding ≥ 50% killing. For both the bactericidal and IgG and IgA total antibodies, the kinetics of primary and secondary responses where higher in groups immunized by the intramuscular route; by contrast, the intranasal groups showed higher anti-OMV titers after the third dose than mice intramuscularly immunized, mainly the BALB/c and H lines. Moreover, the class 5 epitope was only recognized by the IgG response induced by the intranasal priming of Swiss, BALB/c and H mice. These results suggest a better memory induction after the intranasal sensitization.

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Serum bactericidal responses in rhesus macaques immunized with novel vaccines containing recombinant proteins derived from the genome of *N. meningitidis*

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Introduction: Vaccines prepared from outer membrane vesicles (OMV) of *Neisseria meningitidis* are limited by their ability to elicit serum bactericidal antibodies (SBA) against strains that differ by serotype and serosubtype, the primary antigenic determinants. Recent group B genomic sequencing efforts revealed a collection of surface-exposed conserved membrane proteins that elicit SBA in mice. We evaluated the immunogenicity in rhesus macaques of two novel vaccines derived from the genomic approach.
Methods: Three groups of macaques (N=8 per group) were vaccinated intramuscularly with OMV (prepared from strain H44/76), or one of two vaccines containing serogroup B recombinant proteins expressed *in E. coli*. Each animal received three doses in alum administered 1 month apart. A group of unvaccinated animals (N=3) served as controls. Bactericidal activity was assayed using endogenous complement in serum diluted 1:8 against six genetically diverse serogroup B strains that varied by serotype and serosubtype. Macaque sera were also tested in the infant rat protection model. Infant rats were pre-treated with sera and challenged 2 hours later with serogroup B strain 2996.
Results: The Table summarizes the numbers of animals with SBA titers of 1:8 or greater/total number of animals tested in each group.

	2996 2b:P1.5a,2a		BZ232 NT:P1.2		1000 NT:P1.5		MC58 15:P1.7,16b		NGH38 NT:P1.3		394/98 4:P1.4	
	pre	post 3	pre	post 3	pre	post 3	pre	post 3	pre	post 3	pre	post 3
OMV	3/8	1/8	0/8	1/8	1/8	8/8	2/8	8/8	2/8	0/8	0/8	1/8
Rec Vaccine 1	0/8	7/8	0/8	0/8	0/8	0/8	0/8	0/8	1/8	1/8	0/8	0/8
Rec Vaccine 2	0/8	6/8	0/8	2/8	0/8	7/8	0/8	8/8	0/8	5/8	0/8	4/8
Control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
NT= non-typeable with mAbs available.												

The geometric means of the CFU/ml of blood at 18 hours for Vaccine 1 and 2 were 538 (N=32 pups) and 323 (N=32 pups), respectively, vs. 50,131 (N=12 pups) for rats pre-treated with control sera (P<.01).
Conclusions: Recombinant vaccine 2 induced broad cross-strain SBA in macaques. Antibodies induced by both recombinant vaccines provided protection in infant rats against strain 2996. It may be possible to overcome the limitations of OMV vaccines in humans by designing a vaccine consisting of recombinant proteins identified from the genome of *N. meningitidis*.

Identification of human complement donors for use in the measurement of bactericidal activity against *Neisseria meningitidis* serogroups A, B, C

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Introduction: Measurement of complement-mediated serum bactericidal antibodies (SBA) has become a well-accepted serological correlate of protective immunity for *N. meningitidis* serogroup C. Licensure of serogroup C conjugate vaccines was based primarily on the measurement of SBA. Its use stems from a landmark publication demonstrating that pre-existing SBA conferred protection against disease caused by serogroup C (Goldschneider, et al, J Exp Med 129:1307-1326, 1969), where human serum with no intrinsic bactericidal activity was used as a supplemental complement source. In the current study, we report on recent efforts to identify sources of human serum complement for the measurement of SBA against for *N. meningitidis*.
Method: A validated serogroup C SBA assay was adapted for the identification of human serum complement sources, and the measurement of bactericidal activity against a diverse collection of N. *meningitidis* strains. Sera from 60 adult volunteers from Northern California were analyzed for the presence of SBA against six serogroup B strains, two serogroup C strains, and one serogroup A strain. Strains varied by serotype [2b; 4; 15; 16; NT], and serosubtype [1.2; 1.3; 1.4; 1.5; 1.5a,2a; 1.7,1.1; 1.7,16b; NT].
Results: The percent subjects with no endogenous bactericidal activity when screened at 50% serum were 22-60% for the six serogroup B strains tested, 30 and 37% for the two serogroup C strains, and 4% for the serogroup A strain. Of the 60 donors screened at 50% serum, 4 had no bactericidal activity against all six serogroup B strains. The percentage of donors with anti-meningococcal SBA also varied by strain. 7-28% of donors had positive titers (1:4 or greater) against the six serogroup B strains, 27 and 40% against the two serogroup C strains, and 32% against the serogroup A strain. Of the 31 donors that had positive SBA titers against at least one serogroup B strain, 6 donors had positive SBA titers against 4 to 5 of the 6 serogroup B strains, and 4 donors had titers against 3 of 6 serogroup B strains.
Conclusion: Serum sources of human complement were identified for all *N. meningitidis* strains tested. Suitability of donor serum as a complement source varied by strain. Cross-strain SBA was observed among the different serum donors, which was independent of strain serotype and serosubtype.

Vaccination of mice with gonococcal antigens expressed from Venezuelan equine encephalitis viral replicon particles

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There is currently no vaccine against gonorrhea. We have applied a novel vaccine strategy to gonococcal antigens in hopes of developing a vaccine. Venezuelan equine encephalitis (VEE) virus has been adapted by Pushko *et al* (1997) into a non-replicating vaccine delivery system that targets dendritic cells. We successfully expressed two gonococcal antigens, porin (PorB) and transferrin binding protein B (TbpB), from VEE Replicon Particles (VRP's). PorB is a constitutively expressed abundant outer membrane protein in *Neisseria gonorrhoeae*. TbpB is part of the transferrin receptor, and although expressed only when GC are iron starved, is a focus of meningococcal vaccine development. Mice were immunized with VRP's encoding PorB and TbpB with and without a eukaryotic secretion signal. They received two VRP boosts and a final boost with recombinant renatured (rr) protein.

ELISA titers from PorB-VRP immunized mice showed that PorB-specific IgG levels approached those found in rrPorB immunized mice. ELISA titers were substantial, but lower in TbpB-VRP immunized mice. Presence of the eukaryotic secretion signal, especially within the TbpB-VRP immunized group, correlated with higher levels of IgG. Compared to prior VRP boosts, the final recombinant protein boost dramatically raised IgG levels. rrPorB immunization produced similar amounts of IgG2A and IgG1, whereas VRP immunization yielded mostly IgG2A. The latter is consistent with a predominantly T_H1 response, the former with a balanced T_H1/ T_H2 response. Serum from rrPorB-immunized mice preferentially recognized the surface of a homologous GC strain compared to an isogenic strain expressing a heterologous PorB gene. Serum from VRP-PorB-immunized mice recognized both strains equally, suggesting that the immune response to PorB recognized conserved epitopes after delivery of the gene in a VRP. As expected, serum from TbpB-VRP immunized mice bound better to wild-type GC than to a TbpB mutant.

This encouraging immune response by mice to VRP's encoding GC antigens will allow us to test potential vaccines in a mouse model of vaginal-cervical gonorrhea.

Attempts to drive a mucosal immune response to gonococcal PorB in mice

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Despite the apparent lack of naturally occurring protective immunity to *Neisseria gonorrhoeae* and absence of an effective vaccine, a mucosal immune response presumably plays a significant role in protection against gonococcal disease. We have explored means to increase mucosal response to a prototype antigen, gonococcal PorB, focusing on route of delivery and vaccine formulation (adjuvant). Intra-nasal (IN) and subcutaneous routes were compared. The IN immunizations were designed to present the antigens to the nasal associated lymphoid tissues NALT, a technique that has been shown to induce common mucosal immunity. This route of immunization is also the only route to show protection in the estradiol-treated mouse model of gonococcal infection [1]. The subcutaneous immunizations were used as controls.

Three general formulations were examined: 1) recombinant renatured (rr) PorB protein in RIBI R-700 adjuvant; 2) DNA encoding PorB in liposomes; 3) rrPorB in liposomes. Groups of BALB/c mice were immunized three times at approximately three-week intervals with each of these formulations alone or supplemented with the hormonal immunomodulator, 1μ, 25-dihydroxy vitamin D_3 (D3*). D3* is natural steroid hormone thought to directly or indirectly affect the maturation process of antigen processing cells inducing increased levels mucosal immunity[2]. A separate group of mice was immunized with rrPorB in liposomes supplemented with intact cholera toxin (CT), which has also been shown to have potent mucosal immuno-stimulatory effects. Each group had a corresponding negative control group to test for adjuvant effects. One group was immunized intra-nasally with outer membrane vesicles (OMV) similar to those shown to be protective [1]. Antibody responses were measured in weekly vaginal washes and serum samples taken prior to each boost, and two and eight weeks post third immunization. Quantitative IgG and IgA levels in serum and vaginal washes were determined by sandwich ELISA. IgG1 and IgG2a levels were measured in serum to characterize the T_H-1/T_H-2 bias of the response.
By far the strongest response was seen in groups immunized subcutaneously with rrPorB in RIBI adjuvant, IN with OMV, or IN with rrPorB in liposomes supplemented with CT. This was true for vaginal wash and serum IgG and IgA levels. Immunization with DNA liposomes by the IN route generated little or no specific antibody above background levels. Addition of D3* only had small and inconsistent effect on serum and mucosal antibody levels. CT supplementation of IN vaccine delivery had the strongest positive effect on mucosal antibody levels of the immunization regimens investigated.

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Passive protection in the infant rat protection assay (IRPA) by sera taken after vaccination of young adults by two serogroup B meningococcal outer membrane vesicle (OMV) vaccines in Iceland

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Introduction: The Icelandic immunogenicity trial among teenagers suggested that serum bactericidal activity (SBA) assay and ELISA might be insensitive correlates for serogroup B OMV vaccine efficacy. Thus, other *in vitro* and *in vivo* assays have being sought for evaluation of vaccine efficacy. In this study, we have evaluated the immunogenicity of the Cuban and Norwegian OMV vaccines by IRPA and compared the results to those obtained by SBA and ELISA antibody measurements.
Materials and Methods: A 25% stratified subset of Icelandic study sera collected before and six weeks after the second vaccination of teenagers with either the Norwegian (n=37), the Cuban (n=35), or the control, serogroup A/C polysaccharide vaccine (n=20) was analyzed. Groups of six 4-6 days old SPF HsdCpb:WU rats were injected i.p. with 0.1 ml of heat-inactivated, 1:10 diluted serum 1-2 hours before the i.p. challenge of 10^6 colony forming units (cfu)/pup of the rat-passaged Norwegian (44/76-SL; B:15:P1.7,16:L3,7,9) or the Cuban (Cu385; B:4:P1.15:L3,7,9) vaccine type strain. Saline was used as a negative control for protection. Quantitative blood cultures were made from samples taken 6 hours post challenge. The results are expressed as protection index (PI), equivalent to fold decrease in GM blood bacterial density, calculated as follows: GM cfu^{ml-1} for saline treated animals/GM cfu^{ml-1} for serum treated animals. Protection was considered significant if the PI was ≥ 10. A vaccine response was defined as ≥ 10-fold rise in post-vaccination PI compared to pre-vaccination PI.
Results and Discussion: Pre-vaccination sera showed significantly higher background of protective activity against 44/76-SL than Cu385 strain. Before vaccination, 15 (41%), 12 (34%), and 15 (75%) of the Norwegian, Cuban, and control vaccine recipients, respectively, showed protection (PI ≥ 10) against strain 44/76-SL. Six weeks after the second dose, 9 (24%) Norwegian, 5 (14%) Cuban, and 3 (15%) control group vaccinees had responded significantly, and the GM PI increased by 2.0 (P>0.05), 1.4-fold (P>0.05), and 0.8-fold (P>0.05), respectively. Before vaccination, 5 (14%), 3 (9%), and 3 (15%) of the Norwegian, Cuban, and control vaccine recipients, respectively, showed protection against strain Cu385. Six weeks after the second dose, 12 (32%) Norwegian, 5 (14%) Cuban, and none of the control group vaccinees had responded significantly, and the GM PI increased by 4.0 (P<0.05), 2.4 (P<0.05) and 0.8-fold (P>0.05), respectively. The seroconversion rates detected by different assays could be ranked to ELISA>SBA>IRPA. From poor to moderate association between antibody measurements by IRPA and SBA/ELISA were found.
Conclusion: Both study vaccines augmented clearance of 44/76-SL and Cu385 strains but the responses against the former strain were generally low. The high background protection against strain 44/76-SL present already in the baseline serum samples might have lead to underestimation of vaccine responses against this strain.

Are IgM antibodies to meningococcal serogroup B capsular polysaccharide responsible for passive protection by prevaccination sera in the infant rat protection assay (IRPA)?

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Introduction: The Icelandic immunogenicity trial among teenagers might indicate that serum bactericidal assay (SBA) and ELISA are less sensitive correlates for serogroup B outer membrane vesicle (OMV) vaccine efficacy. Thus, we have evaluated the immunogenicity of the Cuban and Norwegian OMV vaccines in IRPA and compared the results to SBA and ELISA measurements. Prevaccination sera from 46% (42/92) of the participants receiving either of the OMV vaccines or the control serogroup A/C vaccine showed protection in IRPA against the Norwegian vaccine strain 44/76-SL that correlated poorly with SBA and ELISA results. Of note, one third (14/42) of the study participants whose prevaccination sera protected in IRPA against strain 44/76 failed to show any detectable SBA activity (titer < 2), and 43% (18/42) had SBA titres < 4. A subset of pre-vaccination sera with convergent/discrepant SBA and IRPA results was therefore analyzed further for functional and specific antibodies. Our aim was to find out whether the poor correlation between IRPA and SBA data with some of the sera could be explained by opsonophagocytic activity (OPA), and to detect possible associations between protection in IRPA and antibody specificities as detected on immunoblots.
Materials and Methods: Four subsets of Icelandic prevaccination sera representing a) IRPA +, SBA +, (n=6), b) IRPA +, SBA - (n=7), c) IRPA -, SBA + (n=6), and d) IRPA -, SBA - (n=7) sera, were analysed. OPA was measured as respiratory burst by flow cytometry with polymorphonuclear leukocytes as effector cells and live 44/76-SL (B:15:P1.7,16:L3,7,9) cells as target. Anti-meningococcal IgG antibodies were quantified with live 44/76-SL in flow cytometry. Specificities of IgG antibodies were analyzed by immunoblotting against OMVs from the same strain. IgM and IgG antibodies to meningococcal group B capsular polysaccharide (B-PS) were measured by ELISA, using B-PS non-covalently complexed to methylated human serum albumin as coating antigen.
Results and Discussion: High SBA and OPA titers were generally reflected in distinct binding to the major outer membrane proteins and/or LPS on immunoblots. However, we could not detect specific antibody patterns on blots that could explain the protection in IRPA. IgM titres to B-PS were significantly higher among IRPA + than IRPA - sera, whereas no significant correlation was observed between IRPA and SBA, OPA or IgG to live meningococci. Only IgM levels to B-PS correlated positively to IRPA for the prevaccination sera.

Cost-effectiveness of meningococcal serogroup C conjugate vaccine: further applications

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In November 1999 the UK introduced the meningococcal serogroup C conjugate (MenC) vaccine into routine infant immunisation and launched a national campaign offering the vaccine to everyone aged under 18 years. Within 18 months, coverage of around 85% had been achieved and the incidence of serogroup C disease in the targeted ages groups had decreased by 80%. The success of the UK campaign has encouraged other high incidence countries within Europe to consider introducing the vaccine. Such policy decisions are increasingly informed not only by estimates of the burden of disease, but also by economic analyses.

In a cost-effectiveness analysis of the UK MenC campaign we estimated the overall cost per life year saved to be around £6,300 ($9,000, € 8,200). The sensitivity analysis showed that the most important determinants for the cost per life year saved were future disease incidence and mortality assumptions, vaccine cost, and vaccine efficacy. The choice of discount rate, recommendations on which vary internationally, was also crucial. The model we developed can also be used to compare the cost-effectiveness of alternative vaccine strategies. For example, in the UK vaccination at 2,3 and 4 months was the most effective, but least cost-effective strategy, whereas vaccination at 1 year was most cost-effective but may have resulted in up to 200 preventable cases in infants under 1 year old.

The EU-MenNet Project provides a resource for modelling and cost-effectiveness analyses within Europe. The UK model can be adapted to examine the cost-effectiveness of MenC vaccination in other European countries by changing the values of the model parameters. Key parameters that would need to be estimated include: age-specific disease incidence and case fatality ratios, hospitalisation and treatment costs, costs associated with outbreak control and costs associated with vaccine delivery. This model can also be extended to explore the effect of waning vaccine immunity. A dynamic model is in preparation (Trotter CL, Gay NJ unpublished), which will be used to estimate the herd immunity effects of vaccination, resulting from reduced serogroup C carriage.

Modelling meningococcal carriage, immunity and disease

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Mathematical models have been used to describe the transmission dynamics of a range of infectious organisms. These models have been used to enhance the understanding of the infectious process and to predict the likely effects of changes to the system, such as the introduction of a mass vaccination programme. The introduction of the meningococcal serogroup C conjugate (MenC) vaccine in the UK has encouraged the development of models describing *Neisseria meningitidis*.

Invasive disease is a rare outcome of infection with *N. meningitidis*, and most transmission is accounted for by episodes of asymptomatic carriage. The relationship between meningococcal carriage and disease is complex, and age-specific patterns of carriage are not proportional to disease incidence. Meningococcal carriage has been shown to be an immunising event, protecting against disease, and it is likely that carriage of *N. lactamica* is also protective. Recently, vaccination with MenC has been shown to reduce serogroup C carriage in targeted age groups. In order to understand and model the likely effect of carriage reduction on the incidence of meningococcal disease, the relationship between carriage and disease must be scrutinised further.
A number of quantitative investigations were carried out to inform the development of a model that would describe and explore the relationships between meningococcal carriage, immunity and disease. Firstly, the average number of episodes an individual would be expected to experience in a lifetime was estimated under a range of assumptions regarding prevalence and the duration of carriage. Secondly, the ratio of cases per carrier was investigated and was shown to vary by serogroup (with serogroup C meningococci being most virulent) and decrease with age. This led to an investigation of the importance of first infections in determining risk of disease, and whether the change in the case per carrier ratio by age be explained by the change in the proportion of first infections by age.

These analyses and a literature review suggested a model framework that is individual based, and simulates the monthly exposure of N individuals to *N. meningitidis* and *N. lactamica*. Carriage is assumed to be an immunising event, such that an individual's risk of acquiring carriage and disease is dependent on their history of carriage. The model draws on three sources of data: age and serogroup specific carriage prevalence, age and serogroup specific disease incidence, and age-specific prevalence of serum bactericidal antibodies to serogroup C meningococci.

Antibody avidity after vaccination with a hexavalent Men B outer membrane vesicle vaccine in toddlers and school children

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Introduction: The RIVM developed two candidate vaccines against N. *meningitidis* serogroup B based on outer membrane vesicles (OMVs) mainly containing porin A (PorA). Both vaccines proved to be safe and immunogenic in toddlers and/or school children. The monovalent vaccine expressing P1.7-2,4 subtype induced a significant antibody avidity maturation[1]. The hexavalent vaccine exists of two OMVs each containing 3 different PorA types. One of the vesicles contains PorA types P1.5-2,10; P1.12-1,13 and P1.7-2,4. After vaccination serum bactericidal activity (SBA) against P1.5-2,10 was 4-6 times higher compared to P1.7-2,4. The aim of this study was to test whether the differences in SBA could be explained by a difference in subtype-specific antibody avidity maturation. The avidity maturation of antibodies against 3 subtypes was evaluated in relation to SBA and compared to the avidity maturation induced by the monovalent vaccine.

Materials and Methods: Serum samples drawn before start of the trial, after 2 vaccinations, before and after a booster vaccination of 121 children were used in these experiments. To assess antibody avidity an enzyme-linked immunosorbent assay (ELISA) was performed on all samples as described earlier[1]. ELISA plates were coated with monovalent OMVs from the isogenic vaccine strain H44/76 expressing either P1.5-2,10; P1.5-1,2-2 or P1.7-2,4 as a subtype. Geometric Mean Titers (GMT), Geometric Mean Avidity Titer (GMAT) and the Geometric Mean Avidity Index (GMAI) was calculated. SBA titers had been assessed earlier[2].

Results and Discussion: The GMT after booster vaccination was 5458 (95% confidence interval (CI) 4790-6221) for P1.7-2,4; 6054 (CI 5353-6847) for P1.12-1,13 and 8392 (CI 7502-9387) for P1.5-2,10. The GMAT ranged from 3368 for P1.7-2,4 to 6022 for P1.5-2,10.

The GMAI increased up to 61% (95% CI 57-64) for P1.7-2,4 and P1.12-1,13 and 70% (95 % CI 67-74) for P1.5-2,10. Correlations between SBA and ELISA are described in table 1.

Table 1. Spearman's rho correlations of the GMAT, GMT and GMAI with SBA

	P1.7-2,4	P1.12-1,13	P1.5-2,10
GMAT	0.45	0.43	0.87
GMT	0.41	0.48	0.85
GMAI	0.24	0.18	0.42

Conclusions: There is a significant avidity maturation induced by the hexavalent vaccine for 3 PorA subtypes. This maturation is most pronounced for P1.5-2,10 (AI = 70%), correlating with the highest SBA titers. The AI for P1.7-2,4 after vaccination with a hexavalent OMV vaccine is significantly lower than after vaccination with a monovalent OMV vaccine (61 % vs.73 %). Generally, the GMAT correlates best with SBA.

References

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Photochemical stability of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B

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Introduction: The meningococcal vaccines produced at NIPH contain vesicles of the bacterial outer membrane proteins. The outer membrane vesicles are adsorbed to aluminiumhydroxide (adjuvant) and stabilised with sucrose. Recommended storage temperature is 2-8 °C. Accelerated stability testing (stressing by heat) shows that the vaccines are stable at 25 °C. However, the vaccines may experience a variable amount of light during production, distribution and storage. Light may have effects on the outer membrane vesicles as well as on the final vaccines.

The aim of the study was to investigate the photochemical stability of outer membrane vesicle vaccines.

Materials and Methods: The stability of two different vaccines was investigated. One vaccine is based on the Norwegian strain 44/76 characterised as B:15:P1.7,16. The other vaccine is based on the New Zealand strain 98/254 characterised as B:4:P1.7b,4.

The samples were exposed to radiant energy including UVB and UVA (310-400 nm) and visible light (400-800 nm) in a SUNTEST CPS (1.8 kW xenon burner) according to indoor daylight conditions (ICH guidelines for photostability testing). The stability of the final vaccines and the outer membrane vesicles has been characterized by antigen pattern (SDS-PAGE).

Results and Discussion: The SDS-PAGE antigen pattern of class 4 protein showed some degradation after exposure to light. The results indicate that the outer membrane proteins might be sensitive to light.

The temperature in the sample vials reached 50 °C during light exposure. Reference samples were therefore incubated at 50 °C in the dark. The outer membrane proteins turned out to be stable when exposed to heat (50 °C).

Further investigations will be carried out to evaluate the photosensitivity of the vaccines including immunoblotting, transmission electron microscopy (TEM), and immunogenicity studies in mice. Results will be presented.

One dose of MenC-T conjugate vaccine in infancy produces higher mucosal IgG and IgA responses than 2 or 3 doses and optimal mucosal booster responses following polysaccharide vaccine at 13 months

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Introduction: Meningococcal group C (MenC) conjugate vaccines have been used in the UK programme which commenced in late 1999. A recent study has shown that the vaccination resulted in a significant reduction in nasopharyngeal carriage of group C meningococcus in students aged 15-17 years. This strongly suggests that the parenteral vaccination could induce effective mucosal immunity against carriage. It is not known whether the commonly used 3-dose vaccination regimen induces optimal mucosal immune responses in infants. We have investigated the primary and booster saliva antibody responses in UK infants.
Methods: Unstimulated saliva samples from 124 infants at 2 month of age immediately prior to commencing primary immunisation with a MenC-tetanus (MenC-T) conjugate vaccine and one month after their primary course. 46 received only one dose (2 month only - group 1), 35 received 2 doses (2 and 4 month, group 2), 43 received 3 doses (2,3 and 4 month – group 3). Samples were also collected just before and one month after a booster dose of unconjugated MenC polysaccharide vaccine at 13 month. Samples were snap frozen on dry ice immediately after collection and held at –70°C until analysis by immunoassay for anti-MenC IgG and IgA.
Results: There were modest but significant ($p<0.01$) increases in salivary IgG after priming in groups 2 and 3 (pre 4.3, post 9.9, 14.8 ng/ml respectively) but significantly higher post-priming concentrations in group 1 who had had only one dose (25.8ng/ml)($p<0.001$). There was a small rise in salivary IgA post priming in groups 2 and 3 (pre 1.5 and 1.8, post- 2.9 ng/ml) but, again, significant rises in group 1 (14.4 ng/ml). Following the booster dose of unconjugated MenC polysaccharide all 3 groups showed a clear rise in salivary IgG at 14 months - group 1 having the highest concentrations (50.4, 25.4, 27.9 ng/ml, respectively). IgA levels at the same time point also rose but once again less in group 2 (12.0, 4.5, 6.4 ng/ml).
Discussion: These surprising data suggest that one dose of this vaccine may induce better mucosal immune responses in infancy than either two doses or three and may also prime better for mucosal memory immune responses to a polysaccharide challenge. Although serum responses – indicating protection against invasive disease – will also be important in predicting optimal future primary schedules, these results suggest it may be possible – and maybe even preferable – to give only one dose of this vaccine to infants in the future. Further studies with this and the other MenC conjugate vaccines will demonstrate how to induce optimal protective mucosal immunity.

DNA immunization of mice with a plasmid encoding *Neisseria gonorrhoeae* PorB protein by intramuscular injection and epidermal particle bombardment

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Gonococcal disease remains a major problem worldwide. Compelling reasons to develop a gonorrhea vaccine include the fact that this disease increases the risk of transmission of HIV, and antibiotic resistance is increasing. DNA vaccines offer the possibility of rapid and inexpensive development of new vaccine candidates, and have the ability to generate a broad range of immune responses. This flexibility provides us a way to test different immunity models to understand what kinds of immune responses are protective.

We began by constructing a plasmid encoding PorB from *N. gonorrhoeae* strain FA1090, and administering the plasmid to BALB/c mice by intramuscular (im) injection and epidermal particle bombardment using the Helios gene gun (gg). For im immunization, 100ug of purified DNA in 50 ul saline was injected into tibialis anterior muscle of each mouse. For gg immunization, 1.5 mg gold microcarrier particles coated with 3 ug plasmid DNA were delivered to the depilated mouse abdomen skin using the Helios Gene Gun System set to a helium discharge pressure of 400lb/in^2. The immunizations were boosted after four weeks. Both methods generated PorB specific antibodies, although the gene gun induced less antibody response. Intramuscular DNA immunization induced an antibody response that was primarily of a Th1-type (IgG2a). In contrast, epidermal particle bombardment generated antibody responses that were indicative of Th2 activation (primarily IgG1). Boosting with either renatured recombinant (rr) PorB protein or PorB expressed from Venezuelan equine encephalitis Virus Replicon Particles (VRP's) significantly increased the antibody responses. Surprisingly, after booster immunization with recombinant PorB protein, there was no switch of the IgG subclasses. (i.e., mice primed by intramuscular DNA injection retained a Th1-type response, and mice primed immunization by gene gun still maintained a Th-2 type response). However, boosting with PorB expressing VRP's enhanced a Th-1 type response. In addition, rrPorB and PorB expressing VRP's helped to generate PorB specific IgA in serum. Whole cell binding experiments showed that a portion of the antibodies recognized the surface of the homologous *N. gonorrhoeae* strain. None of the sera demonstrated a complement-mediated killing in bactericidal assays, but serum from groups with high antibody levels showed some opsonization of the homologous strain using human neutrophils.

In conclusion, we successfully induced anti-PorB specific immune responses by using DNA vaccination. Depending on different immunization routes, we could modulate the immune response toward either Th1 or Th2 type responses. This may allow us to investigate which kinds of immunity are protective in mouse models of gonorrhea.

SESSION VII

Population biology and evolution

Identification of strain-specific genes in conserved locations

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Introduction: Comparisons of the completed genome sequences reveal regions in which different genes were present between conserved flanking genes. *pheS* and *pheT* flank one such region.
Materials and Methods: The sequenced strains *N. menignitidis* strains MC58 and Z2491, and *N. gonorrhoeae* strain FA1090 were compared. Primers were designed to the conserved regions within *pheS* and *pheT* for PCR amplification from a collection of unrelated *Neisseria* spp. strains. PCR products of the similar size were compared by restriction digest to select distinct *pheS-pheT* regions for sequencing.
Results and Discussion: PCR was conducted on 88 strains: 42 *N. meningitidis* serogroups A, B, C, D, 29E, H, W135, X, Y and Z; 32 *N. gonorrhoeae* strains; 7 *N. lactamica* strains; and 7 other commensal *Neisseria* spp. From these strains 7 main groups and 3 subgroups of different sequences between *pheS* and *pheT* were identified. The *phe* region in FA1090 contains four candidate genes, including a restriction modification system. All gonococcal strains tested have *phe* regions similar to FA1090, which is also present in one of the 7 *N. lactamica* strains and *N. polysaccharea*, which contained some sequence variation, but was essentially similar. The *phe* region in Z2491 contains sequence for two hypothetical proteins. The Z2491-like *phe* region, or a subgroup variant of it, was found in 27.3% of the strains tested, present in the widest range of meningococcal serogroups (all but C and X) of all the *phe* region inserts. In MC58 there are three candidate genes encoding a restriction modification system. The MC58-like *phe* region has, thus far, additionally only been found in serogroup X strains. Six of the 7 *N. lactamica* strains had variations of the same *phe* region, containing candidate genes of unknown function. The *phe* region from one of the 6 serogroup C strains assessed contains three candidate genes for a restriction modification system; also present in the now complete genome sequence of *N. meningitidis* strain FAM18. Another putative restriction modification system, encoded by two genes, was found in the *phe* region of a serogroup 29E strain. In addition to identifying strains with different genes, two serogroup B strains and two commensal species were identified in which the intergenic region between *pheS* and *pheT* is just 123 bp, suggesting that the other genes found in this region were transferred into this location horizontally due to the homology with flanking *pheS* and *pheT* sequence, the model for the minimal mobile element.
Conclusion: Seven distinct regions between *pheS* and *pheT* were found in the *Neisseria* spp. These regions contain four restriction modification systems, two sets of genes of unknown function and a short intergenic sequence with no identifiable coding regions.

Phase variation: transcriptional and translational alterations in class 1 protein

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Introduction: Expression of Class 1 protein (PorA) can be altered in multiple ways. Slipped-strand mispairing during replication in the homopolymeric tract of guanidine and/ or thymidine residues between the –10 and –35 domains of the *porA* promoter as well as the homopolymeric tract of adenine residues in the *porA* coding region are the principal mechanisms responsable of the altered PorA expression. In addition, point mutations or insertion of an IS element in the *porA* coding region or deletion of the complete *porA* gene may result in meningococci without PorA expression.

With the aim to analyze the incidence of the mechanisms responsible of an altered expression of the PorA protein, 41 non-subtypable (NST) strains isolated from patients with meningococcal disease in Spain were studied.

Material and Methods: The *porA* promoter and coding region were analyzed by sequencing. Moreover, PorA expression quantification was analyzed by SDS-PAGE and Western blotting (WB).

Results and Discussion: We were able to amplify the *porA* gene from 38 of the 41 strains, finding eighteen different PorA types among them. The remaining three strains were also NST by sequencing. In fact SDS-PAGE analysis showed no PorA presence suggesting the possibility that the *porA* gene was deleted. In two of the 38 amplified genes, the *porA* coding region presented a premature stop codon before the variable region VR1. SDS-PAGE and WB confirmed no PorA expression.

Otherwise, analysis of *porA* promoter region showed a variable homopolymeric tract of 9 to 12 residues. SDS-PAGE showed PorA presence in strains with 10 to 12 nucleotide (nt) residues. We found differences in the level of expression among strains showing 10-12 residues at this region. Strains showing 10 nt presented an intermediate level of expression while strains showing 11nt or 12nt presented the best level of class 1 protein expression.

Moreover, eight strains had mutations in the *porA* promoter spacer, replacing one or two residues in the homopolymeric tract of guanidine residues with adenine. We did not find a clear difference in the level of expression among these strains by SDS-PAGE, suggesting the application of other alternative techniques as quantitative RT-PCR in these strains. Finally, it was remarkable the appearance of two strains with a tract of guanidine of 11nt and 10 nt, respectively, with no PorA expression produced by an additional pair base insertion in the homopolymeric tract of adenine, in the coding region of *porA*. This alteration introduces a frame shift which truncates the coding sequence, in fact SDS-PAGE and WB analysis confirmed no PorA presence.

These findings suggest the importance of these studies for the application of future vaccines in specific countries based in these antigens.

Evolution of the neisserial *tex* gene locus (region E)

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Introduction: The genus *Neisseria* comprises at least 20 species isolated from warm- and cold-blooded animals. Most neisserial species colonize the nasopharynx of their hosts. Among these nasopharyngeal commensals only *Neisseria meningitidis* is associated with human disease. The major pathogenicity factor of meningococci is the polysaccharide capsule. The genes necessary for capsule synthesis (region A), transport (region C), and modification (region B) are clustered on a chromosomal region called *cps*. The *cps* comprises the regions D (LPS biosynthesis) and E (Tex protein), which are also present in gonococci (Petering *et al.*, J. Bacteriol. 178:3342-3345). The Tex protein has been proposed to be involved in regulation of gene expression. *tex* gene variants are present in a variety of bacterial phyla suggesting that the meningococcal *cps* resulted from chromosomal re-arrangements and horizontal gene transfer which took place in the vicinity of the *tex* gene. We therefore analysed the chromosomal arrangement and genetic variability of the *tex* gene in several *Neisseria* and non-*Neisseria* spp.

Materials and Methods: *tex* genes of commensal *Neisseria* were cloned and sequenced. The following isolates were used: *N. animalis*, *N. canis*, *N. cinerea*, *N. iguanae*, *N. lactamica*, *N. macacae, N. sicca*, and *N. weaveri*. The presence of the region D was determined by Southern blot hybridization. From the following non-neisserial species Tex protein sequences were used for comparison: *Agrobacterium tumefaciens*, *Clostridium perfringens*, *Escherichia coli* K12, *Fusobacterium nucleatum*, *Lactococcus lactis*, *Nostoc* sp., *Streptomyces coelicolor*, *Treponema pallidum*, and *Ureaplasma urealyticum*.

Results and Discussion: All *Neisseria* were *tex* gene positive, whereas the region D was only found in meningococci, gonococci, *N. animalis*, *N. cinerea*, and *N. lactamica*. The regions adjacent to the *tex* gene differed among the neisserial species. The meningococcal *tex* genes were highly conserved and related to the *tex* genes of *N. gonorrhoeae*, *N. lactamica*, *N. sicca*, and *N. cinerea*. Similarities of the neisserial Tex protein sequences were 81.2-97.0%. The neisserial sequences were most similar to *E. coli* and *S. coelicolor* (ca. 60%), and least similar to spirochaetal and *U. urealyticum* Tex protein sequences (ca. 33%). Protein sequence alignments elucidated several motifs conserved throughout the phyla.

Conclusion: The region E was demonstrated to be the ancient core of the meningococcal *cps* locus and might be the essential part of the *cps* locus. There was evidence for genomic rearrangements. Sequence comparisons of the Tex protein partly resembled the phylogenetic distances of the species analysed. Several Tex protein motifs were conserved and thus might be under functional constraint. Mutation of these motifs might assist to identify the precise function of the Tex protein in *Neisseria* and other genera.

porB-PCR-AREA, a method to predict *porB* variable region sequences in non-serotypable meningococci

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Introduction: PorB is a meningococcal outer membrane protein with eight surface exposed loops. The majority of variation in PorB is found in four variable regions located at the tips of the surface exposed loops. The use of monoclonal antibodies is the standard method for identifying the PorB type although there are a large number of meningococci that cannot be typed using the serotyping antibodies currently available. Serotyping antibodies recognise epitopes encoded by the *porB* variable region sequences. *porB*-PCR-amplicon restriction endonuclease analysis (AREA) was developed to predict variable region (VR) sequences in non-serotypable meningococci and thus the *porB* type.
Materials and Methods: The amplified *porB* gene from thirty meningococci isolated in New Zealand during 1999 was sequenced to determine the sequence variation in *porB*. Restriction of the amplified *porB* PCR product with *Alu*I and *Ssp*I facilitated differentiation of the common *porB* types. *porB*-PCR-AREA was tested on meningococci with known *porB* type. To validate *porB*-PCR-AREA before it was applied to a large number of non-serotypable meningococci it was applied to ten non-serotypable meningococci and the resultant *porB*-PCR-AREA predictions were confirmed by sequencing the amplified *porB* gene.
Results and Discussion: Sequence data indicated that *porB* from meningococci isolated in New Zealand fell into four groups, which differed according to sequences found in *porB* VR1 and VR2. *porB*-PCR-AREA profiles discriminate between these four groups and enabled prediction of the *porB* VR1 and VR2 sequences and thus the *porB* type. *porB*-PCR-AREA predictions for ten non-serotypable meningococci were confirmed to be accurate by sequencing the amplified *porB* PCR product.
Conclusion: *porB*-PCR-AREA is a rapid, simple typing method that enables the *porB* type to be predicted when serotyping antibodies do not recognise the PorB epitope, serotyping antibodies are not available and for analysis of non-viable cultures.

MLRT, a method to predict the sequence type of meningococci

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Introduction: Multilocus sequence typing (MLST) is used to identify clones within the population of meningococci and assigns sequence types based on the DNA sequence of internal fragments of seven housekeeping genes. MLST relies on the proven theories of multilocus enzyme electrophoresis and is easily standardised and comparable between laboratories. Before the commencement of this study the sequence type (ST) of three meningococci isolated during New Zealand's serogroup B meningococcal disease epidemic was known. These meningococci were ST-42, ST-154 and ST-155, which are subclones of the hypervirulent ST-44 clonal complex. A multilocus restriction typing (MLRT) method was developed to reduce the number of sequencing reactions required to determine the clonal relationships of meningococci causing New Zealand's serogroup B meningococcal disease epidemic.
Materials and Methods: MLRT was developed to predict the sequence type of meningococci based on restriction fragment length polymorphism analysis of MLST PCR products. Restriction enzymes were selected that differentiate between common MLST alleles found in meningococci belonging to the ST-44 clonal complex. MLRT was tested on three meningococci with known sequence type that were isolated in New Zealand. To validate MLRT before it was applied to a large number of meningococci it was applied to twelve meningococci with unknown sequence type. The twelve meningococci were selected according to serological typing results and it was hoped the sample would include meningococci from different clonal origins. MLST was used to confirm MLRT predictions.
Results and Discussion: Restriction enzymes selected to differentiate between common alleles found in meningococci belonging to the ST-44 clonal complex were tested on PCR products amplified from New Zealand's three meningococci with known sequence type. When different MLST alleles were present different restriction profiles were visible on an agarose gel. When MLRT was applied to meningococci with unknown sequence type the majority of meningococci analysed had the same restriction profiles as those found in meningococci with ST-42 and ST-154. Sequence data confirmed that these meningococci were ST-42 and ST-154, as MLRT had predicted. Sequence data confirmed that PCR products that generated different restriction profiles encoded different MLST alleles to those found in ST-42 and ST-154 meningococci.
Conclusion: MLRT is a simple method to predict the sequence type of meningococci and is particularly useful if an automatic sequencer is not available. MLST can be used to confirm the sequence type for a given MLRT profile and results can be compared to the international MLST database.

New Zealand's meningococcal disease epidemic caused by two subclones of the hypervirulent ST-44 complex

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Introduction: New Zealand has been experiencing increased levels of meningococcal disease since mid-1991. The increased incidence of disease is due to serogroup B meningococci with the P1.4 PorA. These meningococci were investigated by assessing their *porB* type and clonal relationships.
Materials and Methods: Serogroup B meningococci with the P1.4 PorA that were isolated from clinical cases of disease in New Zealand during 1989 through 1999 were assessed using serological typing, *porB*-PCR-amplicon restriction endonuclease analysis (AREA), multilocus restriction typing (MLRT) and multilocus sequence typing (MLST). All meningococci were assessed using serological typing and all non-serotypable meningococci were typed using *porB*-PCR-AREA. A randomly selected 10% sample of meningococci expressing the P1.4 PorA was typed using MLRT and results were confirmed using MLST.
Results and Discussion: Serotyping and *porB*-PCR-AREA demonstrated that the common *porB* types in serogroup B meningococci isolated in New Zealand during 1989 through 1999 were 4 (73%) and 14 (9%). Type 14 meningococci were isolated more frequently after 1992 and meningococci containing *porB* other then type 4 and type 14 became more prevalent after 1995. MLRT and MLST were used to assess the clonal relationship of meningococci causing New Zealand's meningococcal disease epidemic. The increased levels of disease are due to meningococci with restriction type 42 (RT-42) and RT-154, which accounted for 52% and 39% respectively of the sample analysed. RT-42 and RT-154 are found in meningococci with sequence type 42 (ST-42) and ST-154, which are subclones of the hypervirulent ST-44 complex. RT-42 and RT-154 profiles were found in meningococci with both *porB* type 4 and 14.
Conclusion: Type 4 and type 14 were the most common *porB* types found in the serogroup B meningococci with the P1.4 PorA causing New Zealand's meningococcal disease epidemic. These meningococci are predicted to be ST-42 and ST-154, which are subclones of the hypervirulent ST-44 complex. Comparison of *porB* type and MLRT predictions indicate that type 4 and type 14 meningococci do not have distinct clonal origins.

High-throughput genetic characterisation of meningococci for large-scale population studies

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Summary: In a multi-centre investigation of meningococcal carriage before and after immunisation with the MenC conjugate vaccine, over 9,000 carried meningococci were isolated from British schoolchildren aged 16-18 years between 1999-2002. Following microbiological identification, high throughput sequencing methods and customised sequence analysis software were developed to characterise this isolate collection genetically at seven loci (Multilocus Sequence Typing). This work is scheduled for completion within a three-year period.

Methods and Results: Following the serological characterisation of isolates at the Meningococcal Reference Unit, killed cell suspensions are transported frozen to the Medawar Building. Samples are each given a unique bar code and entered into an electronic study database, before being dispensed into bar coded 96-well microtitre plates. PCR amplification of target genes and nucleotide sequencing are carried out using a robotic liquid handling platform before the sequences are determined by a 96 capillary automated DNA sequencer. Automated scripts are used to assemble double stranded gene sequences and to assign allele numbers at each locus by interrogating the MLST sequence databases. Output files are imported into the study database, which automatically updates alleles, sequence type and clonal complex of each isolate.

Using these methods, full MLST characterisation at a rate of 95 meningococcal isolates each week - a total of >665kb (332kb double stranded finished sequence) - is possible.

Conclusions: Many questions concerning the populations of highly diverse bacteria, such as *Neisseria meningitdis,* require large nucleotide sequence-based data sets. Semi-automated sequence determination and allele designation provides a rapid, accurate, cost-effective and auditable means of characterising and managing such datasets. These methods are a critical primary step for subsequent genetic and phylogenetic analyses and can also be transferred readily to reference laboratories for routine epidemiological surveillance.

Neisseria meningitidis: genomic diversity and conservation

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Neisseria meningitidis is an important human pathogen, responsible for septicaemia and meningitis, and the infection is often fatal. This bacterial species is known to be very diverse in physical construction of the genome, due largely to its natural competence for picking up exogenous DNA. However, based on our previous observations with enteric bacteria that the basic physical structure of the bacterial genome is highly conserved, especially in genome size and in gene order, we anticipated a comparable level of conservation in *N. meningitidis*. We collected over 200 wild type strains of *N. meningitidis* representing broad geographic regions including the Caugant set of 107 strains. Using the endonuclease I-*Ceu*I, which cuts only in rRNA genes, and pulsed-field gel electrophoresis techniques, we determined genome structure for these bacteria and found striking genome structure conservation. Most of the 200 strains could be divided into one of three genome structures according to their similarity. Strain Z2491 represented about 80 strains of the same genomic structural type (genome type I), with a genome size of about 2200 kb and the four *rrn* operons being spaced 1450 kb, 300 kb, 250 kb and 200 kb from one another. MC58 represented a second genome type (genome type II), with a genome size of about 2270 kb and the four *rrn* operons being spaced 1420kb, 400kb, 200 kb, and 250 kb from one another. A third genome type (genome type III) was represented by strain G2136 of the Caugant set with a genome size of 2130 kb and a spacing of the four *rrn* operons of 1500 kb, 300 kb, 130 kb and 200 kb between them. Other genome types altogether consisted of about 10% of all examined strains. Although Genome type I was seen mainly in Serogroup A strains, it was also seen in other serogroups. Similarly, genome types II and III were seen mainly in Serogroups B and C, respectively, but they were also found in other serogroups. We found phylogenetic divergence among *N. meningitidis* strains in different genome types by 23S rDNA comparisons. We are making correlations of grouping these bacteria by genome structure with groupings by other methods, e.g., multi-locus sequence typing. We are also comparing genomes of *N. meningitidis* with other *Neisseria* spp.

Rapid screening of a single nucleotide polymorphism in the *Neissseria meningitidis fumC* gene using MALDI-TOF

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The ability of matrix-assisted laser desorption-ionisation time of flight (MALDI-TOF) mass spectrometry to quickly and accurately monitor emerging clones of *Neisseria meningitidis* was investigated. A particularly virulent clone of the electrophoretic type (ET)-37 complex, known as ET-15, became more prominent during the 1990's until the introduction of the meningococcal serogroup C conjugate (MenC) vaccine. A single nucleotide polymorphism (SNP) has been identified, at position 640, in the *fumC* gene of meningococci from ET-37 complex strains. This SNP enables the hypervirulent ET-15 strain to be distinguished from other ET-37 complex strains. Potential ET-37 complex strains, collected by the Scottish Meningococcus and Pneumococcus Reference Laboratory, and isolated between 1977 and 2002, were analysed by MALDI-TOF for this polymorphism. MALDI-TOF proved to be an effective method for the detection of the ET-15 *fumC* SNP giving valuable epidemiological information on the emergence and spread of this strain in Scotland over the past twenty-five years.

Dr. SC Clarke is also affiliated with the Faculty of Biomedical and Life Sciences, University of Glasgow.

Analysis of phase-variation of gonococcal *pgtA* from a pool of new DGI isolates

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Introduction: The *pgtA* gene from *Neisseria gonorrhoeae* (GC) codes for the galactosyl transferase that forms the αGal(1à3)GlcNAc bond of pilin glycan. Remarkably, GC *pgtA* can be found in two mutually exclusive allelic forms, with (Pv+ *pgtA*) and without (Pv- *pgtA*) the poly-G mediated phase-variation (Pv). Pv of *pgtA* likely facilitates dissemination of GC because Pv+ *pgtA* and Pv- *pgtA* mainly associate with disseminated gonococcal infection (DGI) and uncomplicated gonorrhea (UG), respectively. All twenty-four DGI isolates that we analyzed earlier carried Pv+ *pgtA*. However, Pv+ *pgtA* was found in only 8 out of 28 UG specimens. In spite of these results, we could not conclude yet that all DGI isolates carry Pv+ *pgtA* as it is possible that some rare DGI isolates may carry Pv- *pgtA*. However, these rare isolates should be identified if a large number of DGI isolates are screened. Therefore, we are currently testing a large collection of new DGI isolates (with ~150 specimens).
Materials and Methods: Isolates were obtained from several hospitals. After growth on agar plates, GC were lysed by boiling and RNA was removed by RNase to obtain the genomic DNA for PCR. Using a *pgtA*-specific primer-pair, a ~450 bp PCR fragment spanning the poly-G was amplified. The *pgtA* amplicons were gel extracted. The Pv- and the Pv+ alleles were then distinguished by restriction analysis of the amplicons using *Bsr*BI enzyme, which cleaves the Pv- amplicon into two fragments but not its Pv+ counterpart because *Bsr*BI site maps at the GGGACGGGG sequence of the Pv- allele.
Results and Discussion: This study is currently under progress and, the results described below are based on the data obtained until now, which is from analysis of only a small fraction of the entire pool of isolates (~ 20% of all isolates to be tested). Therefore, conclusions presented here may change later. However, there is a good possibility that the current trend of our results will be maintained. Until now, the data from our new screening tallies well with our data from the 24 DGI isolates used earlier. We are confident that our entire analysis will be completed well ahead of the dates of the conference and we will present a comprehensive assessment of the role of Pv of *pgtA* in dissemination of GC.
Conclusion: Overall, based on all available results, the Pv of *pgtA* appears to tightly correlate with DGI. To date, all DGI organisms were found to carry the Pv+ *pgtA*. Thus, the role of *pgtA* Pv in dissemination of GC continues to appear very important, if not essential.

Gene-complement comparison of *N. gonorrhoeae* sequence strain FA1090 and laboratory strain FA19, using a *Neisseria* microarray

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Introduction: A microarray has recently been constructed using maximally conserved regions for genes from the complete genomes of *N. gonorrhoeae* strain FA1090, *N. meningitidis* strain MC58, and *N. meningitidis* strain Z2491, as well as additional genes identified from *N. gonorrhoeae* strain MS11. The design of this array lends itself to the investigation of the gene complements of strains other than those to which the microarray was designed. The sequenced strain of *N. gonorrhoeae* is FA1090. We have used the array to assess the similarity of this sequenced strain to strain FA19, a widely used experimental strain.
Materials and Methods: Chromosomal DNA from FA1090 and FA19 was labelled with Cy3 and Cy5, respectively, by direct incorporation of labelled dCTP. Labelled DNA was hybridized to the microarray slide at 65°C overnight, and washed using stringent conditions. The slides were scanned using a GenePix3000, and hybridization patterns were analyzed and compared using GenePix Pro v3.0.
Results and Discussion: Twenty-six probes were identified that hybridized the Cy3 labelled FA1090 DNA, but not the Cy5 labelled FA19 DNA. Due to duplications within the FA1090 genome, this represents 29 genes that are either present in FA1090 but not in FA19, or are too divergent in FA19 to hybridize to the probe under these conditions. These genes include *pilC*1, which is a gene known to show significant strain-to-strain divergence, and probably acts as a measure of hybridization stringency. The remainder of the genes identified cluster into 11 distinct regions that include phage proteins, restriction modification systems, and a type IV pilus-associated proteins. Unlike other arrays that can only address presence or absence with reference to a single index gene set, 68 probes were identified that hybridized to the FA19 DNA, that are not present in strain FA1090. Most of these (61 of 68) are part of the island of gonococcal genes described from *N. gonorrhoeae* strain MS11, from which the probes were designed. The seven additional sequences identified were derived from the meningococcal gene sets, including *lbpB*, which encodes lactoferrin binding protein B, previously reported to be present in strain FA19. The remainder are genes of unknown function.
Conclusion: There are readily detectable gene complement differences between the sequenced gonococcal strain FA1090 and strain FA19. This approach identified regions that can be sequenced directly to fully characterize the detected differences. This approach will be increasingly effective with the addition of strain-specific genes from both meningococcal strain FAM18, and other sources. Although these results are preliminary, they indicate that even for parts of the neisserial population that are considered to be highly clonal by other methods, such as the gonococci by MLST, their gene complements vary and can be used to differentiate strains.

Sequence variation in *Neisseria gonorrhoeae* and *Neisseria meningitidis* in the normally highly conserved division cell wall gene cluster

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Introduction: A cluster of eighteen open reading frames (ORFs) constituting the division cell wall (*dcw*) synthesis cluster has been identified in the pathogenic *Neisseria* spp. Three of these ORFs are not homologous to conserved *dcw* cluster genes in other bacterial species. One of these, *dca*, has been shown to be essential for natural transformation in *Neisseria gonorrhoeae*. The regions of the *dcw* cluster containing the other two atypical *dcw* ORFs, *dcaB* and *dcaC* display unusual inter-strain and interspecies variability.
Materials and Methods: A collection of *N. gonorrhoeae* and *N. meningitidis* strains were investigated for *dcw* feature variability using PCR and sequencing. Transcription was assessed using RT-PCR. Promoter activity was evaluated using the pLES94 promoter reporter plasmid.
Results and Discussion: Immediately 3´ of *dcaB* is a Correia repeat enclosed element (CREE), present in all *N. gonorrhoeae* strains tested but absent in some strains of *N. meningitidis*. This element has been suggested to act as a transcriptional terminator, atypical of *dcw* clusters. This study successfully generated an RT-PCR product spanning the *dcaB-mraY* intergenic region, demonstrating that transcriptional termination does not occur in this location. *dcaC* contains a 108 bp tandem repeat, which is present in different copy numbers in the meningococcal strains examined, although not in the gonococcal strains. There are two putative promoters within the *dcw* cluster, one in the CREE and one 5´ of *dcaC*. The presence of internal promoters other than those at the 3´ end of the *dcw* cluster has not been demonstrated in other species, although one in a similar location to that 5´ of *dcaC* has been suggested in *E. coli*. We find that the putative neisserial promoter 5´ of *dcaC* is not present in all of the meningococcal strains studied, indicating that neither putative promoters are conserved features of the neisserial cluster. The second putative promoter is preceeded by an inverted pair of uptake signal sequences, previously suggested to act as another transcriptional terminator within the cluster. However, successful RT-PCR of the *mraY-dcaC* intergenic region is presented in this study. The two putative promoters were evaluated in a promoter reporter construct for activity.
Conclusion: The first of the unique neisserial *dcw* genes is *dca*. The other two, *dcaB* and *dcaC*, are associated considerable variability between strains and represent the most extensive variation observed to date in this generally highly conserved cluster of essential genes. These elements appear to be more conserved in the gonococcal strains investigated. Variability in the *dcw* cluster of the *Neisseria spp.* indicates a greater capacity for plasticity in this region compared to other species.

Identification by genomic comparison of a genetic island associated with hyperinvasive *Neisseria meningitidis*

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The combination of whole genome comparisons with inferences from population analyses provides a novel approach to the investigation of bacterial pathogenesis. Here, we have used this approach to investigate *Neisseria meningitidis*, a common commensal inhabitant of the human nasopharynx, which nevertheless occasionally causes life-threatening meningitis and septicemia. Genetic analysis of isolate collections derived from cases of invasive disease and asymptomatic carriage have indicated that a limited number of the many lineages of this highly diverse organism are responsible for most cases of meningococcal disease. In order to identify possible genetic determinants that confer a pathogenic phenotype to these 'hyperinvasive' organisms, we made a genomic comparison of 50 meningococcal isolates classified as either hyperinvasive or non-invasive. These comparisons identified a genetic island of approximately 8kbp which was present in 29 of the 30 isolates classified as invasive, but none of the 20 non-invasive isolates examined. The unusually low GC content of this island, together with the presence of an integrase gene, suggested that this may be a mobile genetic element which has been acquired by lateral gene transfer. Determining the functions encoded by this 'meningococcal disease-associated' element, will contribute to an improved understanding of the relationships of commensalism and pathogenicity in the biology of this organism and presents the prospect of targeting disease, rather than asymptomatic carriage, in disease prevention strategies.

SESSION VIII

Antimicrobial resistance and epidemiology

High levels of erythromycin and azythromycin resistance in *Neisseria gonorrhoeae* due to the *mtrCDE*-encoded efflux system

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Introduction: Several mechanisms, such as the action of efflux pumps, target site modification by methylases encoded by *erm* genes or antibiotic inactivation due to enzymatic processes, are involved in macrolide resistance in bacteria. In *Neisseria gonorrhoeae* it has been reported that the overexpression of the *mtrCDE*- encoded efflux pump results in resistance to hydrophobic agents. Included in these agents are the macrolide antibiotics, erythromycin (E) and azithromycin (A). Previous work has shown that mutations in the *mtrR* promoter region, such as a single base pair deletion in a 13 bp inverted repeat sequence or a dinucleotide (TT) insertion, can be responsible for decreased susceptibility of gonococci to both E and A. In strains bearing these mutations, the minimal inhibitory concentration (MIC) E was up to 2 μg/mL and that of A was up to 0.5 μg/mL. Our previous work indicated that Mtr phenotype was common among clinical isolates of *Neisseria gonorrhoeae* in Uruguay. We have recently examined certain isolates (e.g., strain 1011) that display higher levels of resistance to E (4 μg/ml) and A (1.0 μg/ml) to determine what role the *mtrCDE*-encoded efflux might perform. This was considered important because the *ermF* gene has been previously implicated in such resistance levels.
Materials and Methods: We used techniques of transformation, polymerase chain reaction (PCR) amplification of chromosomal DNA, Southern blot hybridizations, DNA sequencing and antibiotic susceptibility testing to conduct the work.
Results and Discussion: Using *ermF*-specific oligonucleotide primers we were unable to amplify an *ermF*-like sequence from strain 1011 or other gonococcal isolates but were able to obtain a product from a *Bacteroides fragilis* isolate known to harbor *ermF*. Southern blot hybridization analysis using *Cla*I-digested chromosomal DNA and the *ermF*-specific probe also failed to reveal the presence of *ermF* in strain 1011. Accordingly, we examined whether the *mtrCDE*-encoded efflux pump might explain the levels of A and E resistance expressed by strain 1011. DNA sequence analysis of the *mtrR* promoter region revealed the presence of a single bp deletion in the 13 bp inverted repeat sequence between the -10 and -35 regions of the mtrR promoter. As this mutation has been previously shown to result in over-expression of the *mtrCDE* operon and increased resistance of gonococci to A and E, we constructed by transformation mutants of strain 1011 bearing an insertionally-inactivated *mtrC* gene. We found that *mtrC::Km* transformants of strain 1011 displayed hypersusceptibility to both A and E. This result suggested that the mechanism of A and E resistance in strain 1011 is due to the action of the MtrC-MtrD-MtrE efflux pump.
Conclusion: This is the first report of a gonococcal clinical isolate that expresses high levels of A and E resistance due solely to the MtrC-MtrD-MtrE efflux pump.

Gonococcal urethritis and cervicitis caused by CZRNG (cefozopran-resistant *Neisseria gonorrhoeae*) – clinical failure of cases treated with expanded spectrum cephems, fluoroquinolones and minocycline

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Introduction: A previous study reported the incidence of clinical failures in gonococcal urethritis treated with cefdinir and aztreonam. For the *N. gonorrhoeae* isolates from such clinical failure cases, high-level MICs of cefdinir, aztreonam, and other beta-lactams were observed. These isolates showed high-level MICs against cefozopran (8-16 mg/L). We have reported that it may well be profitable to use cefozopran to define these isolates. We therefore called these isolates CZRNG (cefozopran-resistant N. gonorrhoeae). We have experienced more clinical failure cases in patients suffering from CZRNG.
Materials and Method: From 1999 onward, this study was intended for 65 episodes detected CZRNG isolates before treatment and performed gonococcal culture test at 3 to 9 days after treatment. The MICs of various antimicrobials against the isolates were determined by the two-fold serial agar dilution method using GC agar with IsoVitalX.
Results: Oral expanded spectrum cephems did not eradicate CZRNG from all 11 cases (4 female, 9 male) treated. The expanded spectrum cephems were cefixime (400mg, 3 days), cefcapene (300mg, 3-14days), cefdinir (300mg, 3days), and cefpodoxime (200mg, 3days). The MICs of cefixime, cefcapene, cefdinir, and cefpodoxime against these CZRNG were ranged 0.25 to 0.5, 2, 1, and 0.5 to 4 mg/L. Fluoroquinolones used in 10 cases failed to eradicate CZRNG. These CZRNG isolates acquired not only Beta-lactams but also fluoroquinolones (MIC of ciprofloxacin against these isolates were 0.25 to 32 mg/L). All 3 cases treated with minocycline showed persistently CZRNG isolates after treatment (MIC of minocycline: 0.5 mg/L). A single dose of 1g of cefodizime eradicated CZRNG isolates from all 27 patients treated. A single dose of 2g of spectinomycin also eradicated CZRNG from all 8 patients treated.
Conclusion: In *N. gonorrhoeae* the emergence of expanded spectrum cephems-resistant isolates was observed, and it is a serious concern. A more serious problem is that these isolates were already resistant to non-beta-lactam antimicrobials, such as fluoroquinolones, tetracyclines. Appearance of CZRNG has made being unable to treat oral antimicrobials in gonococcal infection. Ceftriaxone, cefodizime, and spectinomycin have strong activity against CZRNG.

In Japan ceftriaxone has not been allowed to be used against gonococcal urethritis. Therefore, in Japan, patients with gonococcal infection caused by CZRNG should be administered cefodizime or spectinomycin.

Phenotypes of *Neisseria meningitidis* strains isolated in Cuba (1982-2001)

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Introduction: Cuba suffered a meningococcal disease (MD) epidemic which began in 1976. At the beginning, due to a prevalence of serotype C cases, in 1976 the whole population between the ages of 3 months to 19 years old was vaccinated with the French A-C vaccine. This caused a decrease of group C and an increase in serotype B that was second in frequency[1]. This situation leads to the development of VA-MENGOC-BC®, the Cuban vaccine composed of OMP of meningococcus B and PC of C. A phase III trial that was conducted between 1987 –1988 in children ranging from 10-16 years old, who attended boarding schools, showed an efficacy of 83%. In 1991, the vaccine was included to the National Immunization Program of Cuba.
The characteristics of the meningococcal strains based on the identification of its main surface structures (serotype, sero/subtypes and immunotypes) are a fundamental information for the epidemiological surveillance of MD. In order to make our contribution to this surveillance, we identified and compared the phenotypes of the isolated strains in Cuba taken from patients and carriers during the epidemic and 10 years after VA-MENGOC-BC® was included in the National Vaccination Program.
Materials and Methods: 353 strains from the Culture Collection of the Finlay Institute were studied. Of these, 113 belong to patients and 240 to carriers. Among those belonging to patients, 98 were isolated between 1983-1991, 122 between 1998-1999 and 18 strains from 2001. The identification of genera, species and serotypes was done using conventional methods and the apiNH system (bioMerieux) (4). Whole cell ELISA with monoclonal antibodies was used for the identification of sero/subtype and immunotypes, including AcMP1.19 in strain groups from the epidemic period.
Results: Among the strains from the epidemic period, 3 (0.85%) showed an atypical behavior when challenged with "deficient strains" sugars. During the epidemic there was a prevalence of group B among the patients (67%) and among carriers (68%). Group C was only identified in 4.09% of invasive strains. The phenotype B:4:P1.19,15:L3,7,9 was prevalent among patients and carriers of the epidemic. Due to the low incidence of MD in Cuba during the past years only 15 patient strains have been studied. Of these 83.3% were B.4:P1.15:L3,7,9. The isolated strains from carriers in the past years had a totally different situation. There were diverse phenotypic associations with a prevalence of strains NA, NT and NST. In both groups and stages, the immunotype L3,7,9 showed a higher percentage.
Conclusions: The study with methods not available during the epidemic enabled a greater characterization of strains belonging to patients and carriers. They also enabled a comparison between these and the strains isolated 10 years ago, after VA-MENGOC-BC® was included in the Immunization Program.

Neisseria meningitidis carriers in a day-care center in the city of Havana

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Introduction: The bacterial flora in the superior respiratory tract of humans is variable. The microorganisms appear, are eliminated and reappear during different stages of life. Concerning children in day-care centers, respiratory infections are frequent due to the close contact among them. Also the abusive use of antibiotics contributes to the transmission of respiratory pathogens. Meningococcus carriers are the transmission and dissemination mechanism of this microorganism. Although the incidence of the meningococcal disease in Cuba is currently very small, research on prevalence and characteristics of isolated strains in risk groups has given the opportunity to deepen the knowledge of this disease, this being one of the duties of the Finlay Institute. Due to the fact that studies of carriers and possible risk factors are scare in Cuba during a non-epidemic period, we conducted a study to learn the existing situation in a day-care center in Havana.
Materials and Methods: We attained the approvals of the Ethics Committee, Municipal and Provincial Committees of Public Health and Education and the authorization of parents or tutors. Out of 203 children enrolled, 160 ranging from 8 months to 6 years of age fulfilled the pre-established requirements. A survey was also conducted to detect possible risk factors associated to the meningococcus carrier (age, sex, crowded living conditions, smokers in the family that smoked at home, tonsillectomy, vaccination background, associated bacterial flora and others). Samples were taken from the rear nasal-pharyngeal area with sterile swaps during the months of May and June 2001 and immediately after were incubated in: Agar Mueller Hinton plus bovine fetus blood serum at a 5% and a VCN inhibitor (for *N. meningitidis* and other *Neisserias*), Agar Mueller Hinton with goat blood defibrinated to 5% (for other pathogens) and Agar Brain Heart Infusion with Hemine, NAD and Bacitracine (for *Haemophilus spp).* The identification of *N. meningitidis, N. lactamica, M. catarrhalis, Haemophilus spp* was preformed by the *api*NH system (bioMerieux). Conventional techniques were used to identify microorganisms obtained in Agar Blood. For meningococcus the following were also used: sero-grouping with antisera (Difco), classification of sero/subtypes and inmunotypes by whole cell ELISA with monoclonal antibodies and susceptibility to penicillin (PEN) by the dilution method in agar according to NCCLS.
Results and Discussion: The percentage of carriers (4.3) similar to other countries (5.6) was inferior to the one in Cuba during the epidemic. There was no statistical significance between carriers and risk factors. However, 71.4% were 4 year old children and 85.7% were male. We also found that 71.5% were NA:NT: P1.6:13,7,9 and 85.7% were moderately resistant to PEN (CMI 0.12-1μg/ml). *Haemophilus spp* was also detected (92.5%), *S. pneumoniae* (77.5%), *N. lactamica* (47.5%) and others.
Conclusions: The detection of meningococcal carriers was conducted in Cuba for the first time in a day care center which also included other microorganisms that frequently colonize the nasal- pharynx.

Mechanisms of meningococcal reduced susceptibility to penicillin

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Early antibiotic treatment is highly recommended whenever meningococcal infection is suspected. *Neisseria meningitidis* was extremely sensitive to penicillin up until the late 1980s, since then meningococcal strains with reduced susceptibility to penicillin G (pen^I), with MICs ranging from 0.125 to 1 µg/ml, have been increasingly reported in several countries and penicillin resistant strains (pen^R) with MICs > 1µg/ml are now emerging. This phenotype is thought to be due to changes in target penicillin-binding protein 2 (PBP2), encoded by an altered *penA* gene. Mosaic structures in *penA* result apparently from horizontal DNA exchange by transformation between commensal species of *Neisseria* and *N. meningitidis*. The alterations in *penA* have been shown to lead to a decreased affinity for penicillin of PBP2 and hence explaining the pen^I phenotype. In spite of these data, it is possible that other mechanisms play a role especially in causing high levels of resistance.

PBP1 and PBP3 did not seem to be involved, even for meningococcal strains with MICs > 1µg/ml. Indeed PBP1, encoded by the *pon*A gene, and PBP3 showed no decreased affinity for [^{3}H] penicillin G. The study of the restriction fragment length polymorphism of the *pon*A gene revealed a minor polymorphism which was not correlated with susceptibility to penicillin. At the transcriptional level, no difference was observed among *penA* genes from pairs of isogenic strains with pen^S and pen^I phenotypes. DNA sequencing revealed that *penA* alterations are localized in the 3' half of the gene encoding the transpeptidase activity of PBP2. Alterations in *penA* sequence are hence expected to alter the enzymatic activity of PBP2 resulting in modifications in the bacterial wall. Using biochemical methods (infrared spectroscopy and high performance liquid chromatography), changes in peptidoglycan structures could be observed among pairs of isogenic strains with pen^S and pen^I phenotypes. The modification of peptidoglycan structures may be directly responsible for reduced susceptibility to penicillin G rather than the decreased affinity of the altered PBP2 for penicillin G.

penA gene sequencing in *Neisseria meningitidis*: the key to success in the definition of penicillin breakpoints

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Introduction: Methods recommended for the susceptibility testing of *Neisseria meningitidis* include broth microdilution and agar dilution. However over the last years E-test is frequently used for meningococcal susceptibility analysis. E-test uses a continuous antibiotic concentration gradient and as result Minimun Inhibitorium Concentrations (MICs) obtained are more precise than conventional MICs based on discontinuous two fold serial dilutions.

Penicillin resistant meningococcal strains are extremely rare, and related with b lactamase production. However those defined as intermediate (Pen^i) are relatively frequent. The molecular analysis of these strains (Pen^i) evidenced that these show altered forms of penicillin-binding protein (PBP) 2, due to genetic events of *penA*, the gene which encodes this protein.

So far 0.12 μg/mL has been used as cut off point for Pen^i definition in *N. meningitidis*.

So strains showing MIC ≤0.06 μg/mL are defined as susceptible and when MICs ranged betweeen 0.12 to 1 μg/mL are considered as intermediate. When E-test is used strains possessing MICs between 0.06 and 0.12 μg/mL can be found but so far they can not be properly categorized. Because the molecular basis of Pen^i in *N. meningitidis* is based on detection of mosaic *penA* genes, the aim of this study was to establish the Pen^i breakpoint by *penA* gene sequencing.

Material and Methods:

Strains: Forty-nine meningococcal strains isolated from cases of meningococcal disease were included.

Susceptibility testing: Susceptibility to penicillin G was determined by E-test in Mueller-Hinton agar supplemented with 5% whole defibrinated sheep blood.

penA gene sequencing: A 1.4 Kb DNA fragment encoding the *penA* transpeptidase domain was amplified from the chromosomal DNA of *N. meningitidis* by PCR. The PCR product was purified and sequencing was done in both directions by using Big Dye fluorescent terminators (PE Applied Biosystems).

Results: The results obtained in the susceptibility assay were as follow:

MIC (μg/mL)	Number of strains
0.023	8
0.032	4
0.047	1
0.064	10
0.094	16
0.125	10

All the strains showing MIC ≤ 0.047 μg/mL possessed *penA* alleles previously associated with susceptible strains. Mosaic *penA* alleles were identified in all the strains showing MIC ≥ 0.094 μg/mL. Among those strains with MIC of 0.064 μg/mL (n=10) two groups were defined: five of them had mosaic *penA* alleles and the other ones (n=5) *penA* alleles related with those found in susceptible strains.

Conclusion: According to these results >0.064 μg/mL should be used as Pen^i breakpoint. The heterogeneous situation found among strains with MIC of 0.064 μg/mL determines that isolates showing this MIC should be defined as susceptible in order to not over-estimate the Pen^i meningococci population.

Por variable region typing of *Neisseria gonorrhoeae* using culture and non-culture based specimens

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Introduction: A rapid, simple, reproducible method of typing gonococcal (GC) isolates has been developed. This method specifically differentiates the sequences encoding antigenic regions of the Por (PI) protein, however as a DNA based method, it avoids the problems of monoclonal antibody based assays. To examine the utility of this method as an epidemiologic tool and in investigations of the gonococcal Por protein, a collection of partner strains and disseminated gonococcal infections (DGI) strains were typed. In addition, application to non-culture based samples was explored.
Materials and Methods: Checkerboard hybridization was used to determine the *por* VR types of 101 partner strains with previously determined auxotype and serovar (A/S type), and 53 DGI isolates (1, 2). Inhibiting and non-inhibiting urine spiked with 10 to 10^6 cfu per ml was used to examine the ability to type amplified *por* DNA from urine using single or nested PCR.
Results: The partner strains consisted of 25 PIA strains of 3 PIA *por* VR types (16, 6, and 3 strains respectively) and 76 PIB strains with 20 PIB *por* VR types. The discriminatory power of *por* VR type was the same as A/S type among PIB strains, however the two methods grouped the strains differently. Also, *por* VR type identified 7 discrepant partners compared to 4 based on A/S type. Accuracy of the *por* VR type was confirmed by *por* sequencing. Two common *por* VR types accounted for 33% of PIB strains; 14 *por* VR types were unique to single strains or partners.

42 PIA and 11 PIB DGI strains were typed. One PIA *por* VR type was found in 36 (85.7%) of the PIA strains, the remaining PIA DGI strains had the same or similar sequence type in loops 1 and 6. The 11 PIB DGI strains were more diverse with 7 *por* VR types, however, 9 of 11 strains had closely related loop 5 sequences.

Sufficient PCR product from spiked urine was obtained for *por* VR typing from an estimated single copy (10 cfu/ml) using a nested procedure, and from samples with 10^4 cfu/ml using a single PCR method. Preliminary experiments with clinical samples indicate that *por* VR typing can be conducted using urine from patients identified by LCR as GC + .
Conclusion: *Por* VR typing is an accurate, discriminatory gonococcal typing method that can be conducted when sequencing is not available or is too costly. In addition, specific information about individual regions of the Por protein are obtained which may contribute to studies of structure/function, host immune responses, or transmission. A proposed scheme relating serovar to *por* VR type or *por* sequence has been developed based on these and previous studies, and the published literature.

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Prevalence of bacterial meningitis in Nepal

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Introduction: Apart from epidemics at least 1.2 million cases of bacterial meningitis occur worldwide every year. Of these, 135 000 are fatal. It is estimated that the triad of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* is responsible for over 80% of all cases. To obtain reliable data on the aetiology of meningitis in developing countries is difficult. From Nepal we only have an estimate from a meningococcal epidemic in 1983/84. This study was initiated to determine the relative importance of *N. meningitidis, H. influenzae* and *S. pneumoniae* as causative agents of bacterial meningitis in Nepal and to see whether other agents are causing bacterial meningitis.
Material and Methods: The data collection took place at United Mission Tansen Hospital, Nepal, from 20th of October 2000 to 20th of March 2001. All patients admitted to the hospital, for whom a lumbar puncture was performed and the result gave a pathologic cerebrospinal fluid (CSF), defined as WBC in CSF > 5 /ml, were included in the study. In addition to the methods already employed by the hospital, three other diagnostic tools (C-reactive protein (CRP), bacterial antigen latex agglutination and polymerase chain reaction (PCR) for *N. meningitidis, H. influenzae* and *Streptococcus*) were used.
Results and Discussion: Seventy-five patients were included in the study (age range 11 days-66 years). Sixty-one (79,1%) patients had been ill for 2 days or more and 46 (61,3%) patients (N=74) had been given antibiotics before coming to the hospital. Nine of the 75 patients died. Agglutination test was performed on CSF of 71 patients and one was positive for *S. pneumoniae*. PCR tests were done on CSF of 74 patients. In 5 patients, the PCR was positive, 1 *N. meningitidis*, 3 *Streptococcus* and 1 *H. influenzae,* respectively. The patient with a positive agglutination test was also positive for *Streptococcus* on PCR. The few positive PCR results might be due to the fact that agents other than the three tested are responsible for meningitis in Nepal or that pre-admission treatment with antibiotics had eliminated DNA from the CSF. The interval of time from administration of antibiotics until DNA no longer can be found in the CSF is not known, but 4 of the patients with PCR-positive CSF had been treated with antibiotics for 1-3 days before admission to the hospital. Patients with a positive PCR had CSF WBC counts between 60-2800 cells/ml and CRP values between 10-200 mg/l showing that the diagnostic tools used in industrialized countries might be less useful in other parts of the world.
Conclusion: The use of antibiotics before coming to the hospital in developing countries might be lifesaving for the patient, but makes epidemiological surveillance of causative agents difficult, increases the possibility of antibiotic resistance and makes it difficult to evaluate the value of introducing new vaccines.

Non-culture characterisation of *Neisseria meningitidis* using MLST and its application in outbreak situations

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Introduction: Enhanced surveillance of meningococcal infection is of paramount importance with the future immunisation of large populations across Europe with conjugate meningococcal vaccine and to monitor the emergence of hypervirulent lineages.

Increasing numbers of cases of meningococcal infections are confirmed by non-culture for which no isolate is available. In the year 2000/2001, 46% of laboratory confirmed cases reported by the Meningococcal Reference Unit, of the Public Health Laboratory Service of England and Wales (PHLS), were confirmed by nucleic acid amplification (PCR) only.

Accurate strain identification has been improved by the development of nucleic acid sequence-based technologies, specifically Multilocus Sequence Typing (MLST). MLST is based upon the sequence analysis of approximately 500 bp fragments of seven selectively neutral house keeping genes. MLST is a PCR-based method and can be adapted for non-culture strain identification as required for non-culture confirmed meningococcal infection.

Materials and Methods: A nested touchdown PCR was developed which improved the sensitivity for detecting each of the seven house keeping gene loci to the equivalent of less than one colony-forming unit per PCR. The sensitivity of the nested PCR protocol was estimated using a dilution series of known concentrations of meningococcal organisms. The nested MLST PCR protocol was evaluated using a panel of twenty clinical samples (cerebrospinal fluids, serum/plasma or EDTA blood sample) from culture proven cases and which contained varying concentrations of meningococcal DNA as estimated by quantitative real time PCR. This data was then compared to that obtained from corresponding isolates.

Results and Discussion: The analytical sensitivity of the nested touchdown PCR protocol was estimated as one colony forming unit per PCR. The clinical sensitivity was estimated, using specimens from culture proven cases, to be 1-25 genome copies per PCR. From the analysis of the MLST sequence type (ST) obtained from the clinical sample from the culture proven cases the resultant ST was identical to that obtained from the corresponding culture.

This protocol was further evaluated using material from several outbreaks of meningococcal disease involving non-culture confirmed suspected cases. The nested MLST protocol proved sensitive enough to establish the identity of the causative organisms for each of the cases examined.

Conclusions: These results indicate that the adapted MLST protocol will enable the strain characterisation for cases of non-culture confirmed meningococcal infection. The adaptation of MLST for non-culture strain identification for meningococcal disease is important for enhanced surveillance.

Molecular characterization of the *Neisseria meningitidis* isolates causing meningococcal disease in Croatia in 2000

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Introduction: Based on clinical notifications, there were 41 patients with sporadic meningococcal disease in Croatia in the year 2000 (incidence 0.8/100,000 and mortality 0.04/100,000). Twenty-six cases were laboratory confirmed by isolation of *N. meningitidis* (Men) from blood and/or cerebrospinal fluid. This is the first study aiming to extensively molecularly characterize Croatian Men isolates and assess their relationships to major hypervirulent clonal groups.
Materials and Methods: Twenty-three of the 26 isolates were available for molecular characterization by MEE, PFGE (*Nhe*I), and sequencing of the 16S rRNA gene. Using 10 different primers in forward and reverse orientation, 4-7 fold coverage of approximately 1500 bp of the 16S rRNA gene was obtained.
Results and Discussion: Serogrouping of the isolates identified 17 (74%) to be serogroup B (MenB), four (17%) serogroup C (MenC), one (4%) serogroup W135 (MenW135), while the remaining isolate was nongroupable.

MEE demonstrated that only two of the 17 MenB strains belong to the ET-5 complex; both strains had 16S type 4 characteristically seen in strains of that complex. The remaining 15 MenB isolates were very diverse, exhibiting 12 different PFGE patterns and nine different 16S types; eight 16S types were newly described and identified in one to four isolates, each. Excellent correlation was observed between these three methods: more than one PFGE pattern was seen in isolates with identical 16S type (within or outside of ET-5 complex), but no individual PFGE pattern was associated with more than a single 16S type.

All four MenC isolates had decreased susceptibility to penicillin, and by MEE were shown to belong to the ET-37 complex. By PFGE, two very similar patterns were observed, but all four strains had a novel 16S type 91 that differs from 16S type 13, most frequently identified in MenC strains of the ET-37 complex, by only a single base.

A single MenW135 isolate represented the first invasive meningococcal disease caused by MenW135 strains in Croatia and, by all three methods used, was easily distinguished from the Hajj 2000 isolates.
Conclusion: Molecular characterization of 23 *N. meningitidis* isolates obtained from patients with sporadic meningococcal disease in Croatia in 2000 revealed a substantial level of diversity, with only one quarter of isolates belonging to the ET-5 or ET-37 complex. MEE and 16S rRNA gene sequencing agreed consistently regarding each isolates' association with either ET-5 or ET-37 complex.

Serogroups and susceptibility to penicillin of *Neisseria meningitidis* from cultured-confirmed invasive infection in Portugal

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Introduction: The purpose of this study has been to investigate the serogroups and susceptibility to penicillin of cultured-confirmed invasive strains of *Neisseria meningitidis*, isolated from blood or cerebrospinal fluid from patients in a hospital environment suspected of suffering from meningitis and/or septicemia.
Material and Methods: In all, 35 hospital departments in 27 hospitals throughout different regions of Portugal took part. The investigation was carried out over 12 consecutive months (from the 1 September 2000 to the 31 August 2001), after a two-month pilot study (July and August 2000) in 6 hospitals, which was included in the total analysed. The laboratory study involved 102 strains of *N. meningitidis*; 72/102 strains were isolated from invasive biological products sent to the Antibiotic Resistance Unit (ARU) of the National Institute of Health (NIH), originating from 611 patients; 30/102 strains, originated from a number of patients unknown, were isolated directly in 15 out of 27 hospital laboratories and subsequently forwarded to the ARU. Serogroups were determined by agglutination with specific antisera for all capsular polysaccharides of *N. meningitidis*. The minimum inhibitory concentration (MIC) to penicillin of 95 strains was determined by the agar dilution method according to NCCLS guidelines.
Results and Discussion: Analysis of the results shows that between July 2000 and August 2001 there was a greater frequency of serogroup C in Portugal (52.9%) in comparison with the other serogroups found, B (46.1%) and W135 (1%). Serogroup C predominated in the age groups from 1 to 15 years. Serogroup B predominated in children aged less than one year and in adults aged over 16. The seasonal distribution showed increasing isolation of meningococci in the autumn but also in winter and spring. The number of isolated strains from serogroups B and C was similar in the different geographical regions. The results further suggest that in 54/64 cases in which an invasive infection of *N. meningitidis* occurred there was no previous history of disease, the clinical diagnosis being primarily meningitis in those cases where serogroup C meningococci were isolated (18/37) and meningitis associated with sepsis in the case of serogroup B (15/29). Thirty-four out of 95 (36%) meningococci showed reduced susceptibility to penicillin (MIC≥0.125 mg/L): 26/34 (76%) belonged to serogroup C, 7/34 (21%) to serogroup B and 1/34 (3%) to serogroup W135.

This was the first time that an investigation, with an appreciable coverage of population and geographical areas, has been carried out in Portugal with the aims proposed. The results showed that is important to continue the surveillance of serogroups and penicillin susceptibility of *N. meningitidis* responsible for meningitis or sepsis in Portugal.

Characterisation of novel mutation patterns induced by sub-inhibitory fluoroquinolone exposure in target genes of *Neisseria gonorrhoeae*

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Introduction: Fluoroquinolones have been used extensively in a clinical setting due to their direct inhibition of DNA synthesis, which occurs by interaction of the drug with complexes of DNA and either or both of the target genes, DNA gyrase and topoisomerase IV. In *Neisseria gonorrhoeae*, resistance has been attributed to mutations within the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* gene, resulting in altered GyrA and ParC proteins. These altered proteins are unable to bind fluoroquinolones and the drug is therefore unable to inhibit DNA replication, resulting in decreased susceptibility or resistance.
Materials and Methods: Fluoroquinolone mutants of varying susceptibilities were generated by serial exposure of three clinical gonococcal strains to sub-inhibitory concentrations of ciprofloxacin and/or ofloxacin. Using genomic DNA extracted by the CTAB/NaCl protocol, regions of the DNA gyrase (*gyrA*, and *gyrB*) and topoisomerase IV (*parC*) genes of gonococcal wild-type and mutant strains were amplified by PCR. The presence of mutations in these target genes was determined by direct PCR sequencing and computer analysis.
Results and Discussion: Target genes from wild-type strains demonstrated no amino acid alterations when compared to the reference sequences, however, the GyrB of one strain possessed a pre-existing Asp-419→Asn alteration. While mutations were observed at previously described codons for all three target genes, a number of the alterations appeared to be novel amino acid changes, as well as occurring at codons not previously altered. With respect to GyrA, Asp-95→Asn; Ser-91→Tyr; and or Ser-91→Phe alterations were observed. Previously unreported changes identified at these codons included Asp-95→His and Ser-91→Ala. Alterations at Ala-75, Asp-80, Gly-83 and Val-93, which have not been previously described were observed in mutant GyrA sequences. With respect to the *gyrB* genes, both wild-type and mutant strains possessed the reported 42 bp insert. All G70 and G82 mutants sequenced, displayed an Asp-419→Asn alteration in GyrB. In addition, selected G70 mutants displayed alterations within the 42-bp insert, resulting in Glu-459 being altered to Asp. As has been previously described, only the high-level fluoroquinolone mutants (CIP and OF MICs; ≥ 0.5 – 1.5 and 8 – 32 μg/ml, respectively) had Asp-86→Gly alterations in ParC. Other novel alterations observed in ParC included those at codons Pro-67, Val-68 and Leu-138.
Conclusion: Gonococcal strains appear to acquire varied target gene alterations, regardless of sharing identical serotypes. Apart from *parC* alterations, the acquisition of mutations within *gyrA* cannot be correlated with specific MIC levels. Also, the presence of Ser-91 alterations within GyrA cannot be exclusively linked to cross-resistance to structurally unrelated antimicrobial agents. Instead cross-resistance was demonstrated by a number mutants possessing a variety of target gene alterations. Pre-existing *gyrB* alterations would facilitate the development of high-level fluoro-quinolone resistance and their presence within gonococcal populations should not be underestimated.

Alteration of outer membrane protein genes induced by sub-inhibitory fluoroquinolone exposure of *Neisseria gonorrhoeae* and their role in cross-resistance

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Introduction: Cross-resistance to structurally unrelated antimicrobial agents, including penicillins, tetracyclines, and cephems, has been detected in clinical isolates of fluoroquinolone resistant gonococci but not in gonococcal *in vitro* mutants. Studies involving *in vitro* mutants have not allowed the identification of the mechanisms implicated in this phenomenon, i.e., altered outer membrane proteins (OMPs) and lipooligosaccharides (LOS); instead it has been suggested that this cross-resistance resulted from mutations at chromosomal loci other than the target genes for fluoroquinolones.
Materials and Methods: The MICs to fluoroquinolones and a variety of structurally unrelated antimicrobial agents were determined for three sets of gonococcal mutants, generated by sub-inhibitory fluoroquinolone serial passage. Mutants were characterised with respect to their whole-cell protein, OMP-, and LOS- profiles. *por* and *opa* genes were amplified from mutants demonstrating varying levels of fluoroquinolone susceptibility. The PCR-RFLP profiles of wild-type and mutant *por* genes were obtained after *Cfo*I and *Msp*I restriction. Opa-types of wild-type and mutants strains were obtained following *Taq*I, *Hin*fI, and/or *Hpa*II restriction of *opa* fragments.
Results and Discussion: Fluoroquinolone exposure of the three wild-type strains resulted in mutants displaying varied susceptibility profiles. However, in all instances increased penicillin G and tetracycline MICs were observed. Whole-cell protein profiles of mutants belonging to the G82 subset displayed an Opa protein with an increased molecular weight compared to the wild-type strain. Examination of OMP profiles of all three groups revealed profiles, which were not significantly altered. While only a single G70 mutant displayed an altered Por protein, many mutants appeared to have increased expression of penicillin-binding proteins (PBPs) 2 and 3. LOS profiles of all three mutant sets were not significantly altered from their respective wild-type strains, with only the G70 mutants displaying varying expression of the high-molecular weight LPS molecules. *por* PCR-RFLP profiles allowed the identification of four mutants belonging to the G56 and G70 subsets mutants with altered *por* genes. This could be correlated with increased penicillin, tetracycline, and cefotaxime MICs. An altered *por* gene was sometimes accompanied by altered *opa*, however, this was not the rule. Although no *por* alterations were observed for G82 mutants, they displayed altered Opa-types, which could be correlated with the alterations observed in whole-cell protein profiles.
Conclusion: Sub-inhibitory fluoroquinolone exposure does result in altered *por* genes, which play an important role in cross-resistance to structurally unrelated antimicrobial agents, independent of active efflux. Cross-resistance in the absence of an altered *por* gene may be attributed to increased expression of PBPs 2 and 3. Whether the Opa and LPS alterations play a role in the cross-resistance phenotype or are simply involved in increased/decreased bacterial adherence and infection remains unclear. However, given its role in adherence, altered *opa* may facilitate the survival of mutants *in vivo*.

Induction of active ciprofloxacin efflux following exposure of *Neisseria gonorrhoeae* to sub-inhibitory fluoroquinolone concentrations

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Introduction: The increasing worldwide incidence of fluoroquinolone resistant-*Neisseria gonorrhoeae* strains is complicating the treatment of gonococcal infections. Since the MIC levels cannot be correlated solely with target gene alterations, it has been suggested that a number of mechanisms (decreased outer membrane permeability, and/or active efflux) function synergistically in order to produce fluoroquinolone resistance phenotype, although the mechanisms involved have not been fully elucidated.
Materials and Methods: A fully-susceptible wild-type gonococcal strain (G56) was serially passaged on sub-inhibitory concentrations of ciprofloxacin and/or ofloxacin. The accumulation of ciprofloxacin was assayed, with CCCP being used to identify active efflux. Mutants generated were characterised with respect to their whole-cell protein-, OMP-, and LOS-profiles. Genotypic characterisation included identification of GyrA, GyrB, and ParC alterations; *por* PCR-RFLP; Opa-typing; and PFGE analysis.
Results and Discussion: Of the 21 mutants generated (CIP and OF MICs; ≤0.002 – 0.032 and 0.003 – 0.094 μg/ml, respectively), three strains presented decreased fluoroquinolone susceptibility as well as cross-resistance not only to structurally related fluoroquinolones but to unrelated antimicrobials such as penicillin G, tetracycline, cefotaxime, erythromycin, and novobiocin. No significant alterations were observed in whole-cell protein-, and LPS profiles of mutants. Strain G56 O1G (ciprofloxacin and ofloxacin mutant) possessed a 66 kDa OMP, not observed in other mutant or wild-type profiles. Mutant G56 O1G possessed three alterations in *gyrA*, while G56 C1N possessed only a single alteration. Both these strains possessed only a single *parC* alteration. With respect to *por* and *opa* genes, only two of the three strains (i.e., G56 C1M and G56 C1N) had altered *por* and *opa* fingerprints, as well as an altered *Xba*I PFGE profile compared to wild-type and other mutant strains. Accumulation assays revealed that mutants accumulated levels of ciprofloxacin similar to that accumulated by wild-type strain, both in presence and absence of CCCP. Mutant G56 O1G, however, demonstrated active efflux of ciprofloxacin in the presence of CCCP. The presence of an inducible active efflux mechanism would thus account for the multiple antibiotic resistance (Mar) phenotype demonstrated by this strain, in concert with *gyrA* and *parC* alterations. Although strains G56 C1M and G56 C1N also demonstrated a weaker Mar phenotype, the mechanisms involved included altered *por* and *opa* genes in tandem with alterations in *gyrA* and *parC*, rather than active efflux.
Conclusion: Combined sub-inhibitory fluoroquinolone exposure resulted in induction of a broad-substrate range efflux pump. The MtrCDE system may have acquired mutations allowing it to efflux ciprofloxacin or the Mar phenotype results from a single substrate ciprofloxacin pump working in tandem with the MtrCDE efflux pump. The interplay of a number of low-level resistance mechanisms could thus be the "stepping-stone" leading to the proliferation of gonococci demonstrating decreased fluoroquinolone susceptibility and cross-resistance to structurally unrelated antimicrobial agents.

Meningococcal sequence typing using microarrays

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Introduction: Multi locus sequence typing (MLST) has become the gold standard for meningococcal typing. The extensive use of MLST created a large electronic database containing a high proportion of alleles and polymorphisms occurring in nature. DNA arrays have been used for typing of single nucleotide polymorphisms (Cheng *et al.*, Genome research 9:936-949). We therefore attempted to utilise the data being made available by MLST for establishing a microarray-based sequence typing system of meningococci.
Materials and Methods: Oligonucleotides with a C6 amino linker modification at the 5' end for covalent attachment to the slide surface were spotted on QMT epoxy slides (Quantifoil) using the Affymetrix 417 Arrayer (MWG-Biotech AG). DNA/DNA hybridization was performed using PCR products or chromosomal DNA labeled with Cy3-dCTP.
Results: We optimised the length and melting temperature of oligonucleotides spotted on the glass slides as well as hybridization conditions. Using labeled PCR products, one mismatch per oligonucleotide could be detected. Hybridization with chromosomal DNA was less specific and reproducible and will be subject to further experimental evaluation.
Discussion: The goal of sequence typing using microarrays must be to discriminate between DNA sequences differing only in a single base pair. This goal could be achieved by the use of labeled PCR products as probes, whereas the use of chromosomal DNA was less reproducible. In the future, we will set-up the bioinformatics needed for data management. Furthermore, speed, accuracy, and costs of sequence typing via microarrays will be compared to MLST.

Detection and identification by PCR of microorganisms causing bacterial meningitis

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Introduction: Bacterial meningitis (BM) is a threat for life; it affects the central nervous system (CNS) and it is reported among the main causes of deaths in children of less than five years. It is quite important to carry out a quick and exact diagnosis, as well as an effective treatment for this disease.

Microbiological diagnosis of bacterial meningitis is not enough sensitive because its efficiency can be affected by the therapeutic regimen given to the patient. Polymerase chain reaction (PCR) can provide a more sensitive diagnosis and allows us to get an earlier result.

This work describes new primers, derived from the gene *16S rRNA*, for detection and identification of the microorganisms that more frequently cause BM: *Neisseria meningitidis*, *Haemophilus influenzae* type b, *Streptococcus pneumoniae* and *Streptococcus agalactiae*.

Objective: To evaluate a set of primers derived from the *16S rRNA* gene for the detection of bacterial meningitis in CSF samples.

Design: We tested reference strains and 24 CSF samples by PCR with the primers E1 (5' GAATTCCA(C/T)GTGTAGC(A/G)G 3') and E2 (5' TACGACTTCACCCCA(G/A)TCAT 3') to detect species causing bacterial meningitis and previously reported primers to identify *Neisseria meningitidis,* Streptococci and the primer HI (5' CTTGTGCCTTCGGGAACT 3') for *Haemophilus influenzae*. Specific PCR detection was made for *Streptococcus agalactiae* (5' GCTTTCTCTTCGGAGCAG 3') and *Hae*III restriction patterns were obtained from PCR products.

Results: The PCR with E1 and E2 results in the amplification of a 0.82 kb DNA fragment in strains of *N. meningitidis, H. influenzae*, Streptococci, *Escherichia coli*, *Salmonella* and *Staphylococcus aureus*. This product is shorter than others described and was obtained in 9 of 10 cases with bacterial meningitis, 2 of them culture negative, including one with previous treatment. The second PCR detected 9 of the 10 samples, (4 *N. meningitidis,* 4 Streptococci and 1 *H. influenzae*). The negative case was identified as *S. aureus*. The 4 Streptococci were analyzed by PCR and 2 were identified as *S. agalactiae* by the amplification of a 0.47 kb fragment. The rest of the samples included 3 negative CSF and 11 CSF of viral meningitis and they gave no false positive results. The *Hae*III restriction of the E1-E2 PCR products was specific for all the species tested while the *Hae*III digestion of the specific PCR could not distinguish between the two Streptococci.

Conclusions: Primers E1 and E2 allow the detection of bacterial species in CSF samples with sensitivity of 0.9 and specificity of 1.0. The second PCR identifies all *N. meningitidis*, *H. influenzae* and Streptococci in the samples tested and the *Hae*III restriction of E1-E2 PCR products provides a confirmation tool for the species identification.

Acquired macrolide resistance, *mef*(A)and *erm*, genes in *Neisseria gonorrhoeae* isolated between 1975-1987

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Introduction: The *erm* genes code for rRNA methylase genes, which produce resistance to macrolide-lincosamide-streptogramin B antimicrobials and have been identified in a number of gram-positive and gram-negative genera since the 1950's. In contrast, the *mef*(A) gene, first found in streptococci in1986, codes for macrolide resistance using an efflux mechanism. These genes have low G+C content, suggesting that their origins may have been from gram-positive species. Previously we have shown that some *Neisseria gonorrhoeae* isolated during the past 10 years from both the United States and Uruguay carry *erm* or me*f*(A) genes. We sought to determine the frequency of *erm* or me*f*(A) genes in *N. gonorrhoeae* during the period 1940-1987 in diverse geographic settings.
Materials and Methods: Seventy-two *N. gonorrhoeae* isolated between 1940-1987 in North America, Asia, Europe, and New Zealand were examined for the presence of *mef*(A), *erm*(B), *erm*(C) and *erm*(F) genes using DNA-DNA hybridization and PCR analysis. Probes from the 7 *orf* genes from Tn*1207.1* were used to further characterize *mef*(A) positive isoaltes. The presence of the 1 bp *mtrR* was determined in a sample of 7 *erm* or *mef*(A)-containing isolates by sequence analysis.
Results: *erm* or *mef*(A) genes were detected in isolates from each geographic region studied and were detected in isolates recovered as early as 1975. Twenty-six (36%) of the 72 isolates carried *erm* or *mef*(A) genes: 18 had one gene and 8 had multiple genes. Among 20 of these isolates, the 1 bp deletion in *mtrR* was detected in 3 strains which did carry *erm* and/or *mef*(A) genes. In the 26 positive isolates, most commonly found genes was the *erm*(F), 13 [50%], followed by the *mef*(A), 12 [46%] isolates, *erm*(B), 6 [23%] isolates and the *erm*(C), 6 [23%] isolates. The *mef*(A) positive strains also carry some of the *orf* genes from Tn*1207.1* previously found in all *mef*(A) carrying streptococci.
Discussion: *N. gonorrhoeae* with increased MIC to erythromycin have been described since the 1960's and often attributed to the 1 bp *mtrR* deletions. In this study, 36% of the *N. gonorrhoeae* isolated between 1940-1987 carried one or more of the 4 macrolide resistant genes examined, suggesting that macrolide resistance may be due to a variety of determinants in different isolates. Currently, we are sequencing these genes from gonococcal isoaltes recovered in 1975.
Conclusion: The earliest known isolates to carry the *mef*(A) and *erm* genes have been identified in *N. gonorrhoeae*. These acquired macrolide resistance genes may predate the presence of the TEM β-lactamase and predate the *tet*(M) in *N. gonorrhoeae* and deserve further study.

Characterisation of *Neisseria meningitidis* strains by Pyrosequencing™

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Introduction: *Neisseria meningitidis* is a major cause of morbidity and mortality world-wide. The bacterium is characterised phenotypically through the analysis of its capsule and outer membrane proteins. However, sequence-based methods are now also being used for the genotypic characterisation *of N. meningitidis*. We show for the first time that Pyrosequencing ™ can be used as a tool for typing meningococci based on phenotypic markers and also shows that the procedures can be fully automated.

Materials and Method:

a) Meningococcal capsular gene analysis by Pyrosequencing ™

The main capsular serogroups associated with invasive meningococcal disease are A, B, C, W135 and Y. In Scotland and the rest of Europe, the main serogoups are B and C followed by W135 and Y. Four of these serogroups can be characterised genotypically by DNA sequencing or restriction length polymorphism analysis (RFLP) of the *sia*D gene. In serogroup B and C strains the RFLP is the restriction site TaqI and in serogroup W135 and Y strains is XbaI. The restriction sites only occur in serogroup C and Y strains. These four serogroups can therefore be characterised by an SNP Pyrosequencing ™ methodology by making use of the RFLP's in the *sia*D gene. Primers have been designed for external amplification of the RFLP for each allele and used to identify the serogroups of different strains by Pyrosequencing ™.

b) Meningococcal outer membrane protein gene analysis by Pyrosequencing ™

Meningococcal outer membrane proteins (OMP) are used for serotyping and sero-subtyping strains. The genes encoding two of these OMP's are *por*B and *por*A respectively. Both genes are exposed to the immune system of the infected host and are therefore antigenically variable. The genes possess particularly variable regions that are used in the typing scheme. The *por*B gene has four variable regions namely VR1, VR2, VR3 and VR4, which are approximately 50, 40, 50 and 50 nucleotides in length respectively. The *por*A gene has three variable regions namely VR1, VR2 and VR3, which are approximately 60, 50 and 20 nucleotides long respectively. The VR's within the *por*B and *por*A can be characterised by a SQA Pyrosequencing ™ methodology. Primers were designed external of each variable region to identify the sequence within each VR. This subsequently provides the serotype and sero-subtyping of the organism.

Results: SNP determination of the *sia*D gene from all meningococcal serogroups B, C, Y and W135 isolated from 1999, 2000 and 2001 have been successful. SQA analysis of *porB* and *porA* has also been successfully performed on all the sample isolates.

Conclusion: It has been demonstrated that Pyrosequencing ™ can be used as a rapid tool for the characterisation of *N. meningitidis* strains using capsular and outer membrane protein genes as targets for typing.

Dynamics of meningococcal carriage in teen-agers

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Aims: Studying the transmission/acquisition of meningococci in a longitudinal study.
Methods: In total, 4783 throat swabs of 1910 pupils at 19 schools in 6 Northrhine-Westfalian towns were examined for *Neisseria meningitidis* by culturing on Martin-Lewis medium. The teen-agers (14-19 years old) were screened for colonization with meningococci three times at intervals of approximately 10 weeks between February to September 2000. 981 students had participated in all 3 sampling rounds (i.e. 3 pharyngeal swabs and 3 filled in questionnaires) and the results were used to determine acquisition rates. For determining carrier rates all throat swabs taken were used. All isolated meningococcal strains were typed serologically, 93% of them using macrorestriction analysis (pulsed-field gel electrophoresis PFGE, restriction endonuclease *Nhe*I). The genetic relationship of the meningococcal strains of each town was demonstrated in dendrograms (program GelCompar, cluster analysis UPGMA).
Results: The overall carriage rate was 18.8% (901 meningococcal isolates in 4783 throat swabs). Of the 981 students with a complete data set, 270 (27.5%) carried *N. meningitidis*. In 101 (37.4%) of these participants meningococci were isolated only at one sampling date. 72 (26.7%) teen-agers carried meningococci during the whole 7-month study period. 84.8% of them were colonized at each of the 3 samplings with a genetically identical strain. 4.5% of them had lost the old and acquired a new strain between the first and second and 9.1% of them between the second and the third sampling. One pupil carried a new different isolate at all 3 samplings.

97 (35.9%) participants were carriers at 2 sampling dates: 46 students at the first and the second sampling, 43 at the second and the third and 8 at the first and the third sampling. In 91.2% their 2 strains examined were genetically identical. 8.8% of students had lost the old and acquired a new strain during the 2 samplings.
Conclusion: 169 (62.6%) of the 270 teen-agers tested positive carried meningococci at more than one sampling date so the duration of their carriage is considered to be at least 10 weeks. In 88.5% their strains of the different samplings were genetically identical. 13.0% of the 981 participants were newly colonized by meningococci during the study period. Furthermore, additional 11.5% of the participants colonized had lost a strain and acquired another one between the samplings.

Phenotypic characterisation of *Neisseria meningitidis* isolates collected from UK 15-18 year-olds at the time of introduction of serogroup C polysaccharide conjugate vaccine and one year post vaccine

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Background: The introduction of meningococcal serogroup C conjugate (MCC) vaccination for the under 18 year-old population of the UK in November 1999 prompted efforts to collect representative *Neisseria meningitidis* isolates to determine the effect of any selective immune pressure that may be exerted by the introduction of MCC and also for *N. meningitidis* population genetic studies.
Methods: A multi-centre swabbing study was carried out as the vaccine was given to 15-17 year-olds in schools and colleges. The centres were: Bangor (N. Wales), Cardiff (S. Wales), Glasgow (Scotland), London, Nottingham, Oxford, Plymouth and Stockport. Collections of throat swabs and isolates were repeated in 2000 and 2001 at the same schools and colleges. All Neisseria species collected were characterised using conventional serological and biochemical methods at either the PHLS MRU (England and Wales) or SMPRL (Scotland).
Results: To date the complete results are available for 1999 and 2000 when the total number of swabs collected was 15,206 and 18,273 respectively yielding 2,809 (18.6%) and 3,410 (19.1%) presumptive *Neisseria* species (isolates). Carriage rates of presumptive Neisseria species isolated at individual centres ranged from 8-25%. The phenotypic analysis of *N. meningitidis* isolates (2,347 from 1999 and 2,971 from 2000) form the basis of this presentation. The percentage of carriage of the *N. meningitidis* serogroups identified respectively from 1999 and 2000 were: serogroup C (0.4% and 0.1%), B (3.8% and 3.8%), W135 (1.0% and 1.3%), Y (0.7% and 0.9%), 29E (0.7% and 0.6%), X (0.2% and 0.2%), Z (0.1% and 0.1%), Z/29E (0.02% and 0.03%) and Non-serogroupable (8.3% and 9.3%). Twenty-three and 16 serogroup C phenotypes were identified from the 1999 and 2000 isolates respectively, among which serotype 2a and sero-subtypes P1.5 and P1.2 predominated. Serogroup B meningococci exhibited a great diversity of phenotypes in both 1999 and 2000. The predominant serogroup B phenotype was B:1:P1.14 (13% and 21% of serogroup B isolates in 1999 and 2000 respectively). Phenotype B:4:P1.4 accounted for 8% of serogroup B meningococci in 1999 and 2000. Sero-subtype P1.4 was detected in 11% of the serogroup B isolates in 1999 and 2000. There was a reduction in B:2a meningococci from 3 isolates to 1 isolate (in 1999 and 2000 respectively). Similar numbers of non-serogroupable (NG) isolates expressing serotypes 2a or 2b were detected in both 1999 and 2000 (equivalent to 1% carriage in both years). Phenotype W135:2a:P1.5,P1.2 (2 isolates) was only observed in 2000.

Conclusion: A significant reduction in serogroup C meningococci was observed. No evidence of "capsule switching" from serogroup C to B was observed. Future determination of the isolate genotypes will include MLST, *por*A sequence types and the *sia*D serogroup specific sequences. This will not only enable population genetic studies but also determine whether down-regulation of capsule expression is occurring among serogroup C meningococci.

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Epidemiological markers of epidemic strains of *Neisseria meningitidis* isolated in Cuba

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Introduction: The search for genotype markers is currently being conducted for the purpose of studying the genetic feasibility and the population structure of meningococcal strains isolated from epidemics. These offer a greater information on the epidemiology of the meningococcal disease (EM) and broaden the data that are provided by phenotype markers. To understand the characteristics of the Cuban epidemic strains is a function of the Finlay Institute. Having this information will provide a better understanding of the dynamics of this disease and the focus on the strategies for the attainment of vaccines against this microorganism.
Materials and Methods: Ninety-one Cuban epidemic strains isolated from patients between 1985 –1992 were studied. The identification of genera, species and serotype was performed by conventional methods. Whole cell ELISA with monoclonal antibodies were used for the classification of sero/subtypes and for the first time in Cuba the electrophoretic type (ET) was detected by electrophoresis of multilocus enzymes (EMM). The antimicrobial susceptibility was determined using the method of dilution in agar according to NCCLS to: Penicillin (PEN), Ceftiaxone (CFTX), Rifampicine (RFA), Choraphenicol (CMP), Ciprophloxacine (CP), and Sodium Sulphadiacine (SU). ET are associated to the rest of the detected epidemiological markers.
Results and discussion: Twenty-six major clonal complexes were identified with ET-5 complex being predominantly (67.0%). The rest of the strains belonged to cluster A-4 (3.3%) and to ET not associated to any predominance. These were called "others" and showed great differences in the allelic profile of the 14 enzymes under study. All strains were group B, with a predominance of the serotypes 4 (67.0%) and 15 (27.5%) associated to the subtypes P1.15, P1.10 and P1.16. There was a large percentage of strains sensitive to PEN (57.1%) and 42.8% showed moderate resistance to this drug. A hundred percent of the strains were sensitive to the rest of the antimicrobials.
The use of more sophisticated epidemiological markers could show that one clone alone could be responsible of epidemics and that it can spread to other regions of the world. Strains of the ET-5 complex were responsible for the Cuban epidemic. Even though ET-5 complex was described in 7 Cuban strains isolated during the epidemic and sent to Norway, up to the moment, there has been no verification of its predominance in a larger number of strains during the epidemic period. Epidemic outbreaks due to this complex were also present in Europe, Chile, Brazil and the United States.
Conclusions: The clone character of the Cuban strains under research is shown in this paper. The majority belonged to the ET-5 complex, associated to the phenotype B:4:P1.15 and strains resistant to SU.

W135 meningococcal disease in the EU associated with the Hajj 2000, 2001 and 2002

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Introduction: In 2000, outbreaks of W135 disease meningococcal disease were observed in a number of European countries amongst people returning from the Muslim pilgrimage to Mecca in Saudi Arabia (the Hajj), and their contacts. Prior to Hajj 2000, different vaccines had been recommended for pilgrims in each EU country, some using AC polysaccharide vaccine with others using quadrivalent (A/C/Y/W135) vaccine. In 2001, many countries changed to recommend quadrivalent vaccine for all. In 2002 the Saudi Arabian authorities required all pilgrims to be vaccinated with QV before Hajj visas were issued. In September 2000, the EC-funded meningococcal disease surveillance network established a sentinel rapid reporting system for the outbreak strain in the EU. The objectives were to monitor the spread of the W135 outbreak strain in Europe and to inform future interventions within Europe and in relation to travel.
Materials and Methods: A rapid reporting surveillance network was established in September 2000 as part of the EC-funded meningococcal disease surveillance network in the EU. Sentinel national reference laboratories in the European Union reported weekly any cases of W135:2a:P1.2, 5 or compatible strains. Data was analysed at PHLS Communicable Disease Surveillance Centre and published monthly in Euro-surveillance Weekly.
Results and Discussion: Between week 36 in 2000 and week 20 in 2002, a total of 149 cases of W135:2a:P1.2,5 (or compatible strains) were reported from the six sentinel reference laboratories in the EU. Of these, 7 cases had been Hajj pilgrims in 2000, 2001 or 2002, 31 were contacts of pilgrims, and 111 cases had no known link to a Hajj pilgrimage. A marked reduction has been seen in the number of cases in Hajj pilgrims and contacts following Hajj 2002, the year vaccination with quadrivalent vaccine became a requirement by Saudi Arabian authorities. However, cases of meningococcal disease caused by the outbreak strain are still being recognised in individuals with no link to the Hajj. Europe wide surveillance indicates that W135 became the third most common serogroup identified in the majority of EU countries in 2000. The CFR in cases infected with this strain of W135 is 15.4%, higher than any serogroup across all the EU countries.
Conclusion: Surveillance through our rapid sentinel reporting system indicated that circulation of the outbreak strain in Europe continued throughout 2001 and 2002. Data from one of the sentinel countries suggests that most cases occur in the Muslim population, and therefore suggesting limited circulation of this strain. High coverage with QV vaccine among pilgrims to the Hajj 2002, as a result of new Saudi Arabian requirements, resulted in a reduction in Hajj associated cases. The flexible rapid reporting system within the wider meningococcal surveillance network has shown to be an important asset in informing intervention policies.

Epidemiology of invasive meningococcal disease in the European Union, 1999-2001 – the need for conjugate Men C vaccine

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Introduction: European Commission Decision No. 2119/98/EC for setting up a network for the epidemiological surveillance and control of communicable diseases in the European Community stated that 'bacterial meningitis' aswas a priority. Invasive disease due to *Neisseria meningitidis* comes within this priority. Building on the surveillance networks already in existence within Europe, a surveillance network for *N. meningitidis* disease was established in all 15 EU countries and 2 non-EU countries. The aims of the surveillance network are:

– to improve the epidemiological information on invasive meningococcal disease within the European Union,
– to improve the laboratory capacity to accurately characterise the isolates of *N. meningitidis*.

Materials and Methods: Questionnaires on the surveillance system(s) and laboratory diagnostic methods used were completed by participant countries. An agreed minimum dataset was supplied 6-monthly by each contributing partner. Standard case definitions are used. Analysis of age-specific incidence rates, temporal trends and diversity of *N. meningitidis* are compared.

An external quality assurance scheme (EQAS) of the participating reference laboratories was undertaken using standard micro agents.

Results and Discussion: The incidence of culture confirmed invasive meningococcal disease varied widely between the participating network countries over 1999-2001. In 2000, overall incidence ranged from 0.27 – 5.93 /per 100,000 population. This reflects genuine differences between countries in the epidemiology of the infection and probable differences in case ascertainment.

Variation was seen across participant countries in the proportion of meningococcal disease due to serogroup C infection (in 2000, 6-56%). Unlike the incidence data, this variation is likely to reflect true differences in epidemiology. Resultantly, a wide range in the incidence of serogroup C infection is observed across Europe (in 2000, 0.06-3.34 /per 100,000). A decline in serogroup C incidence has already been observed in countries using conjugated meningococcal disease group C vaccine.

The majority of cases and highest incidence of serogroup C disease are seen in pre-school children. Highest incidence is in infant population, with a secondary peak in 15-19 year olds. The case fatality rate (CFR) is higher in older age groups. The impact of vaccination is most marked in the age groups targetted for MenC in the UK and Ireland.

Predominant serogroup C serotypes differ by country (2a versus 2b), and changes have been observed in countries over 1999-2001. Such changes may be significant, as C2a is part of the ET-37 complex, a hyper-virulent clone associated with high

incidence and high CFR. Changes within one country may prelude changes in neighbouring countries and are important to monitor at a European level.
Conclusions: This project demonstrates the successful development of a surveillance network towards the objective of providing high quality information on meningococcal infection in the EU and neighbouring countries. Identification of changes at a European level is important, as it may predict changes that will subsequently take place in neighbouring countries.

Confirmation of a cluster in Styria caused by C:2b strains with the help of multi-locus sequence typing (MLST)

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In February 2001, two C:2b:1.2 cases of meningococcal disease in the same village in the Southeast of Styria were reported to the Austrian Reference Centre for Meningococci in Graz. The first case was a 1 year old boy with septicaemia and the second case a 14 year old boy with meningitis. Both cases were admitted to the local major Hospital on the same day. Chemoprophylaxis was immediately given to the families. The investigation of district public health authorities found no connection between the two families. 10 days later, a 60 year old man from the neighbouring town died in consequence of a purulent pericarditis and meningitis. Again a C:2b strain was isolated. Also here no contact between the families could be found. The strains were examined with pulsed feld gel electrophoresis (PFGE). C:2bstrains from different years and geographic locations were used as controls. The PFGE results showed no difference in the bands of all C2b: strains run on the gel. The situation was further complicated through 2 further cases of C:2b: in the larger area. Therefore we decided to ask the Manchester reference unit for help. The 5 recent strains and 16 C:2b strains from 1995 –2000 were sent there with the request for Multi-Locus Sequence Typing (MLST).

Materials and Methods: Boiled lysates were prepared for each isolate. Following PCR amplification, the *porA* VR1 and VR2 regions and 400-500 bp fragments for each of the seven MLST gene loci were analysed by automated nucleic acid sequence analysis using the Beckman Ceq™ 8000 Genetic Analysis system and Ceq™DCTS Dye terminator cycle sequencing reaction kits. Allele calling and Sequence Type and clonal complex assignations were performed by electronic interrogation of the *N.meningitidis* MLST database (www.mlst.net)

Results: The analysis of the sequence data shows that the 21 C:2bs from Austria belong primarily to the ST8 complex/Cluster A4. The complete allelic profile was determined from the 5 possible cluster strains and 7 other C:2b strains.10 Strains belong to MLST type ST66 and 3 strains are ST 8. From the possible cluster strains, the cases 1, 2, 3 and 5 are ST 66 and case 4 is ST 8.

Discussion: The C:2b strains in Austria from 1995 -2001 show a very limited variation in MLST. This makes the public health management of possible clusters much more difficult. Where the clusters occurs in a small defined community as in the first 3 cases, the intervention is much more easily justified. In situations where the cases are distributed over a larger area as by all 5 cases, the differentiation between cluster case or sporadic case is not possible and the management becomes extremely difficult.

Prevalence of gonorrhoea and antimicrobial susceptibility of *Neisseria gonorrhoeae* in rural Madagascar

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Introduction: An on-going study is carried in a *Schistosoma haematobium* endemic rural area in Northern Madagascar to assess the prevalence of sexually transmitted infections (STIs) and uro-genital schistosomiasis and the associated morbidity. The preliminary findings related to the prevalence and antimicrobial susceptibility of gonococci (GC) are presented.
Materials and Methods: A total of 656 inhabitants (318 men, 333 women) aged 15 to 49 years old from five neighbouring villages were examined. At baseline one sampling was performed prior to STI treatment followed by a control sampling three weeks later. All individuals, whether symptomatic or asymptomatic, were given a single dose of ciprofloxacin 500 mg orally, except pregnant and breast feeding women who were given ceftriaxon 250 mg i.m. Urethral swabs from men and cervical swabs from women were stored in tubes in liquid nitrogen until culture for GC. The E-test was used for MIC determination, and a Nitrocephin test to detect penicillinase production.
Results and Discussion: GC were cultured from 29 (4.5 %) individuals: 10 (3.1 %) men and 19 (5.7%) women (NS). The GC prevalence was significantly higher in the age group 15-24 years (10.1%) than among those aged 25-49 years (1.6%) ($P<0.01$). In the age group 15-24 years GC were found in 8 (8%) of 101 males and in 14 (12%) of 117 females (NS). Burning urination and/or urethral discharge were reported by 8 (80%) of the GC positive men and by 108 (35%) of the GC negative men ($P<0.01$), whereas burning urination and/or vaginal discharge were reported by 10 (53%) of the GC positive women and by 115 (38%) of the GC negative women (NS). At follow-up 18 of the 29 GC positive individuals were re-sampled, and all were GC negative. Only one (0.3%) of 286 previously GC negative individuals was then cultured positive. Twenty-one (70%) of the 30 GC isolates were resistant to penicillin and were penicillinase producing. All 30 GC strains were sensitive to azithromycin, ceftriaxone, ciprofloxacin, kanamycin, and spectinomycin, and 28 to chloramphenicol. Only one (3.3%) strain was sensitive to tetracycline.
Conclusion: The prevalence of gonorrhoea was significant higher in individuals aged 15-24 years suggesting that this group in particular should be targeted in STI control programs. A relatively high proportion of GC positive individuals were asymptomatic. There was a high frequency of resistance to penicillin and tetracycline, both of which are often used for STI treatment in Madagascar. Ciprofloxacin is a suitable alternative to the old regimens.

Outbreak of meningococcal disease by PorA deficient meningococci

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Introduction: The outer membrane protein (OMP) PorA is an important component in first generation protein based vaccines against meningococcal disease. Stable expression of PorA in meningococci during infection is a prerequisite for PorA based vaccines to be effective. In this study an outbreak of meningococcal disease caused by nonsubtypeable meningococci in 2001 was examined.
Materials and Methods: Meningococcal isolates were characterised in the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) by serotyping, multilocus sequence typing and sequencing of the variable regions of *porA*, encoding the PorA epitopes, as well as sequencing of whole *porA*. The OMP fraction of the isolates was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.
Results: In the first week of August 2001 the NRLBM received meningococcal isolates from seven patients, residents of a confined area in the southwestern part of the Netherlands. Patient 1 - 5 belonged to the same age group (9 to 11 year) and lived within the same social community. These patients had visited the same community swimming pool on July 24th. Patient 6 and 7 were two and 23 years of age, respectively. They lived in other villages nearby. An epidemiological connection could not be established between these patients or between them and patient 1 - 5.

All isolates of the clustered cases were sequence type 11 by multilocus sequence typing, which is commonly found among isolates of the ET-37 complex. The seven isolates were all C:2a, but differed in serosubtyping. The isolates from patients 1 - 5 were non-serosubtypeable, whereas the isolates from patients 6 and 7 were serosubtyped as P1.5. However, sequencing of the *porA* variable regions of the seven isolates revealed identical PorA epitopes, P1.5-1,10-8. SDS-PAGE and Western blotting of the OMP fraction of the isolates demonstrated P1.5 specific PorA expression in the two serosubtypeable isolates, while PorA was absent in the five non-serosubtypeable isolates. Sequence data showed a premature stop codon in *porA*, which was caused by a single base pair substitution C→T at position 259 of the *porA* coding region in the five PorA deficient isolates.
Discussion: PorA deficient meningococcal isolates have previously been identified. However, those cases were sporadic, so it is possible that patients acquired infection with a meningococcus expressing PorA and that a mutation in *porA* occurred during the course of disease. In such cases disease would be preventable by a PorA based vaccine. The five PorA deficient outbreak isolates had an identical stable *porA* mutation. It is unlikely that by chance, these five isolates obtained the same *porA* mutation.
Conclusion: It is probable that PorA deficient meningococci were transmitted and caused invasive disease, indicating that PorA based meningococcal vaccines will provide only limited protection.

Neisseria meningitidis serogroup W-135 isolated from healthy carriers and patients in Sudan after the Hajj 2000

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Introduction: The first epidemic in the world of meningococcal disease due to serogroup W-135 was reported during the Hajj year 2000 with subsequent spread. The aims of the present study were to investigate if the Hajj year 2000 *Neisseria meningitidis* serogroup W-135 clone also had been carried to Sudan, the Eastern part of the African meningitis belt, by examining healthy Sudanese pilgrims, their family members and if the strain was causing meningitis.
Materials and Methods: W-135 meningococci from Sudanese carriers (n=5) in 2000 and patients (n=2) in 2001 were investigated together with documented Hajj 2000 associated W-135 strains. In order to phenotypically characterize the meningococcal isolates they were examined by serogrouping, serotyping/subtyping, and antibiogram. The isolates were further investigated with the combination of two molecular techniques, sequencing of the *porA* gene for variable regions (VR1, VR2 and VR3) and pulsed-field gel electrophoresis (PFGE) of the whole genome (digested with *Spe*I and *Nhe*I).
Results and Discussion: The phenotypic character of the W-135 meningococci isolated from Sudanese carriers in 2000 and patients one year later was similar to the Hajj 2000 associated W-135 strains. The present study shows that the Hajj year 2000 serogroup W-135 clone (P1.5,2,36-2 of the ET-37 complex) most probably was introduced into Sudan by pilgrims who visited Hajj year 2000. This is suggested by the findings of the carrier strains that had the same genosubtype and indistinguishable PFGE fingerprint pattern as the Hajj clone. The two meningococci isolated from patients in 2001 also had the same Hajj genosubtype and PFGE fingerprints that were indistinguishable to the Hajj pattern after *Nhe*I digestion but only closely related (one band missing) using *Spe*I.
Conclusion: The Hajj year 2000 serogroup W-135 clone was most probably introduced into Sudan after Hajj 2000. The results may also reflect that a minor genetic event in the genome of *N. meningitidis* clone of the Hajj year 2000 strains has occurred during about one year. Whether this has happened within or outside Sudan is an open question, but this strain has not been identified before in Sudan.

Close epidemiological surveillance is required to catch a possible new emerging meningitis situation.

Molecular characterisation of *Neisseria meningtidis* group A isolated in Sudan 1985-2001

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Introduction: To obtain a thorough characterisation of *N. meningitidis* it is necessary to use a combination of methods, both genetic and phenotypic. The aims of the present study were to characterise *N. meningitidis* group A isolates from Africa (Sudan) and follow clonality and possible smaller alterations over time.
Materials and Methods: A total of 31 *N. meningitidis* group A isolates were included. They were collected between 1985 and 2001 from Sudanese patients suffering from meningococcal meningitis. In order to characterise the isolates traditional serological methods with serogrouping, serotyping, serosubtyping and antibiogram were used in combination with molecular techniques like genosubtyping, pulsed-field gel electrophoresis (PFGE) with restriction enzymes *Spe*I and *Nhe*I, and multilocus sequence typing (MLST).
Results and Discussion: Three clones of group A meningococci were identified. One before 1988 (sulphadiazine sensitive, serotype 4, genosubtype P1.7,13-1,35-1, and ST-4); the next during and after the 1988 epidemic (sulphadiazine resistant, serotype 4, genosubtype P1.20,9,35-1, and ST-5); the last causing the 1999 epidemic (sulphadiazine resistant, serotype 4, genosubtype P1.20,9,35-1, and ST-7).
The first clone shows major differences compared to the other two. The second and third clone had major similarities with differences in a single gene in the MLST (47 of the 450 bp of the *pgm* gene). Significant differences were seen with PFGE. Within the clones genosubtyping and MLST gave identical results (except one base substitution in the *aroE* gene in one isolate). The PFGE patterns showed changes over time within the clones where *Spe*I gave somewhat more diversity then *Nhe*I.
Conclusion: It is earlier known that the rate of variation for group A *N. meningitidis* is low and the population structure is clonal, i.e. clones persist for long periods, an idea that was supported by the results of the present study. The use of genetic methods made it possible to show that the *N. meningitidis* group A strains circulating in Sudan between 1985-2001 are relatively clonal, and that two clonal shifts have occurred during these 16 years.

Clonal affinities of meningococcal isolates from patients and their contacts

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Introduction: Although many studies of the clonal affinities of invasive isolates of *N. meningitidis* have been made, carrier isolates are less well studied. Since 1986 we have collected carrier isolates from the contacts of patients and compared these with the patient isolate. In this study we have investigated clonal affinities within these carrier isolates and made comparisons between patient isolates, isolates from carriers of the patient isolate (associated carriers) and carriers of other isolates (non-associated carriers).
Materials and Methods: 285 *N. meningitidis* isolates from 1443 contacts (N=239) and 46 patients (N=46) were investigated. 43 carriers isolates were identical to their respective patient isolate (associated carrier isolates). Isolates were first classified by RFLP analysis of the *dhps* gene. Within each *dhps* class, isolates were compared by DNA fingerprinting and PFGE. Serotyping was performed on 41 patient isolates and 56 non-associated carrier isolates.
Results: 17 clones were identified. Their characteristics are summarised in table 1.

	Patients	Associated contacts	Non-associated contacts		
Clone	N	Typical phenotype	N	N	Typical phenotype
1	17	B:15:P1.7,16	14	11	NG;15;P1.7
2	8	C:2a:P1.2,5	9	7	C:2a:P1.2,5
3	6	B:16:P1.2,5	3	1	-
4	1	NT: P1,16	1	2	B:NT:-
5	2	Y:14,19.	6	9	NG:14,19:P1.5,2,6
6	1	W135:16: P1.7.	1	8	W:16:P1.3,6
8	2	C:2b: P1.2,5	3	0	-
9	1	B:4:P1.16	1	6	B:1,19:-
10	1	B:4:P1.14.15	1	9	-:4,7:-
B1	0		0	4	Variable
B2	0		0	3	B:7,17:-
C2	0		0	5	NG:4,7:-
C3	0		0	4	NG:4,7:-
G	0		0	17	NG:15:P1.6
K	0		0	9	Variable
R	0		0	4	Not tested
Y	0		0	6	B:8,10,19:-

Eight clones included only carrier isolates. Nine clones included patient isolates and associated and non-associated carrier isolates. One clone included only patient isolates and associated carrier isolates. Seven patient isolates (15%) and 90 carrier isolates (38%) could not be assigned to any clone. Most, but not all, clones were phenotypically homogeneous. Clones with phenotypic similarity to ET5 and ET37 were abundant both among patient and carrier isolates. Patient isolates of ET5 were typically B:15:P1.7,16 while non-associated carrier isolates were typically NG:15:P1.7. PCR-RFLP analysise of *dhps* gene alone identified clones 1, 2, 5, 8, 9 and 10 with high specificity.

Conclusions: We identified clonal affinities in 62% of carrier isolates from patients' contacts. 25% of carrier isolates belonged to clones that did not contain invasive isolates. Thus, as for patient isolates, clonal expansion is an important aspect of the population biology of carrier isolates of *N. meningitidis*. The patient's contacts may be carriers of invasive clones other than the patient strain. Where these can be readily identified, by for example PCR-RFLP analysis, treatment seems advisable.

The MtrCDE efflux pump promotes increased survival of *Neisseria gonorrhoeae* in the murine genital tract

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Introduction: The presence of hydrophobic, membrane-damaging compounds on mucosal surfaces is an important component of the host innate defense. Active efflux of such substances is likely to be an important bacterial adaptation mechanism. The gonococcal MtrCDE efflux system is responsible for conferring high level resistance to bile salts, fatty acids and antibacterial peptides such as protegrins, as well as lipophilic antibiotics such as erythromycin and azithromycin. In contrast, efflux of long chain fatty acids such as those found on rectal mucosa occurs via the gonococcal FarAB,MtrE efflux system. The isolation of gonococci with increased resistance to hydrophobic agents from rectal cultures due to mutations affecting the *mtrR* repressor promoter region and/or structural gene supports a role for multidrug resistance efflux systems during rectal infection. The contribution of these systems with respect to adaptation to the genital tract, the most common site of gonococcal infection, however, has not been explored. We tested genetically defined efflux pump mutants of *N. gonorrhoeae* in a female mouse model of gonococcal genital tract infection to assess the role of these systems in adaptation of *N. gonorrhoeae* to the genital tract.
Methods and Results: Gonococcal mutants KH14 (*mtrD*::Km^R), and RD1(*mtrE::aphA3*) lack functional MtrCDE and FarAB,,MtrE efflux systems due to the shared requirement for the MtrE outer membrane component by these pumps. Mutant EL1 (*farB::* Km^R) is deficient in the FarAB,MtrE efflux pump due to a nonpolar insertional mutation in the *farB* open reading frame; the MtrCDE efflux system in mutant EL-1 is not affected. To compare the survival of these mutants in the murine genital tract with that of the wild type strain, mixed inocula containing defined ratios of the parent strain (FA19) and mutants KH14, RD1 or EL1 were inoculated intravaginally into estradiol-treated BALB/C mice. A dramatic decrease in the recovery of mutants KH14 and RD1 occurred early in infection, with no mutant bacteria isolated after 3-6 days. In contrast, mutant EL1 was not attenuated. Mutants KH14 and RD1, but not EL1 were sensitive to progesterone in vitro, suggesting gonadal steroids may inhibit *N. gonorrhoeae in vivo* in the absence of a functional MtrCDE efflux system. Consistent with this hypothesis was the demonstration that mutant RD1 was more rapidly cleared from intact mice than from ovariectomized mice during mixed infection experiments with the wild type strain.
Conclusions: This is the first direct evidence that a multidrug efflux system promotes bacterial survival in the genital tract. Although multiple inhibitory factors appear to be present in the genital tract, the evolution of the MtrCDE pump may in part have been driven by the need to protect *N. gonorrhoeae* against gonadal steroids and/or their effects.

DNA sequence typing of *por*A variable regions of invasive meningococcal isolates from England and Wales to inform vaccine development

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Background: The successful introduction of meningococcal C conjugate vaccination in the UK has highlighted the continuing high incidence of serogroup B infection. The variable regions (VRs) on cell surface exposed loops of class 1 porin (PorA) proteins elicit production of antibodies. Monoclonals (mabs) prepared against some of these antigens are used in an international typing scheme to determine serosubtype. These antibodies have also been shown to be among the limited number which have direct bactericidal activity on meningococci. PorA antigens are therefore regarded as potential vaccine candidates, particularly for situations such as prevention of serogroup B infection where there are concerns and practical difficulties regarding the use of capsular polysaccharide. Only a minority (<10%) of clinical serogroup B isolates from England and Wales can be fully characterised at both VR1 and VR2 using the current panel of mabs. This is partly as a result of newly emergent antigens and partly due to small variation in established antigens making them unreactive with the mabs. To obtain better characterisation of these important targets, DNA sequencing of loci encoding VRs1 and 2 has been performed on clinical isolates from October 2000. Results to April 2002 have been analysed.
Methods: Submitted strains are all phenotypically characterised using poly- and monoclonal antibodies. *Por*A sequencing is carried out on a random selection of 50% submitted clinical isolates and one in five of these also has full multilocus sequence typing performed to establish clonal origin. Sequencing of extracted bacterial DNA is performed using the Beckman Coulter CEQ8000 and identity confirmed using the porA VR database on www.mlst.net or www.neisseria.org.
Results: About 1,110 clinical isolates have been *por*A sequence typed in accordance with the algorithm over 18 months, of which 780 were serogroup B meningococci. There were 79 different VR1:VR2 combinations among the B isolates tested; 52 were seen on only one occasion. The 'top 10' combinations were responsible for 78% while the five commonest caused 66% cases. The 'top 5' were 7-2:4 (31%), 19-1:15-11(11%), 22:9 (9%), 22:14 (9%) and 19:15 (6%). Novel alleles have been identified and added to the VR database.
Conclusion: Sequencing of the VR loci is providing valuable information to inform porA based vaccine formulation and design. There are wide differences in the ability of mabs to identify porAs encoded by only minimally changed DNA sequences. The information gleaned is being used to design DNA arrays.

Etiology of bacterial meningitis in Moscow

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Surveillance of meningococcal infection (MI) and bacterial meningitis (BM) during 2000-2001 in Moscow showed three main etiological agents: *Neisseria meningitidis* – 65,5% (269 cases out of 411), *Streptococcus pneumoniae* – 23,1% (95 cases out of 411), and *Haemophilus influenzae* type b –5,7% (22 cases out of 411). Out of 269 cases of confirmed meningococcal infection – 33,8% (91 cases out of 269) belonged to serogroup A, 39,8% (107 cases out of 269) – to serogroup B and 25,3% (68 cases out of 269) – to serogroup C.

Clinical diagnosis was confirmed in 77% of cases among the patients with MI (269 cases out of 349) and in 64,5% of cases among patients with other forms of BM (142 cases out of 220).

The laboratory diagnostic methods included bacterial culture, antigen detection (counter immune electrophoresis and latex agglutination) and specific antibody detection (gemagglutination). The application of an original technique (method of diffusion in agar by analogy with method Kirby-Bauer) has allowed to define antimicrobial activity of cerebrospinal fluid (CSF), taken from the patients with diagnosis MI and BM at the stage of receipt in hospital and to reveal parameters influencing on productivity of laboratory confirmation of the MI and BM diagnosis. For the period from April till September 2001 80 CSF samples were investigated. In the majority of the samples, antimicrobial activity was not revealed (46 cases – 57,5%). At the same time 8 samples (10%) contained very high concentration of antimicrobial preparation (more than 16 ED/ml). Out of 80 samples 26 (32,5%) contained significant (but not very high) concentration of antibacterial preparation (from 0,13 ED/ml to 12,5 ED/ml). The comparison of the results of various methods of diagnostics for BM and parameters antimicrobial activity in CSF showed the high importance (up to 75-100%) of non-culture methods of diagnostics among CSF samples with large degree of antimicrobial activity.

It is expedient to use non-culture methods of diagnostics for MI and BM because of high-sensitivity of the agents to the action of penicillin and cefalosporins. Thus, the basic activators of BM are found out in a viable condition only in CSF samples with negative antimicrobial activity.

Epidemic of *Neisseria meningitidis* W135 in Burkina Faso

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Introduction: Following the outbreaks due to *N. meningitidis (N.m.)* W135 that coincided with international travels (Hajj pilgrimage) in the Kingdom of Saudi Arabia in 2000 and 2001, countries in various parts of the world reported cases of meningococcal disease due to *N. m*. W135 among pilgrims, their contacts and among the general population.

To be able to assess the importance of the emergence of *N.m*. W135 as a public health problem and with a view to better support the response to eventual epidemics in the countries of the meningitis belt, WHO put in place an enhanced surveillance study in Burkina Faso, Mali and Niger. This study is two folds. One fold aims at following up at least three districts that have crossed the epidemic threshold and characterising all pathogenic strains of cases occurring during the epidemic season 2001-2002. The second fold aims at identifying the strains causing disease in 5-10 cases of any district having reached the alert threshold in the country.
Only Burkina Faso experienced an epidemic in 2002 and the results are reported here.

Methods: Cerebrospinal fluid (CSF) was collected from 5-10 patients when the alert thresold (5cases per 100,000 population per week) was reached in any district in Burkina Faso (routine study). In addition, 10 CSF from patients coming from 5 districts where the epidemic threshold (10 cases per 100,000 population per week) had been reached were collected on a weekly basis and until the epidemic in these districts was finished (longitudinal study).

For the longitudinal study, the CSF were inoculated in two Trans-Isolate (T-I) bottles; one was processed in the national laboratory and the second was sent to the WHO collaborating centre in Oslo ensuring the quality control, the confirmation and the molecular characterisation of the isolates. For the routine study CSF were collected in one T-I processed in the national laboratory.

Results: From week 10 to week 17 (4 March to 28 April 2002), a total of 504 CSF (from both longitudinal and routine studies) were collected and processed in Burkina Faso. From these, 426 were completed; 35% gave growth to meningococci and 83.81% (93% for longitudinal study and 67.21% for routine study) were W135.

Of the T-I (all from the longitudinal study) sent to the collaborating centre in Oslo, 34,4% gave growth to meningococci and 94,3% were serogroup W135. The W135 strains

tested so far in Oslo were serotype 2a, serosubtype P1.5,2 and sequence type(ST) 11.

The strains were sensitive to penicillin (MIC: 0.047-0.064 mg/l), chloramphenicol (MIC:0.75 mg/l), ciprofloxacin (MIC:0.003 mg/l), ampicillin (MIC:0.032-0.047mg/l) and ceftriaxone (MIC<0;002mg/l), but resistant to sulfonamide (MIC>256mg/l).

Conclusion: Most of the cases for whom a laboratory diagnosis was possible were confirmed as due to *N. meningitidis* W135. This strain is identical to the one identified in most of the *N.m* W135 cases that occurred in Saudi Arabia in 2000 and 2001. The implications of the Burkina Faso epidemic are considerable for several reasons. On one hand, it outlined the importance of laboratory-based surveillance for guiding the epidemic response. On the other it signals the possibility of emergence of the N.*m* W135 and thus, the need to improve the availability of meningococcal vaccine protecting against this serogroup to countries most at risk.

MLST of *Neisseria meningitidis* directly from cerebrospinal fluid

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Introduction: Two new molecular methods were introduced in the National Reference Laboratory for Meningococcal Infections in Prague in 2000: polymerase chain reaction (PCR) for non culture diagnosis of invasive meningococcal disease (IMD) and multilocus sequence typing (MLST) of *Neisseria meningitidis*. In 2001, we attempted meningococcal MLST directly from clinical material. This direct MLST was successfully developed in our laboratory and is used for IMD cases confirmed by PCR only since 2001.

Materials and Methods: CSF samples were centrifuged at 10,000 rpm for 10 min. DNA was extracted from the supernatant using the QIAamp kit (QIAGEN, Germany). For direct MLST from CSF two amplifications were performed.

The first amplification of seven alleles (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC* and *pgm*) was performed in an Amplitrone II thermocycler using Hot start. The primer pairs used for the PCR amplification of internal fragments of these genes were identical to those presented on the MLST website (http://neisseria.mlst.net) and were prepared by GENERI BIOTECH, Czech Republic. For the first amplification from CSF more DNA (25 µl) was used than for "classical" MLST of meningococcal strains (10 µl). Second amplification of the same alleles was performed from 1 µl of amplified products and after this a purification of the product was performed with 20% PEG.

The sequencing reactions were performed in PCR tubes with the BigDye terminator cycle sequencing kit (PE Biosystems) and subsequently analysed with an ABI PRISM 377 automated DNA sequencer (Perkin Elmer). The final sequence of each locus was determined with the LASERGENE software package (DNASTAR, Madison, Wisconsin). Housekeeping alleles and sequence types (STs) were assigned by reference to the MLST website (http://neisseria.mlst.net).

Results and Discussion: Direct MLST was performed on six samples of CSF from patients with IMD. In all cases sequence types were identified and allowed us to assess epidemiological relationships of IMD cases.

Conclusion: Meningococcal MLST directly from clinical material was successfully developed and is used for selected IMD cases with only PCR positivity. Its routine use improves surveillance of IMD.

Acknowledgement: Part of the work was supported by grant NI6882-3 of the Internal Grant Agency of Ministry of Health of the Czech Republic. This presentation made use of the Multi Locus Sequence Typing website (http://neisseria.mlst.net) developed by M.-S. Chan and K. Jolley and located at the University of Oxford. We thank Dr. K. Jolley for assistance with editing of the text.

Complex use of molecular methods in epidemiology of invasive meningococcal disease

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Introduction: Strains of *Neisseria meningitidis* are characterised in our laboratory by classical and molecular methods. Complex use of all methods allows us to assess epidemiological relationships in individual epidemiological situations of invasive meningococcal disease (IMD) and some of these situations are presented.
Materials and Methods: Six individual epidemiological situations were analysed using several characterisation methods of *N. meningitidis*: five cases of IMD in a small village within 6 months (the morbidity 416,7/100000 population); IMD confirmed by PCR and contacts of IMD case with positive cultivation of *N. meningitidis* from the throat swab (two individual IMD cases); outbreak of four cases of IMD with two deaths within 8 weeks; IMD of two cousins within three weeks living in different districts; six cases of IMD caused by *N. meningitidis* with unusual phenotype Y:2c:P1.2,5.
Results and Discussion: A strong increase of IMD in a small village was caused by *N. meningitidis* B:4:P1.16, ET-5 complex variant. Two cases were confirmed by PCR only, which revealed *N. meningitidis* B. Direct MLST from clinical material showed an identical ST as MLST of strains from IMD.

Two individual IMD cases were confirmed by PCR which revealed *N. meningitidis* B. Direct MLST from clinical material and MLST of *N. meningitidis* from the throats of contacts allowed us to identify contacts in epidemiological relation to IMD cases.

The outbreak of four cases of IMD with two deaths within 8 weeks was caused by *N. meningitidis* C:NT:P1.2,5, ET-37 complex, ST-11. The strains isolated from IMD cases showed a variation in PFGE analysis. MLST and PFGE of strains from IMD cases and their contacts allowed us to identify contacts in epidemiological relation to IMD cases.

Two cousins living in different districts suffered fom IMD within three weeks. Both visited the same discotheque (no strain available from there) and IMD of both of them was caused by *N. meningitidis* C:NT:NST, identical by MLEE, PFGE and MLST.

Six IMD cases were caused by *N. meningitidis* with the unusual phenotype Y:2c:P1.2,5. The question arised as to which ST complex they belonged to. Investigation of strains by MLEE and PFGE showed their similarity. Investigation by MLST is in progress as this abstract is submitted.
Conclusion: Molecular methods are very important in the assessment of epidemiological relationships among individual IMD cases and their contacts. Performing MLST directly from clinical materials improves the characterisation of individual epidemiological situations.
Acknowledgement: Part of the work was supported by grant NI6882-3 of the Internal Grant Agency of Ministry of Health of the Czech Republic. This presentation made use of the Multi Locus Sequence Typing website (http://neisseria.mlst.net) developed by M.-S. Chan and K. Jolley and located at the University of Oxford. We thank Dr. K. Jolley for assistance with editing of the text.

Epidemiology of invasive meningococcal disease in the Czech Republic

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Introduction: An emergency epidemiological situation of invasive meningococcal disease (IMD) started in the Czech Republic in 1993, when ET-15, a new clone of the ET-37 (ST-11) complex occurred. Strains belonging to this complex had not been found in the Czech Republic at least since 1970. Since 1993, the new meningococcal complex caused increased IMD morbidity and case fatality rates in the country.
Materials and Methods: Faced with this new epidemiological situation in 1993, the National Reference Laboratory (NRL) for Meningococcal infections started a nation-wide programme of active surveillance of IMD in collaboration with epidemiologists, microbiologists and clinicians. The guidelines for epidemiological measures to be taken in the focus of IMD and vaccination strategy were implemented in 1994. Meningococcal strains isolated from patients and healthy carriers were sent from the field microbiological laboratories to the NRL for detailed characterisation. The polymerase chain reaction (PCR) for non-culture diagnosis of invasive meningococcal disease was introduced in 1999.
Results and Discussion: The emergency situation peaked in 1995 and since then a decreasing trend in IMD morbidity was noticed. However, the case fatality rate persisted at relatively high figures. After seven years of high prevalence of serogroup C among the strains isolated from patients, which was caused by the ET-15/37 clone, we have noticed the prevalence of serogroup B in 2000 (58%), which is typical of the endemic situation. Serogroup C reached only 15% in 2000 and 26% in 2001. We expect this trend to continue, i.e. prevalence of serogroup B among patients´ strains in the coming years. We found a decrease of strains belonging to the ET-15/37 clone in 2000 (only 28%) compared to its high prevalence which culminated in 1997 (75 %). However, in 2001, its frequency increased (31%), particularly in the age group 15-19 years. A strategy of targeted vaccination (using A+C polysaccharide vaccine) of part of the population at highest risk was adopted in 1993. This targeted vaccination has been used less frequently now, as we have noticed a decreasing incidence of IMD and a decreasing percentage of group C. For this reason, massive vaccination with meningococcal conjugate C vaccine is not planned.
Conclusion: The emergency situation in IMD caused by meningococci of the ET-15/37 clone is over in the Czech Republic. However, the case fatality rate remains relatively high. Ongoing active surveillance of IMD is needed to recognize any epidemiological change as soon as possible and to introduce relevant epidemiological measures.
Acknowledgement: Part of the work was supported by grant NI6882-3 of the Internal Grant Agency of Ministry of Health of the Czech Republic. We thank Dr. K. Jolley for assistance with editing of the text.

Comparison of automated ribotyping, serogrouping/serosubtyping and *porA* gene sequencing as methods for differentiating strains of *Neisseria meningitidis*

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Introduction: Differentiation of *Neisseria meningitidis* strains is important for a number of reasons including identification of outbreaks, comparison of index case and secondary case strains, analysis of clonal expansion over periods of time and/or geographic regions. The availability of a set of monoclonal antibodies (originally developed in the Netherlands, RIVM) for classifying organisms with respect to their reactivity to epitopes of class 1 and class 2/3 porins was an important step forward. More recently, *por*A and *por*B gene sequencing (ST:SST) has been used to compare hypervariable regions at the DNA level for class 1 and 2/3 proteins. Ribotyping (RT) is an automated, rapid, high throughput and reproducible method for differentiating and grouping bacteria by comparing genetic fingerprints against a database of known isolates. Sophisticated software is used to analyse the resulting patterns allowing both identification and characterisation of the isolates.
Materials and Methods: A total of 53 clinical isolates (43 from the SENTRY Project and 10 from South Australia, 2001) with no repeats or duplicate patients with invasive meningococcal disease were tested. The SENTRY isolates were derived from Australia, South Africa and Singapore. Automated ribotyping was performed using a RiboPrinter (Dupont,Qualicon) with data analysis using BioNumerics v2.5 software. Phenotyping (PorA and PorB proteins) was performed using a dot blot methodology, probing the filters with 19 monoclonal antibodies (RIVM Serosubtyping Kit, Netherlands). Serogrouping was performed by slide agglutination using Murex *N.meningitidis* antisera. *por*A sequencing as described by Tribe (University of Melbourne) was used with identification of the sequence type by comparison with the VR1 and VR2 database at www.neisseria.org/nm/typing/pora/.
Results: Ribotyping yielded 34 ribogroups with a number of possible clusters identified. All the common serogroups were present (A, B, C, W135 and Y). Phenotyping was able to identify serotype/serosubtype for most isolates while *por*A sequencing classified all isolates tested, again with possible clusters identified. Ribotyping and subtyping identified many sporadic cases although some correlation between methods was observed. Four isolates, from one institution in Australia, had identical ribogroup, porA phenotype, *por*A sequence type and serogroup. Uniqueness was demonstrated by automatic ribotyping with isolates from geographically remote regions showing distinct ribogroups.
Conclusions: RT demonstrated it is useful for differentiating and identifying strain relatedness or uniqueness. Rapidity, reproduceability and portability across the globe make it suitable for both long and short term epidemiology. *Por*A/*Por*B sequencing is important for identification of outbreak strains while serogrouping remains important for identification of strains where there is a potential vaccine use.

The evolution of serogroups of *Neisseria meningitidis* in Romania during 1992-2002

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Objectives: The aim of this study was to characterize *Neisseria meningitidis* strains received by the National Reference Center for Meningococci during the last ten years.
Introduction: Meningococcal disease has not been a major public health problem in Romania during the last ten years. A very interesting finding was the evolution of serogroups in this period. After the epidemic of 1987 with 2,623 cases and morbidity rate of $11.4/10^5$, due to serogroup A (isolated in 84.5%), the incidence of meningococcal disease decreased dramatically, reaching $0.58/10^5$ in 1995 and $1.76/10^5$ in 1999.
Material and Methods: The National Reference Center for Meningococci collected 217 strains of *Neisseria meningitidis* isolated from CSF and blood (141 cases) and from nasopharynx and sputum (76 cases). The strains were identified to the species level by standard methods. Serogroup determination was carried out by slide agglutination using antisera produced by Cantacuzino Institute.
Results and Discussion: *Neisseria meningitidis* strains isolated from clinical cases were more frequently serogroupable (83.68%) than those isolated from carriers (35.52%). Following the fluctuation curve of serogroup A, one can observe that the moments of maximum incidence coincided with the two big epidemics of meningococcal disease in Romania (96% in 1970 and 84.5% in 1987). The very low frequency of serogroup A during 1992-1995 was followed by an increase from 1996 (58.33%), but the incidence decreased to 16.16% in 2000. Immediately after the epidemic of 1987 when serogroup A decreased dramatically, a significant increase of serogroup B was noticed, reaching 90.90% in 1992 and 66.66 % in 2000. During this period an interesting evolution was noticed in serogoup C. It was absent or having a very low frequency between 1994-1999 then reached in the following years 33.33% in 2001 and 50% of the strains during the first half of 2002. Non-groupable *Neisseria meningitidis* strains were frequently isolated especially in carriers (42% in 1992).
Conclusions:

- The frequency of serogroup A responsible of the two great epidemics of meningococcal meningitis in Romania was relatively low during 1992-2002 (17.5%).
- Serogroup B, which was absent during 1986-1990 will become the predominant serogroup during 1992-2002 (42.39%).
- Beginning with 2001 an increase of the number of cases of meningococcal disease due to serogroup C was noticed (50% from strains in the first half of 2002). An epidemiological situation similar was recorded during the last years in the countries located in our neighbourhood.

Risk factors for carriage of *Neisseria meningitidis* in British teenagers

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Introduction: In response to increasing rates of serogroup C disease, all people in the United Kingdom 17 years and under were offered meningococcal group C conjugate (MCC) vaccine in a national immunisation programme which commenced in November 1999. As part of a 3 year multicentre study to assess the effect of MCC vaccination on meningococcal carriage in teenagers we examined risk factors associated with carriage.
Methods: Students aged 15-19 attending school or college from 8 participating centres around the UK (Glasgow, London, Nottingham, Oxford, Plymouth, North Wales (Bangor), South Wales (Cardiff) and Stockport) were recruited to the study during October – December 1999. An oropharyngeal swab was obtained for meningococcal culture and students completed a questionnaire assessing risk factors for carriage. Oropharyneal swabs were cultured locally using standard microbial techniques. Oxidase positive, gram negative diplococci were frozen as putative meningococci and sent to the meningococcal reference laboratories in Manchester and Glasgow for serotyping and serosubtyping. A swab was considered positive if *N. meningitidis* was confirmed by the reference laboratory. Data were analysed by logistic regression using S plus.
Results: 15,103 swabs were obtained with a matching questionnaire. For this preliminary analysis, 190 were excluded because they were outside the predefined age range or age was unknown leaving 14,913 (98.7%) individuals (48.6% male) for analysis. 2,306 samples (15.5%) were confirmed as *N. meningitidis*. Carriage rates varied from 7.7% to 23.6% indicating significant heterogeneity in carriage between centres ($p<0.0001$). There was also significant heterogeneity in carriage by school within most centres. Carriage varied by school year (yr12, 15.1%; yr13, 18.2%). Other factors significantly associated with increased carriage were active smoking (Non smoker 14.0%, 1-5 cigarettes 24.8%, 6-10 cigarettes 27.4%, 11-20 cigarettes 29.0%), passive smoking (Other smokers in household: No 15.0%; Yes 19.3%), attendance at pubs or clubs in the last week (No of nights: None 10.2%, one 17.1%, two 20.9%, three 23.7%, four 26.7%, five 31.5%) and kissing (No of partners: None 12.4%, one 21.4%, two 21.4%, three 28.8%). Current and recent antibiotic use protected against carriage (Current 9.4%, Within last month 13.5%, None in last month 17.2%). All results were highly significant in single and multivariable analyses ($p<0.0001$). Associations with gender, household size, sharing a bedroom, and previous meningococcal polysaccharide vaccination were not statistically significant.
Conclusions: This large multicentre carriage study has clearly demonstrated that school year, antibiotic use, active and passive smoking, attendance at pubs and clubs and kissing were all independently associated with carriage of *N. meningitidis* in healthy teenagers.

The effect of ethnicity on rates of invasive meningococcal disease

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Introduction: It has been suggested that in the UK, children of Indian subcontinent (ISC) ethnic origin have a lower rate of invasive meningococcal disease (IMD) than others.[i] However, that study only looked at children less than five years old. The current study was designed to test whether these findings could be replicated, and whether a similar difference occurred in older children.
Methods: Notifications of all cases of IMD were obtained for two health districts population 1.17 million) from April 1992 to March 1999. Cases were divided into those where there was laboratory confirmation of the diagnosis and those where IMD was considered the most likely diagnosis by the clinician managing the case. The subjects' ethnic origins were determined from the first names and surnames.[ii] Two observers assessed each name independently, then the assessments were compared. The populations of people of ISC origin and others were determined from the 1991 census. Disease rates were calculated for people of ISC origin and others in four age bands. These were compared using χ^2 tests, or Fisher's Exact Test where appropriate.
Results: A total of 445 cases of IMD were reviewed. In one case the assessors could not agree on the origin of the name and this case was excluded from further analysis. Disease rates in those of ISC origin were significantly lower for both clinical and confirmed cases in the under five age group and significantly higher in the 16 to 25 year olds. No significant differences were found in the age groups 5 to 12 years or 25 years and over.
Discussion: The rates of IMD in people of ISC origin were found to be lower than that of the rest of the population under the age of five. However in the 16 to 25 year olds the rate was higher. There are two possible explanations for these differences: they may be due to genetic differences or to patterns of social mixing. If the difference were due to genetics, it would be expected that it would be consistent over different age groups. However, differences in social mixing could account for the pattern seen. Cultural differences may mean that meningococci are introduced into ISC families less often resulting in lower disease rates in younger children. However, the lower exposure to meningococcal carriage may result in lower levels of immunity, resulting in higher disease rates in early adulthood. Studies of carriage rates in populations of different ethnic origins may help to elucidate these points.

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Relative stability of the PorA protein in the context of New Zealands epidemic

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Introduction: New Zealand is in the 11th year of an epidemic of meningococcal disease caused by meningococci with phenotype B:4:P1.4. Serotyping and genetic analysis of isolates is important for examining the burden of disease of serogroups or particular subtypes, for detecting changes in strain types and for informing on vaccine use. Trials with a subtype-specific vaccine, produced by NIPH in association with Chiron Vaccines, started in June 2002. We sought to determine PorA protein subtype diversity among case-isolates from 1991-2001.
Materials and Methods: Reported are the results of serotyping and genetic typing of case-isolates. Standard Monoclonal antibodies (Mabs) from RIVM, The Netherlands were used for serotyping and serosubtyping. Isolates not serosubtypable were examined by *porA* PCR amplification followed by DNA-DNA hybridisation and/or sequencing.
Results: In 1991 an increase in serogroup B isolates with the serosubtype P1.4 was noted. Meningococcal disease cases, in 1991, totalled 78 of which 18 (23.7%) were B:4:P1.4. In 2001, meningococci with the P1.4 serosubtype represented 92.4% of serogroup B isolates and caused 80.5% of all disease regardless of serogroup. 2001 was a record year with 650 cases reported, giving a population rate of 17.4 per 100 000.

Over the 11 years there has been some variation in serotypes associated with the P1.4 serosubtype (Refer Poster - Dyet, Simmonds and Martin). Serotypes 1, 14, and 15 have been identified. Isolates of phenotype B:4:P1.4 have dominated representing 77.9% (1516/1945) of all serogroup B isolates. B:14:P1.4 isolates represented 4.7% and other serotypes less. Of 42 isolates defined as B:4:nst, six were shown to have the P1.7b,4 epitopes by sequence analysis. Four others had deletions in the sequence encoding the VR2 P1.4 epitope but had the expected sequence for expression of the VR1 P1.7b epitope. All of the remaining non-serosubtypable isolates had distinct PorA subtypes for which monoclonal antibodies were unavailable.
Discussion: Sequence variations in the *porA* gene are largely confined to the VR1 and VR2 regions of the PorA protein. Amino acids at the apices of the loops are the most important for immune recognition. The VR1, the longest loop, contains the linear epitope for P1.7b but a three amino acid deletion causes a shortening of the VR1 loop masking the P1.7b epitope from immune recognition. Thus, only the P1.4 epitope on the VR2 loop is recognised by monoclonal antibodies. Five isolates were identified which showed genetic variation within the VR2 P1.4. None of the variants were linked by time nor geographic region. The significance of such variation with respect to immune recognition by serum bactericidal antibodies produced in vaccinees will be investigated.

Antimicrobial susceptibility of *Neisseria meningitidis* strains isolated in Cuba during 1985-2000 period

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Introduction: The antimicrobial susceptibility studies of meningococcus strains are of great importance for many countries. The moderate resistance (MR) to penicillin (PEN) is reported in Europe for over a decade and is also currently described in America. Chemo-prophylaxis used to avoid the appearance of secondary cases is also studied. Among these, Sulfonamide resistance (R), closely linked to strains producing outbreaks and epidemics, is an important epidemiological marker. There are R reports to Rifampicine (RFA) and Cloramphenicol (CMP). The latter is the antibiotic of election for the treatment of MD in some regions of the world. Because it provides data of clinical and epidemiological value for Cuba, this paper presents the behavior of strains isolated from patients and carriers when challenged with drugs used in treatment and prophylaxis of the MD.
Materials and Methods: During 16 years (1985-2000) 1262 isolated strains were studied: 1042 (invasive), 2002 (carriers). The minimum inhibitory concentration (MIC) was determined by the agar dilution method (NCCLS) and strains were identified as sensitive (S) to MR and R when challenged to PEN, CMP, RFA, Cefriaxone (CFTX), Ciprofloxacine (CP), and Sodium Sulphadiacine (SU).
Results and Discussion: Prevalence of S strains to PEN (81.5%, CMI ≤ 0.06 μg/ml) was found and although MR was detected (18.5%, CMI 0.12-1 μg/ml), this one was greater among invasive strains (20.8%) than among carriers (7.3%). There were no R to PEN (CMI ≤ 2 μg/ml). A high percentage turned out R to SU (84.4%, CMI ≤ 10 μg/ml) having both groups similar figures: patients (85.2) – carriers (80.9). In invasive strains alone we detected MR to CMP (27.5%, CMI=2 μg/ml); 100% were found sensitive to CFTX and CP. The MR to PEN is of great clinical importance because it is the drug of election for the treatment of MD in many countries. In Cuba, MR strains to PEN were also reported in previous studies. Although there was a high percentage of resistance to SU, it was higher in invasion strains and at the beginning of the epidemic. Similar to the results of other countries, CFTX and CP showed great efficacy when challenged to meningococcal strains.
Conclusions: These results provide important data on strains isolated in Cuba and advise to be vigilant in order to guide, when necessary, any changes in clinical, therapeutically and prophylactic procedures. The increase in MR to PEN strains isolated demands the use of molecular biology techniques that could show the existent relationship between the changes in *penA* genes and this decreased sensitivity.

Correlation of epidemiologic trends with the genotypes of *Neisseria meningitidis* strains causing invasive disease in Maryland, USA

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Background: The incidence of meningococcal disease in persons 15-24 years of age increased in Maryland from 1990-97, then declined in 1998-99. An increase in this age group is characteristic of a clonal outbreak of meningococcal disease. From 1990-99, the incidence of meningococcal disease steadily increased among persons ≥ 25 years old.
Methods: Pulsed-field gel electrophoresis (PFGE) was performed on *N. meningitidis* isolates obtained from active, laboratory based surveillance during 1992-99. Genetic relatedness between serogroup C strains was calculated using the mean of the Dice coefficients. PFGE-based clonal groups were defined as strains ≥ 80% genetically related by dendrogram. Multilocus sequence typing (MLST) was performed on a subset.
Results: The serogroup was known for 84% (246/293) of the cases. Thirty percent (19/62) of the 15-24 years old were infected with a serogroup Y strain compared to 44.1% (41/93) of the ≥ 25 years old ($p = 0.09$). Likewise, 46.8% (29/62) of the 15-24 years old were infected with a serogroup C strain compared to 21.5% (20/93) of the adults ≥ 25 year old ($p < 0.01$). Seventy-six (58/76) percent of the serogroup Y strains were in 2 clonal groups (1 and 2) and all belonged to the ST-23 complex. The proportion of clonal group 2 strains increased from 11% (1/9) in 1992 to 57% (12/21) in 1999 ($p = 0.01$); this trend was seen among adults ≥ 25 years old, but not in the <15 and 15-24 year age groups. From 1992-97, the mean of the Dice coefficients of serogroup C strains was 83.7 for persons 15-24 years old versus 67.1 for children <15 ($p < 0.01$) and 66.8 for adults ≥ 25 years old ($p < 0.01$). During 1999, 88% (7/8) of the serogroup C infections in persons 15-24 years old were due to a unique PFGE pattern which was not previously seen. Two serogroup C outbreaks were caused by 2 different PFGE-types that belonged to ST-11.
Conclusion: During the rise in meningococcal incidence in persons 15-24 years old, serogroup C strains were more genetically related in this age group than in the other two age groups. Interestingly, during the decline of meningococcal disease, a serogroup C clone emerged in persons 15-24 years old. Among adults ≥ 25 years old, the increase in meningococcal disease was partially due to an increase in one serogroup Y clonal group.

The epidemiology of meningococcal disease in Scotland 1998 – 2001

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Following virtual elimination of *Hib* Meningitis by *Hib* conjugate vaccine, *Neisserria meningitidis* is now the leading cause of bacterial meningitis in the UK, with around 300 notified cases per year in Scotland. Of the two serogroups which account for over 90% of all cases in Scotland (and the UK as a whole) serogroup C generally has a worse prognosis and in the mid to late 1990's accounted for a steadily increasing proportion of cases (reaching 43% of confirmed infections in 1998 compared with 40% for serogroup B). The late 1990s also saw a sustained rise in the overall numbers of infections after a long period of relative stability, together with an increasing burden of infections in older age groups. The recent availability of a new conjugate polysacharide vaccine for group C meningococcal disease (MenC) offered a new means of intervention to reduce the burden from this disease. In November 1999 the UK and Scottish Executive departments of Health, incorporated MenC vaccine into routine infant immunisation and launched a nationwide campaign to offer the vaccine to everyone aged under 18 years old. After 1999, the number of confirmed group C cases began to decline markedly, especially in those age groups targeted by the new vaccine. The year on year increase in the number of cases also began to stabilize after 1999 when it had almost certainly been exacerbated by a severe influenza outbreak in winter 1999/2000. At the end of 2001, the total number of cases had decreased by 23% over the total for the previous year (from 355 in 2000 to 271 in 2001) and the trend towards a higher disease burden in older age groups had also halted. A continued source of concern however is the high case fatality rates (CFRs) for older age groups, which continues to diverge from that seen for younger patients (e.g.: the CFR for over 20yr olds in 2001 was nearly 15%, compared with less than 1% in the under 5s). It is expected that extension of the MenC vaccine programme to the 20 to 24 year old age group will help reduce meningococcal infections in older age groups, who in general have a higher risk of death or serious long-term sequelae.

Prevalence of super multi-drug resistant *Neisseria gonorrhoeae* CZRNG (cefozopran-resistant *N. gonorrhoeae*) in Japan

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Introduction: We have reported the incidence of clinical failures in gonococcal urethritis treated with cefdinir and aztreonam. The *N. gonorrhoeae* isolates from such clinical failure cases exhibited high-level MICs to them and other beta-lactams. We have also reported that the gonococcal infection caused by the resistant isolates were prevalence in Japan. These resistant isolates were clearly separated from the isolates exhibiting a low level of beta-lactam-resistance by the MIC distribution of cefozopran. We therefore called these expanded spectrum cephems-resistant isolates CZRNG (cefozopran-resistant *N. gonorrhoeae*). In order to investigate the prevalence of CZRNG isolates in Japan, we examined the susceptibility of *N. gonorrhoeae* isolates from patients with gonococci.

Materials and Methods: In this study, we used 418 *N. gonorrhoeae* isolates from different cases during August 1998 to December 2001 in Japan. The MICs of various antimicrobials against the isolates were determined by the two-fold serial agar dilution method.

Results: 171 of 418 isolates (40.9%) were the CZRNG (MIC of cefozopran were 4 to 32 mcg/ml). The 171 CZRNG isolates were resistant to benzylpenicillin, cefuroxime, and aztreonam of which MICs were >1, >8, and >4 mcg/ml, and had high-level MIC to cefixime, cefpodoxime, and cefepime (0.125 to 0.5, 0.5 to 4, and 1 to 16 mcg/ml). However they were susceptible to ceftriaxone and cefodizime of which MICs were less than 0.25 mcg/ml. Moreover, most of the CZRNG isolates acquired resistance to fluoroquinolones and tetracyclines. The 35.1% of (60/171) CZRNG isolates were low-level resistant to ciprofloxacin (MIC 0.125 to 0.5 mcg/ml), and the other CZRNG isolates (111/171) were high-level resistant isolates (MIC $\geq$1 mcg/ml). The 98.8% of CZRNG isolates (169/171) were low- or high-resistant to tetracycline (MIC $\geq$ 0.5 mcg/ml). The 95.3% of CZRNG isolates were susceptible to spectinomycin (MIC $\leq$32 mcg/ml).

Conclusions: In Japan, most of patients suffering from *N. gonorrhoeae* are treated with oral antimicrobials, such as beta-lactams, fluoroquinolones, tetracyclines. Therefore, the emergence of CZRNG isolates that acquired super multi-drug resistance is of serious concern. We have reported that the CZRNG isolates in gonococcal infection were eliminated by single dose treatment of ceftriaxone (1g i.v.), cefodizime (1g i.v.), or spectinomycin (2g i.m.). In Japan, ceftriaxone has not been allowed to use against gonococcal infections. We therefore recommend to use cefodizime and spectinomycin as the first line treatment for gonococcal infection.

Changing clonal distribution of Czech *Neisseria meningitidis* isolates related to partial replacement of the ET-37 complex by distinct serogroup B complexes

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Introduction: By the prevalence of serogroup B and C characteristics among isolates from previous decades (cumulatively >97%), Czech *Neisseria meningitidis* population suggests in general for that of the Atlantic regions of Europe. Due to a hyperendemic increase of the disease incidence related to spread and predominance of the ET-15 strain of the ET-37 (ST-11) complex over the country, representation of serogroup C prevailed in 1993-1999 period. Recently, significant shift in clonal distribution of meningococcal population has been detected.
Materials and Methods: Clonal feature of all *N. meningitidis* isolates, submitted to the Laboratory and being acquired from the majority of cases of invasive infection in the Czech Republic, is assessed continually. MLEE is used for routine screening of the isolates and MLST is employed for an additional specification of nature of representatives of clonal lineages.
Results and Discussion: In parallel to the decline of the ET-37 (ST-11) complex action, endemic occurence of distinct hypervirulent complexes related to serogroup B has increased since the end of the 1990s. The ET-5 (ST-32) complex together with the major complex related to serotype 22 meningococci (ST-18 complex) have been responsible in particular for the clonal shift. Occurence of lineage III and cluster A4 meningococci was rare. Representation of ST-11 complex organisms among isolates belonging to major hypervirulent clonal complexes of Czech meningococci has dropped from the 90% level in 1995 to less than 50% in 2000, but has increased rather, in part due to spread of a minor variant of the ET-15 strain, afterwards. Within the same period, representation of the ST-32 complex among Czech hypervirulent isolates has reached the 25% level. Occurence of the ST-18 complex culminated in 2000 (29% representation).

In opposite to ST-11 complex isolates, expressing typically the C:2a:P1.5,2 or C:2a:P1.2 phenotype, ST-18 complex isolates showed a meaningful (40%) extent of serologically "NT" or "NST" patterns, disabling effective serological assessment of isolate clonal relevance. Besides the hypervirulent ST-32 complex, the B:4:P1.15 pattern was common also for lineage IV isolates, showing lower virulence despite of their attribution to the ST-44 complex.

As the host protection by the A+C vaccine was not adopted in a wide schedule, being targeted to the limited population at the highest risk of infection, the partial clonal replacement detected is considered to be the consequence of natural process and not of the vaccination.
Conclusion: At the recent time, natural clonal replacement of the ST-11 complex was determined within *N. meningitidis* population in the Czech Republic. In spite of the current endemic incidence of the disease (1.0/100,000), overall antigenic characteristics of isolates strengthen the importance of routine assessment of isolate clonal nature by a multilocus genotyping approach, allowing precise monitoring of spread of serogroup B hypervirulent lineages.

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Differences in carriage rates of meningococci between ethnic groups is partly explained by social mixing

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Introduction: Recent work from the UK has suggested that the pattern of meningococcal disease is different among different ethnic groups. Low rates of invasive meningococcal disease have been demonstrated in children under the age of 5 of Indian sub-continent origin but not in teenagers and adults. Also, there have been cases related to specific strains amongst people associated with the Hajj.
Materials and Methods: Nottingham is one of the centres in a UK wide study of the effects of the recently introduced conjugated serogroup C meningococcal vaccine and meningococcal carriage in 16-18 year olds. A detailed epidemiological questionnaire was administered and a naso-pharyngeal swab taken as part of this study. The questionnaire includes questions of age, sex, ethnic group, antibiotic use, smoking, social mixing, household size and occupancy.

Risk factors for carriage were analysed using multiple logistic regression (SPSS for Windows V10) with carriage as the dependent variable and carriage adjusted for social and behavioural factors.
Results: 2341 swabs were taken in 2000 and 2586 taken in 2001. Of these 453 and 412 meningococci were obtained. Overall carriage rates were 17.6%.

Carriage of meningococci was lower in young adults of Indian sub-continent 7.1% (odds ratio = 0.37, 95% CI = 0.2 - 0.6, $p < 0.001$), South-East Asians 4.8% (odds ratio = 0.27, 95% CI = 0.1 - 0.9, $p < 0.008$) origin compared to other ethnic groups. A greater difference was seen in females and overall numbers were small in the South-East Asian group. There was a trend for carriage in people of Pakistani origin to be lower than those of Indian origin but this was not significant.

Carriage was also associated with social and behavioural factors: visits to pubs and night clubs, smoking and number of people kissed as well as gender, antibiotic use and age.
Conclusion: Carriage rates vary by ethnic group and are also dependent on social factors. Some of the difference in carriage in ethnic groups is likely to be due to unresolved confounding but further work is required in this area to explore these differences.

This work was a local study done as part of the work of the UK MENINGOCOCCAL CARRIAGE GROUP (UKMCG).

An evaluation of multilocus sequence typing for non-culture strain characterisation of invasive *Neisseria meningitidis* infections from England and Wales

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Introduction: In England and Wales approximately 46% of cases of meningococcal infections are now confirmed by detection of meningococcal nucleic acid alone. Furthermore, a growing number of case clusters involve both culture and non-culture confirmed infections. The accurate characterisation of the causative organisms for non-culture confirmed cases is essential for surveillance for meningococcal infection in England and Wales following the recent introduction of the MenC conjugate vaccine in the United Kingdom. One of the major advantages of the recently developed Sequence Typing scheme (*porA* and MLST) for N*eisseria meningitidis* is that it is PCR-based and can be applied to non-culture cases. Both *porA* and MLST PCR assays have been adapted by the PHLS meningococococcal Reference Unit to carry out sequence type analyses from non-culture confirmed cases of meningococcal infection.
Materials and Methods: Specimens from every tenth PCR-only positive case for the three month period October –December 2000 were analysed for *porA* and MLST sequence types. Previously extracted *ctrA* gene and or *siaD* gene PCR positive cerebrospinal fluid, serum/plasma and whole EDTA blood specimens were analysed. Following two rounds of PCR amplification for the *porA* VR1, VR2 and seven MLST gene loci were analysed by automated nucleic acid sequence analysis using the Beckman Ceq™ 8000 Genetic Analysis system and Ceq™DCTS Dye terminator cycle sequencing reaction kits. Allele calling and Sequence Type and clonal complex assignations were performed by electronic interrogation of the *N.meningitidis* MLST database (www.mlst.net). In several instances where case clusters were suspected involving both culture proven and non-culture confirmed cases both cultures and clinical specimens were analysed.
Results and Discussion: A total of 39 specimens were analysed, 31 confirmed as *siaD* serogroup B and 7 as *siaD* serogroup C. The strain composition for the population sample of non-culture confirmed cases as identified by Sequence type and clonal complex did not differ significantly from that of culture proven cases. The predominant clonal complexes associated with serogroup B and C isolates were ST-44 (lineage III) and ST-11 (ET-37) respectively. Several case cluster investigations involving either non-culture confirmed cases only or mixtures of culture proven and non-culture confirmed cases demonstrated the usefulness of both *porA* and or MLST sequence typing to establish the relatedness between isolates.
Conclusions: This evaluation has established the utility of Sequence Typing for strain characterisation for cases of non-culture confirmed meningococcal infection. The ability to perform definitive characteristion of the causative isolates in a representative sample of non-culture confirmed cases has confirmed that the isolates involved in non-culture confirmed infections do not differ significantly from the culture proven cases. Non-culture Sequence Typing for meningococcal strain characterisation using MLST is an essential reference activity for enhanced surveillance for meningococcal infection in England and Wales.

Crystal structure of penicillin-binding protein 2: structural mechanism of penicillin resistance

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Introduction: Chromosomally mediated resistant *Neisseria gonorrhoeae* (CMRNG) strains arise from the stepwise acquisition of multiple antibiotic resistance genes. The first resistance determinant is the *penA* gene, which encodes altered forms of penicillin-binding protein 2 (PBP 2) that have a lower rate of acylation by penicillin. The development of low affinity forms of PBP 2 in penicillin-resistant strains arose through multiple amino acid substitutions and an insertion of an aspartic acid residue into the transpeptidase domain of PBP 2 at position 345a (D-345a) that confers the greatest reduction in penicillin affinity. To understand the structural mechanisms by which these alterations lower the rate of acylation by penicillin, we solved the crystal structures of PBP 2 and PBP 2-D345a to 2.4 and 3.0 Å resolution, respectively.
Results: PBP 2 is an elongated molecule with two contiguous domains: an N-terminal domain of unknown function that comprises mostly ß structure, and a C-terminal domain that is immediately recognizable as a transpeptidase domain. The active site cavity is on the side of the protein near the top of the molecule, at the opposite end of the presumed N-terminal membrane-spanning region (removed in our construct). As with other PBPs, the conserved boxes found in all proteins that interact with penicillin, including the S^{310}-X-X-K^{313}, S^{362}-X-N^{364}, and K^{497}-T-G motifs, are clustered in the active site. A comparison of the PBP 2-D345a mutant structure with the wild type reveals that, in addition to the region around amino acid 345, structural changes are observed in the loop containing the catalytic triad S^{362}-X-N^{364}.
Conclusion: These data suggest that the insertion of D-345a into the PBP 2 sequence alters the conformation of the S^{362}-X-N^{364} active site loop, leading to a lower rate of acylation by penicillin.

Chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* is due to mutations in the S10/*rpsJ* ribosomal gene

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Introduction: Tetracycline resistance in *Neisseria gonorrhoeae* can be either plasmid- or chromosomally mediated. Whereas high-level plasmid-mediated tetracycline resistance ($MIC_{tet} \geq 16$ µg/ml) is due to expression of the TetM determinant, the mechanism of chromosomally mediated tetracycline resistance ($MIC_{tet} \geq 2$ µg/ml) has not been elucidated. Early work suggested that the gene encoding tetracycline resistance mapped to a cluster of ribosomal protein genes, but the identity of this gene was not determined. To understand more clearly the origins and mechanism of chromosomally mediated tetracycline resistance, we cloned the gene conferring tetracycline resistance from the clinical isolate, FA6140.
Results: The gene encoding tetracycline resistance was identified from a mini-library of size-selected *ApoI* fragments by measuring the transformation frequency of a recipient strain (FA19 *penA mtr penB*; MIC_{tet} = 1 µg/ml) to high-level tetracycline resistance (MIC_{tet} = 4 µg/ml). The tetracycline resistance gene (*rpsJ*) encodes the ribosomal protein S10 with a single bp mutation that changes Val-57 to Met. Amplification and sequencing of the *rpsJ* genes from five distinct tetracycline-resistant clinical isolates with tetracycline MICs between 2 and 8 µg/ml revealed identical Val to Met mutations, suggesting that this particular mutation is widely disseminated within the gonococcal population. Site-saturation and site-directed mutagenesis of Val-57 in S10 identified several additional mutations (Leu, Gln, and Ile) that also were capable of conferring tetracycline resistance to the recipient strain. These data suggest that large, uncharged amino acids are necessary to confer resistance. The high-resolution structure of the 30S ribosome reveals that S10 is an elongated molecule with one end of the molecule jutting into the ribosome very near the A site of the ribosome. A model of the 30S ribosome with bound tetracycline shows that the mutation in S10 is present at the vertex of a loop that extends into the ribosome and is within 6 Å of the antibiotic.
Conclusion: The location and identity of the mutations in the S10 protein suggest that large, hydrophobic amino acids are capable of decreasing the binding affinity of tetracycline by either steric hindrance or by altering the local RNA structure at the tetracycline binding site.

Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*

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Introduction: *Neisseria gonorrhoeae* has two porins, PIA and PIB, which are alleles of a single *por* locus. We recently have shown that mutations at positions 120 and 121 in PIB, which reside in loop 3 that forms the pore constriction zone, confer intermediate-level resistance to penicillin (1.0 μg/ml) and tetracycline (1.0 μg/ml). In this study, we investigated the electrophysiological and solute and antibiotic permeation properties of PIB proteins with these mutations (G120K, G120D/A121D, G120P/A121P, and G120R/ A121H).
Materials and Methods: To determine whether these mutations in PIB alter the electrophysiological properties of the porin, recombinant wild type and mutant porins were reconstituted into planar lipid bilayers and ion conductance and ion selectivity were measured. Liposome swelling assays with wild type and mutant porins were also performed to determine the permeation of sugars with varying molecular weights and β-lactam antibiotics with differing degrees of hydrophobicity.
Results: Whereas the anion-selectivity of each of the PIB variants was similar to wild type in planar lipid bilayers, the predominant conducting state of each of the porin variants was less than 30% of wild type. However, liposome swelling experiments revealed no apparent differences in the permeation of sugars or β-lactam antibiotics through wild type or PIB variants. Moreover, our data were consistent with earlier studies showing that in addition to the PIB mutations, increased expression of the Mtr efflux pump, the second determinant in chromosomally mediated resistance in *N. gonorrhoeae*, is necessary to observe porin-mediated increases in antibiotic resistance.
Conclusion: These data suggest a synergistic interaction between *mtr* and *penB* resistance determinants in the development of antibiotic resistance in gonococci.

In 2000 and 2001, most of meningococci isolated in countries belonging to the African meningitis belt were serogroup A (sequence type 5 or 7) or W135 (ST-11)

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Introduction: The WHO collaborating centre in Marseilles collects meningococcus strains from countries belonging to the African meningitis belt. That permits to follow clones circulating in Africa and predict what would possibly happen in close countries. Our laboratory data can help African Ministries of Health in the preparation of adapted responses to meningococcal diseases. In 2000 and 2001, most of meningococcus strains isolated in African countries were serogroup A or serogroup W135.
Material and Methods: At the WHO collaborating centre in Marseilles all the meningococcus strains are serogrouped, typed and subtyped. Multilocus sequence typing (MLST) permits to characterise each strain by its sequence type (ST). Pulsed field gel elecrophoresis (PFGE) is used to assess clonality of epidemic strains. Antibiotic susceptibility is determined using Etest.
Results: Serogroup A meningococci were isolated in 2000 and in 2001 during the Chad and Niger outbreaks. ST-7 characterised the strains isolated from Chad, but in Niger ST-5 and ST-7 coexisted. Serogroup W135 meningococci were isolated from sporadic cases in Algeria, Cameroon, Chad, Senegal and Central African Republic. Numerous W135 meningococci were isolated at the end of the outbreak concomitantly with serogroup A in Burkina Faso. Some of these W135 strains, characterised by MLST, were ST-11. Using PFGE, 5/10 showed indistinguishable fingerprint patterns with the strains responsible for 2000 outbreaks in Saudi Arabia and in France.

Antibiotic Susceptibility: all these meningoccci were susceptible to penicillin, amoxicillin, ceftriaxone, chloramphenicol and rifampin. More than 75% were resistant to sulfonamide (MIC > 10µg/mL)
Discussion: Since 1988 meningitis outbreaks and sporadic cases in countries belonging to the meningitis belt were mostly due to serogroup A meningococci belonging to ST-5. After clonal expansion of ST-5, ST-7 emerged in 1997 and seemed to replace ST-5. However in Niger ST-5 and ST-7 coexisted. In 2000, the global outbreak that began in Saudi Arabia was due to W135:2a:P1.5,2 belonging to ET-37 complex and ST-11. Our results show that in Africa indistinguishable strains belonging to the epidemic clone were isolated. Some close related W135 strains would correspond to strains that already existed or to an evolution of this clone transformed by foreign DNA.
Conclusion: It is very important to constitute and maintain strain collections in reference laboratories to follow clones over the world and to survey their susceptibility to antibiotics. In 2000 and 2001 the meningococcal meningitis outbreaks in Chad and Niger were due to serogroup A meningococci. Isolation of serogroup W135 meningococci in many African countries is a great concern for the future as the 2002 outbreak in Burkina Faso shows the epidemic potential of the W135 clone. Antibiotic susceptibility surveillance of meningococcal strains allows, until now, the treatment of meningococcal meningitis during outbreaks with chloramphenicol.

Pharyngeal carriage of serogroup W135 *Neisseria meningitidis* in Hajjees and their family contacts in Morocco, Oman and Sudan

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Introduction: In March 2000, a meningococcal disease outbreak coinciding with international travel began in Saudi Arabia (Hajj). It was followed by a global spread in returning pilgrims and their close contacts. The outbreak was caused by a W135:2a:P1.5,2 strain of *N. meningitidis* belonging to ET-37 complex and to sequence type (ST) 11. In 1987, a serogroup A:4:P1.9 meningococcus belonging to subgroup III was introduced in the African meningitis belt by pilgrims returning from Mecca and was responsible for most of outbreaks in the region since 1988. It is thus a concern that introduction of the W135: 2a:P1.5,2 clone might lead to an epidemiological shift in these countries. For this reason, WHO supported studies of meningococcal carriage in Morocco, Oman and Sudan to determine the spread of the W135 strain.
Materials and Methods: Nasopharyngeal swabs from pilgrims and their family contacts in Morocco, Oman and Sudan, were taken 3-6 months after the Hajj 2000. Meningococci identified in these countries were then sent to Marseilles (France), Orebro (Sweden) and Oslo (Norway) respectively where they were further characterised. The strains were serogrouped, typed/subtyped, and their clonal assignment was determined using multilocus enzyme electrophoresis, multilocus sequence typing, sequencing of the variable region of *porA* gene and pulsed-field gel electrophoresis (PFGE).
Results: In Morocco 1050 pilgrims and contacts were swabbed three times. Ninety-seven meningococci were isolated. PFGE showed that 32 strains (33 %) were indistinguishable from the clone identified during the outbreak in Saudi Arabia. In Sudan 5 serogroup W135 that were identical to the outbreak clone were obtained from sampling 250 family members of 35 families with at least one member having participated to the Hajj. In Oman 18 (4,5%) meningococci were isolated from 50 pilgrims and 349 family contacts and 11 of these (61%) belonged to the 2000 outbreak clone.
Discussion: The epidemic W135 clone was carried asymptomatically by returning pilgrims and their family members in these 3 countries and it was the predominant strain in Morocco and Oman. The carriage was of long duration, as these studies were performed several months after returning from Saudi Arabia. In Sudan no W135

isolates had been previously identified, it is thus likely that the strain was introduced by returning pilgrims.

Conclusion: There is evidence of an important pharyngeal carriage of the W135 epidemic clone among returning pilgrims and transmission to close contacts in Morocco, Oman and Sudan. The 2001-2002 outbreak in Burkina Faso confirms the epidemic potential of this clone. That is of great concern for countries of the meningitis belt where the disease burden is greatest. It is thus urgent to reinforce laboratory-based surveillance, to adapt and plan strategies of responses and to work with manufacturers to make available the appropriate vaccine to the most in need.

Genetic basis of rifampicin-resistance in *Neisseria meningitidis* and consequences on fitness *in vitro*

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Introduction: In routine laboratories of developed countries the number of meningococcal strains isolated displaying resistance to rifampicin is comparatively low, whereas in developing countries the rate may be significantly higher [1]. On the other hand one step acquisition of rifampicin-resistance in meningococci is associated only with a single point mutation within a defined subgenic region of *rpoB*, the gene locus coding for the prokaryotic DNA-dependent RNA polymerase [2, 3, 4]. For high level resistance aspects of membrane permeability as alternate mechanisms are still under debate [5].
Material and Methods: Three high-level rifampicin resistant strains (MIC >256 µg/mL) of *N. meningitidis* (two were selected towards resistance *in vitro*, one strain was already resistant upon isolation) were kept in a long term liquid culture to find out more about the stability of rifampicin-resistance *in vitro*. The meningococci were cultured in RPMI1603 cell culture medium for at least 36 days (previous experiments have shown a mean generation time of app. 2.3 hrs). The experiment was done in duplicate i.e. one set of strains was cultured under an atmosphere of 7.5% CO_2, the other under normal atmosphere, but both under permanent rotation. The cultures were passaged daily.
Results: The three strains were found to harbour a single point mutation in the well characterised subgenic *rpoB* fragment leading to an amino acid substitution known to confer resistance [2, 3, 4]. PCR of the *mtrR* promoter region (carrying an insertion sequence in meningococci) did not reveal any change in length of the expected amplicon. During long term culture one of the strains reverted to a sensitive phenotype. Sequence analysis of the *rpoB* fragment belonging to that strain revealed that the mutated site was changed to the wild-type, i.e. the resistance conferring mutation was simply reverted. Tested together in co-culture the rifampicin-sensitive, revertant strain displayed a shorter generation time as the resistant one.
Discussion: The data indicate that high-level rifampicin resistance is spontaneously revertible under conditions applied *in vitro* (no obvious selection pressure was applied although the strain which lost resistance grew under normal atmosphere). The finding that this type of resistance can be lost in the same stepwise manner as it can be acquired indicates that only a single point mutation causes resistance. The observed lower generation time *in vitro* as well as the easy reversion mechanism may account for the low number of resistant strains isolated in developed countries where a low selection pressure on rifampicin-resistance can be assumed.

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An increase in prolyliminopeptidase-negative isolates of *Neisseria gonorrhoeae* in the United Kingdom is associated with the spread of one clonal strain of the organism

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Introduction: The laboratory identification of *Neisseria gonorrhoeae* is commonly carried out using a kit, (API NH, Gonochek II or Neisseria PET), which relies on the detection of the enzyme prolyliminopeptidase (PIP). This preformed enzyme is found almost universally in strains of gonococci. A previous survey (Dealler *et al.*, 1991 *J Clin. Pathol.* 44:376-9) demonstrated that only 2 of 398 isolates of *N. gonorrhoeae* gave PIP-negative results, and between 1988-2000 only 16 such strains were submitted to the Gonococcus Reference Unit (GRU) for analysis. In contrast, between October 2001 and April 2002 60 PIP-negative isolates were referred to the GRU for confirmation of identity. We have investigated the epidemiology of these isolates and the genetic nature of their PIP-negative status.
Materials and Methods: Isolates that gave negative PIP test results in the API NH or Gonocheck tests were confirmed by the GRU as *N. gonorrhoeae* using the sugar utilisation test in CTA medium (Becton Dickinson, Oxford, UK) and the Phadebact Monoclonal GC Test (Launch Diagnostics, Longfield, UK). Isolates were also auxotyped. A selection of 30 isolates were opa-typed and the presence of gene coding for PIP was investigated by PCR and sequencing. Epidemiological data (gender, sexual orientation, country/city of acquisition of the infection) was requested from sending laboratories.
Results: Isolates were received from geographically dispersed towns and cities in England and Wales (27 from London, 14 from other areas in the South East, 9 from the North West, 3 from each of the South West, and North East of England, 2 from Wales and 2 from the Midlands. Fifty-one patients were male, four were female and two were of unstated gender. Most specimens (33) were urethral swabs, 18 were from the rectum, seven from the throat, one from the vagina, and one from the cervix. Demographic data is as yet incomplete but of eleven of known sexual orientation, 10 patients were homosexual, and one was heterosexual. With one exception which was serogroup WI, all isolates belonged to the WII/III serogroup, and 50 isolates demonstrated a non-requiring auxotype (ten isolates were not auxotyped). 30 phenotypically identical isolates (29 from males and one from a female) were characterised further. All 29 isolates from the male patients gave indistinguishable opa profiles whereas the isolate from the female had a different opa profile. The gene coding for PIP was detected by PCR in all 30 strains; details of sequence differences between PIP-negative isolates and the published sequence from a PIP-positive strain will be presented.
Conclusion: These results suggest the widespread dissemination of one highly related PIP-negative strain of *N. gonorrhoeae* around England and Wales, predominantly within the homosexual community.

Ultrasound enhanced latex agglutination test (USELAT) vs. conventional latex agglutination test (LAT) for the detection of meningococcal antigen in clinical cerebrospinal fluid (CSF) samples

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Aim: to evaluate the diagnostic characteristics of USELAT.
Methods: USELAT and LAT were applied to the CSF samples from 36 patients with meningococcal meningitis. Slidex Meningite kit 5 (bioMerieux) was used for both assays. The diagnosis was confirmed by serogroup-specific PCR with primers to *siaD* and *mynA* genes (19, 9, and 8 cases caused by serogroup A, B, C, respectively); 18 CSF samples were culture-positive (7, 8, and 3 samples positive for serogroup A, B, C, respectively). The number of meningococcal genome copies in the same samples was determined by quantitative PCR with primers to *ctrA* gene.
Results: The sensitivity of USELAT and LAT in comparison to PCR was 26/36 (72%) and 14/36 (39%), respectively, when undiluted CFS samples were used. The specificity of USELAT and LAT was 100% (no false-positive serogroup-specific results as well as false-positive results with latex for detection of pneumococcal and Hib antigens). Last positive dilution (titre) of a CSF sample in USELAT was determined which was equal 1:1000, 1:100, 1:10, 1:1 for 4, 8, 7, and 7 positive samples, respectively. LAT gave positive results only with undiluted CSF samples.

Last positive USELAT titre correlated highly with the number of meningococcal genome copies in a sample (Spearmen correlation coefficient 0.82). If the number of genome copies was less than 10^3/ml USELAT results were negative. USELAT titre 1:1 was observed when the number of copies varied from 10^3/μl to $2 \cdot 10^4$/μl (median value $6 \cdot 10^3$/μl). USELAT titre 1:100 or 1:1000 corresponded approximately to 10^5 copies/μl (range 10^4/μl - 10^7/μl). The detection limit of LAT corresponded to $3 \cdot 10^5$ copies/μl. Thus USELAT provided at least 30-fold enhancement of detection limit. Additional analysis revealed that the sensitivity and detection limit of USELAT and LAT was better for the detection of serogroup A antigen when for the detection of serogroup B antigen. In Slidex Meningite kit 5 serogroup A latex is bound with polyclonal antibodies whereas serogroup B latex is bound with monoclonal antibody. Both the number of genome copies and last USELAT positive titre correlated significantly with severity of meningococcal meningitis.
Conclusion: USELAT is a rapid, low-cost non-cultural method which can be used for meningococcal diagnosis and serogrouping in laboratories where PCR-based facilities are not available.

Disappearance of epidemic serogroup A subgroup III meningococci in Moscow 1998-2001 after the outbreak in 1996

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Epidemiological Background and Observation: In 1993-1995 the incidence of systemic meningococcal disease (SMD) caused by serogroup A meningococci in Moscow was about 0.8/per 100,000 /per year. The cases were randomly distributed at Moscow territory, 22% of cases occurred in Northern or North-Eastern administrative districts. Multilocus sequence typing (MLST) of isolates from patients with SMD demonstrated that most strains belonged to subgroup VI (10 of 14 = 71%) or subgroup X (1 strain) [1]. Subgroup VI and X are considered endemic in Eastern Europe. Two subgroup III, sequence type 7 (ST-7) strains were also found in Moscow for the first time. In 1996 the incidence of serogroup A SMD increased dramatically to 2.3/100,000/year or 201 cases. 67 cases occurred among Vietnamese migrants but serogroup A SMD affected also the native Muscovite population. Most cases were observed in Northern and North-Eastern districts where the migrants lived. When MLST and fine typing were applied to isolates of 1996, 17 of 18 meningococcal serogroup A strains belong to subgroup III, genocloud 8, possibly imported in 1993-94 from China as a part of genocloud 8 pandemic spread [1].
Methods: MLST of meningococcal strains isolated from patients with SMD in 1997-2002. Epidemiological surveillance of SMD in 1997-2001.
Results: The incidence of serogroup A SMD decreased to 0.5-0.55/100,000/year in 1997-1999 and rose slightly in 2000-2001 (1.0/100,000). The proportion of cases in Northern and North-Eastern districts was again about 22% of all Moscow cases. 3 of 5 strains from 1997 belonged to subgroup III. However, in 1998-2002 there were found 7 isolates of subgroup VI, 2 of subgroup X, and one rare variant of group A meningococci. MLST of additional group A strains is underway.
Discussion: Although very preliminary, these data suggest that the circulation of subgroup III, genocloud 8 meningococci has decreased substantially in 1998-2001 in Moscow. This decrease correlated with the decrease of serogroup A SMD incidence. Active mass vaccination with serogroup A meningococcal polysaccharide vaccine were undertaken in 1996-1999 in Moscow. In 1996 about 3000 Vietnamese and 35000 of Muscovites from Northern districts were immunized. In 1997-1999 about 250,000 children of 1.5 to 7 years of age and adults from risk groups were immunized. The protective effect of vaccination is well-known but it is unclear how serogroup-specific vaccination may influence the clonal distribution of meningococci. In 1976-1996 in Moscow SMD was successively caused by four independent clonal groupings which changed each other briefly due to unknown reasons [1].

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Evaluation of a diagnostic PCR for *Neisseria meningitidis* in North America and field experience during an outbreak

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Meningococcal infection has a high public profile because of the dramatic presentation, high fatality and propensity for outbreaks and clusters of cases to occur. Use of diagnostic polymerase chain reaction (PCR) enhances laboratory confirmation of cases and could guide the public health response in North America.

To assess the performance of PCR diagnosis of meningococcal disease following implementation of its use in a North American setting for detection of prevalent bacterial isolates we undertook a laboratory evaluation of sensitivity and specificity of PCR and an observational study of a series of cases comparing molecular diagnosis against criterion standard laboratory diagnostic tests.

Children and adults presenting with suspected meningococcal disease in British Columbia, Canada were included in the study and PCR was compared with conventional laboratory testing in field conditions for diagnosis of meningococcal infection in clinically suspected cases.

Results: PCR correctly identified all of 38 Canadian isolates of *N. meningitidis* and correctly assigned the serogroup to each isolate. None of 57 other Gram positive or Gram negative bacteria or yeasts were detected by the PCR. In a clinical evaluation PCR for diagnosis of meningococcal disease had a sensitivity and specificity of 91% and 76% respectively against conventional methods of diagnosis. Laboratory confirmation of clinically suspected cases rose by 36%. During an outbreak PCR allowed serogroup determination of 3 of 7 cases, aiding in the public health decision to launch an immunization campaign.

Conclusions: PCR is more sensitive than conventional methods of diagnosis of meningococcal disease and enhanced surveillance may help direct the public health response to the changing epidemiology of disease in North America.

Sulphonamide resistance in isolates of commensal *Neisseria* can be explained by variations in the dihydropteroate synthase

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Development of sulphonamide resistance in *Neisseria meningitidis* has been suggested to involve horizontal DNA-transfer from a commensal *Neisseria* species. In this study, we isolated commensal *Neisseria* from throat specimens and examined the isolates with respect to sulphonamide resistance. Three resistant clones were identified and the resistance phenotype could be explained by amino acid variations in their dihydropteroate synthase, the target molecule for sulphonamides. Some of these variations occurred in positions corresponding to previously detected variations in resistant *N. meningitidis*, supporting the hypothesis of transformational spread of resistance determinants. The sulphonamide resistant commensal *Neisseria* were isolated from an environment not exposed to sulphonamides, suggesting that resistant *Neisseria* has become a natural part of the commensal throat flora.

Trends in an emerging strain of *Neisseria meningitidis* serogroup C in Victoria

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In Victoria, in 1999, cases of invasive meningococcal disease increased significantly (137 cases, 95 culture-positive, population attack rate 2.9/100,000)) compared with the preceding 7 years (at 60 to 90 cases per year). Much of this increase was serogroup C disease.

Prior to 1999, serogroup B disease was twice as common as serogroup C disease, but in 1999 serogroup C disease became as common as serogroup B disease. The introduction of molecular biological identification techniques in 1999 improved the detection and characterisation of meningococci causing invasive disease. The proportion of serogroup C disease continued to increase during 2000 and 2001, and has continued into 2002, with about 1.5 confirmed serogroup C cases for each serogroup B case.

A new recombinant strain of *N. meningitidis* (C:2a:P1.7-2,4, MLST sequence-type 11) was identified in 1999, when it caused 16/44 (36%) of the serogroup C cases. Very few cases of the strain have been reported from other Australian states. In 2000 it caused 41/72 (57%) of serogroup C cases and in 2001 39/54 (72%). We have studied this strain in detail, and describe the changing epidemiology of an emerging strain over four years.

The recombinant clone strain of *N. meningitidis* that emerged in 1999 is now the cause of the majority of cases of invasive serogroup C meningococcal infections in Victoria. Although young adults predominate in absolute numbers, the age distribution has shifted towards younger and older age groups. Microbiological and epidemiological surveillance of serogroup C meningococcal disease is critical to decisions on implementing and monitoring vaccination programs that aim to control serogroup C meningococcal disease.

Invasive meningococcal disease in Switzerland, trends 1999–2001

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Introduction: Among the European countries, the incidence rate of meningococcal disease (MD) observed in Switzerland is considered as being mid-range. In the period 1999/2000, it was one of the countries with a high proportion of cases due to serogroup C (SC) [1].
Material and method: Physicians and laboratories report cases of MD to the SFOPH and all isolates should be sent to the National Laboratory. Only confirmed (isolation of *N. meningitidis* from a normal sterile site) and probable cases (compatible illness with detection of *N. meningitidis* from blood or cerebrospinal fluid by PCR or immunoassay, or cerebrospinal fluid with Gram-negative diplococci or leucocytosis, or Hennoch-Schönlein Syndrom) are considered here.
Results: From 1999 to 2001, 435 confirmed and 77 probable cases of MD were reported, with an incidence rate of 2.3/100'000 in 1999 and 2001 and a peak of 2.5/100'000 in 2000. One quarter of all cases occurred among children <5 years and another quarter among teenagers from 15 to 19 years of age. Children <2 years of age experienced the highest incidence rate in 1999 (19.8/100'000). Among children from 2 to 4 years and teenagers, a peak was observed in 2000 (10.7/100'000 and 10.8/100'000). Meningitis and sepsis were both reported in 70% of cases. The overall case fatality rate was 8% (serogroup B: 8%, serogroup C: 7%), with the highest rate observed among one year old children (22%).

The National Laboratory analysed 399 isolates, with 209 strains of serogroup C (serotypes C:2a: 33%, C:2b: 58%), 159 of serogroup B (serotypes B:4: 30% and B:15: 26%), 17 of serogroup W135 and 14 of serogroup Y. Subtypes P1.2,5 and P1.4 accounted for 37% and 10% of all isolates. The MLST 8 and 11 were seen in 26% and 16% of all strains.

The incidence rate of SBMD varied from 1.1/100'000 in 1999 to 0.9/100'000 in 2000/2001. For SCMD, a peak incidence rate of 1.5/100'000 was observed in 2000, followed by a decrease to 1.3/100'000 in 2001. In 1999, the highest incidence rate of SCMD was seen among children <2 years (10.4/100'000). In 2000 however, a shift to children 2-4 years old and teenagers was observed (7.2/100'000 and 8.5/100'000). Whereas in 1999, the proportions of cases attributable to serogroup C were similar in cases <2 years and ≥2 years of age (50% vs. 43%, $p>0.05$), they differed in 2000 (37% vs. 64%, $p<0.05$) and 2001 (25% vs. 55%, $p<0.05$).
Conclusion: Switzerland experienced a peak in the incidence rate of MD during the year 2000, attributable to an increase of SC cases in children between 2-4 years of age and teenagers. Children under two years were more affected by serogroup B MD. These variations must be considered, when the use of the conjugated vaccines in children is discussed.

Reference

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A molecular investigation of the long-term effect of an outbreak intervention on the capsular status of meningococcal carrier isolates

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Introduction: Following intervention for community outbreak of meningococcal disease it is not currently known whether replacement by virulent or less virulent meningococcal isolates may occur. In 1995, 16,000 people ages 2 to 18 years were given meningococcal prophylaxis over a six week period as part of a community outbreak of 8 cases of group C disease centred on two schools. The intervention consisted of meningococcal AC polysaccharide vaccine and either rifampicin or ciprofloxacin.
Methods: Carrier isolates were collected from subjects in the two affected schools, and from two adjacent non-intervention control schools matched for socio-economic status. Nasopharyngeal swabs were taken from participating individuals at 6 and 11 months after intervention. Isolates were tested by PCR to detect the presence of the capsular transport gene *(ctrA)* as a surrogate marker for capsular status.
Results: At six months and 11 months following the intervention, 43 and 89 non-serogroupable isolates were obtained respectively. At 11 months following intervention a higher percentage of *ctrA* positive isolates ($p < 0.001$) was observed amongst meningococcal strains obtained from those sampled in non-intervention schools than from those sampled at intervention schools. The difference was not significant at 6 months ($p = 0.105$).
Discussion: The *ctrA* negative isolates warrant further investigation of their genotypic organisation since such avirulent strains may be important in conferring natural protection against invasive disease. Following mass antibiotic prophylaxis, recolonisation occurs preferentially with non-pathogenic meningococcal strains. This has implications for assessment of the benefits of mass antibiotic and vaccination programmes for outbreak control. The lack of significance at 6 months is due to the small number of isolates available for testing.
Conclusion: Previously expressed concerns of increased risk due to removal of protective flora may have been overstated.

Meningococcal disease cases produced by B:2a:P1.5 strains in Spain: a capsular switching event?

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In the early 1990s an increasing number of meningococcal disease (MD) cases produced by serogroup C strains was observed in Spain. The increase in the incidence of MD was associated with the emergence of C:2b:P1.2,5 isolates that belonged to the A4 lineage.

As consequence of this increase, there was a mass immunization campaign using the polysaccharide A+C vaccine in most of the country between 1996 and 1997. Three years later, a new C-conjugate vaccine was introduced in routine in autumn 2000 because a tendency of increase in the incidence of serogroup C cases was again detected. After these immunization campaigns, a specific surveillance to detect possible changes in the phenotypic expressions distribution was initiated. Over the 2000-2002 period the number of C:2b:P1.2,5 strains decreased, ranging from a percentage of 48.5% of the serogroup C isolates in 2000 to 21.8% in 2002. On the other hand, in the same period, the number of isolates showing the antigenic expression C:2a:P1.5 increased reaching a percentage of 47.4% in 2002. During the first quarter of 2001, meningococcal cases produced by B:2a:P1.5 strains were detected. In this situation a capsular switching event between C:2a:P1.5 and serogroup B strains was suggested. Finally, seven and sixteen B:2a:P1.5 strains were received in our laboratory during 2001 and the first five month of 2002 respectively. Most of these strains were isolated in the Basque country, an autonomous community of the north of Spain. More specifically these strains were found in Vizcaya, a defined area of this region. This finding was associated with an increasing number of cases in this location. We received 16 serogroup B meningococcal strains from Vizcaya during 2001 being 5 of them characterized as B:2a:P1.5. Over the five first months of 2002 twenty one serogroup B isolates have been analysed and fifteen showed this antigenic combination. In addition three meningococci with these characteristics were also isolated in Cantabria (two strains isolated during 2001) and Valencia (one strain during 2002).

To analyze the genetic relationship between C:2a:P1.5 and B:2a:P1.5 isolates molecular typing methods were applied: Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Tying (MLST).

Both C:2a:P1.5 and B:2a:P1.5 isolates showed a common sequence type (ST11) belonging to the ET37 clonal complex. A more detailed characterization by sequencing the *fum*C gene including position 640 identified the strains as ET15 variant. PFGE analysis in twenty three B:2a:P1.5 and fifty five C:2a:P1.5 strains showed four closed related pattern profiles grouping most of the C:2a:P1.5 and all of the B:2a:P1.5 meningococci.

These results suggest that a capsular switching event has originated these B:2a:P1.5 strains that might maintain the epidemic potential of the precursor.

Epidemiology of *Neisseria meningitidis* in Queensland 1994– 2001

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Introduction: Meningococcal disease continues to be an important cause of morbidity and mortality. The disease is notifiable in Queensland and over the eight year period 1994 – 2001 sterile site isolates were sent for identification, serogrouping and sensitivity testing.
Methods: All isolates were serogrouped and from 1997 isolates were also serosubtyped using monoclonal reagents. A standardised national antibiotic susceptibility procedure was used to determine sensitivity to penicillin, ceftriaxone, rifampicin and ciprofloxacin. Pulsed Field Gel Electrophoresis was used from 1995 to determine epidemiological relatedness of isolates.
Results and Discussion: There was the expected peak in incidence in children aged four and younger with a secondary peak in adolescents and young adults. The youngest patient was 16 days old and the oldest was 91 years. Males accounted for 53.6% and females were 46.3% for cases. Mortality ranged from 6% - 11% of cases.

Isolations by site: blood 367(59%), CSF 199(32%), blood/CSF 37(6%), synovial fluid 8 (6%) and four from other sites (pericardial fluid, post-mortem brain swabs). The ratio of CSF:Blood was 0.54:1. Serogrouping showed that over the eight year period: serogroup B (65%) was the most common followed by C (29%), Y (2.9%), W135 (1.7%) Z (0.3%) and A (0.17%). Serosubtyping showed serogroup B to have a large degree of heterogeneity. The phenotype B:4:P1.4 although increasing in numbers did not assume the dominance it has in other countries. Serogroup C was more homogeneous with C:2a:P1.5,2 causing two small clusters in 1994. These were of the ET-37 complex. The first ET-15 case was seen in 1996 and this phenotype has now become established in Queensland. A small cluster of C:NT:P1.15 occurred in 2001.

In 1994, 54% of isolates were less susceptible to penicillin, while in 2001, 69% isolates were less susceptible (MIC 0.06 – 0.5 mg/l). All isolates were susceptible to ceftriaxone and ciprofloxacin. Twelve isolates for the period were resistant to rifampacin but no trend was evident.

In 2000, PCR testing was introduced. This has resulted in a 20% increase in diagnosis of meningococcal cases. These cases were previously listed as probable cases as they were culture negative. Serogrouping by PCR has not shown a statistically significant difference between culture diagnosed and PCR diagnosed cases.

Continued epidemiological monitoring is required to determine trends and to determine the effect of the new C vaccine which was recently licensed in Australia but has not, so far, been implemented into the vaccination schedule.

Molecular analysis of the most common meningococcal phenotypes in Italy

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Introduction: Among the 7 most common phenotypes isolated in the country, B:14:P1.13-B:4:P1.13- B:15:P1.7,16- B:4:P1.4-B:15:P1.4-C:2aP1.5 and C:2b:P1.5, genotypic analysis by MLST and PorA VR typing was performed to delineate relationship among the strains during a long low endemic period. Fluorescent Fragment Length Polymorphism (FAFLP) was also included in the analysis in order to assess the discriminative power of this technique.
Materials and Methods: DNAs from a subset of 36 *N. meningitids* invasive strains, isolated from 1994-2001, were analyzed. MLST protocol was that described in the web site www.neisseria.mlst.net. PorA VR typing (including VR3) was set up following the procedures described by Mölling et al., 2000. FAFLP was carried out after double digestion of 10 ng of total DNA with *Eco*RI and *Mse*I. Primers *Eco*RI/0 and *Mse*I/C were used in the amplifications reactions following manufacturer procedures (PE Biosystem). Dice Coefficents of similarity were computed and cluster analysis was performed by the unweighted paired group method with arithmetic averages (RAPD Distance Program Neighbor Joining Tree).
Results: ST 44/lineage III was represented by MenB strains with antigenic formula 14:P1.13 and 4:P1.13. Among them three different STs were identified (ST40-ST42-ST1127). Among B:15:P1.7,16 strains ST32 and a new ST named 1859 were found both belonging to the ET5 complex. Meningococci of phenotype C:2a:P1.5 (ST 11) currently circulating in Italy, were demonstrated to belong to cluster 37 together with the new ST1860 found also among strains C:2b:P1.5. A4 complex was represented by C:2b:P1.5, ST 8 . B:15:P1.4 strains had ST1403 not included in any known complex.
PorA VR typing showed that 89.5% of the P1.13 strains analyzed had VR1,VR2 and VR3 sequences identical and corresponding to 7-2, 13-2, 35a. P1.4 serosubtype, distributed between B4 and B15, had the same type 7-2, 4,37. No sequence variations, except for VR1, were detected among all MenB P1.7,16 (7-2, 16-2, 35). PorA types P1.5-2-36b and P1.5,2-1-36b were the combination found among MenC 2a and 2b.
Conclusions: Among the 7 phenotypes most frequently isolated from cases of meningococcal disease in Italy 4 major complexes have been identified which are present also in other European countries. Two new STs were found, 1 from MenB and 1 from MenC invasive strains. At least 2 PorA sequence variations were found among P1.13 and P1.7,16 subtypes. Among P1.4 , P1.5 and P1.2 only one variant was detected. Preliminary results obtained on B:14:P1.13 strains by FAFLP analysis were in agreement with those obtained by MLST analysis, confirming the clonality of these isolates.
Acknowledgements: This study made use of the MultiLocus Sequence Typing website (http://neisseria.mlst.net) developed by Dr. Man-Suen Chan, cited at the University of Oxford and funded by the Wellcome Trust.

Characterization of *Neisseria meningitidis* strains isolated from invasive meningococcal disease cases in Canada in 2001

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Objective: An increase in invasive meningococcal disease (IMD) activity was observed amongst several provinces during the winter months of 2001. This study describes the characteristics of *N. meningitidis* involved in these IMD cases.
Method: The National Microbiology Laboratory of Health Canada receives isolates of meningococci from all Provincial Public Health Laboratories in Canada, and characterizes them for the following information: serogroup, serotype, serosubtype, and electrophoretic type.
Results: The types of meningococci isolated from patients in the different provinces appeared to differ. Although serogroup C meningococci were responsible for all the outbreaks reported from 5 provinces, another serogroup (Y) also appeared to be causing a significant number of disease cases in Ontario. Also a unique serosubtype of group C meningococci characterized by the antigenic formula of C:2a:P1.1,7 was causing a large number of IMD cases in both Quebec and Ontario. This serosubtype of meningococci has never been reported in such large numbers in Canada before.
Conclusion: IMD continues to cause significant morbidity and mortality in Canada. The types of meningococci involved in IMD cases in some regions also appeared to show unqiue characteristics.

Comparison of serological and genetical typing of *Neisseria gonorrhoeae*. Consequences for future characterization

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Introduction: Conventional characterization of *N. gonorrhoeae* is mostly based on phenotypical methods exhibiting some inherent limitations. Serovar designation is based on antigenic diversities in the outer membrane protein PorB, which are detected by monoclonal antibodies (mAbs). The two different groups of this protein, PorB1a and PorB1b, are encoded by the mutually exclusive alleles of the *porB* gene, *porB*1a and *porB*1b respectively. For several of the widely used mAbs the antigenic epitopes of PorB are not identified. Thus, the prospects of developing a genetical typing comprising a congruent translation of the serovar designation seem to be limited.
Aims: To compare serovar designation based on PorB with *porB* gene sequencing, and to examine the genetic heterogeneity in the *porB* gene of different serovars of *N. gonorrhoeae*.
Materials and Methods: *N. gonorrhoeae* reference strains (n=34) and clinical isolates (n=72) from different geographic localities representing 28 different serovars and two non-serotypeable were included. Serotyping was performed using the Genetic Systems panel of mAbs. The *porB* gene was amplified by PCR and sequenced. Phylogenetic trees were constructed using the maximum-likelihood method.
Results: For the majority of the mAbs used in the serovar designation the PorB epitopes were difficult to map. A few isolates of different serovars even comprised identical *porB* gene sequences.

In the alignment of the *porB*1a sequences, 7% (68/924) of the nucleotide sites were polymorphic. Gene segments encoding 1oop 2 (21% of the nucleotide sites were polymorphic), 8 (19%), 1 (19%), and 4 (17%) of the mature proteins were the most heterogeneous ones, whereas regions encoding loop 7 (11%), 6 (10%), 3 (10%), and 5 (7%) were more conserved.

Among the *porB*1a isolates (n=35), 22 unique *porB*1a sequences were identified. Analysing only the four most variable gene segments (a total of 174 bp) gave the same discrimination between isolates.

In the alignment of the *porB*1b sequences, 15% (147/999) of the nucleotide sites were polymorphic. Regions encoding 1oop 5 (38% of the nucleotide sites were polymorphic), 8 (28%), 6 (28%), 3 (24%), 1 (22%), and 7 (19%) of the proteins exhibited the most heterogeneity, whereas regions encoding loop 4 (7%) and 2 (5%) were more conserved.

Among the *porB*1b isolates (n=71), 65 unique *porB*1b sequences were identified. Analysing only the six most variable gene segments (a total of 351-363 bp) identified 64 genetical variants.
Discussion and Conclusions: The difficulties of identifying the antigenic epitopes of PorB are probably due to the existence of conformational epitopes as well as inherent limitations of the serological characterization, for instance problems concerning the reproducibility. Sequencing of shorter highly variable regions of the *porB* gene generates sufficient discriminatory ability as well as fast, objective, portable and reproducible data in the characterization of *N. gonorrhoeae*.

Sharp increase in the incidence of group C meningococcal disease in the Netherlands in 2001

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Introduction: During the period 1996 – 2000, the Netherlands Reference Laboratory for Bacterial Meningitis (RLBM) yearly received isolates from 500 to 600 patients with meningococcal disease. In that period the proportion of serogroup C fluctuated between 10 to 20% of all meningococcal isolates.
Materials and Methods: Meningococcal isolates were characterised in the Netherlands NRLBM by serotyping, and sequencing of the variable regions of *porA*, encoding the PorA epitopes.
Results and Discussion: In 2001 the NRLBM received 33% more meningococcal isolates than in 2000; 717 and 539, respectively. Of 717 isolates in 2001, 421 (59%) had serogroup B and 276 (38%) serogroup C, while of 539 isolates in 2000, 417 (77%) were of serogroup B and 105 (19%) of serogroup C. This means that the increase of meningococcal disease in 2001 was exclusively due to the increase of serogroup C.

In 2001, the incidence of meningococcal disease was 4.49 cases per 100,000 inhabitants. The age specific incidence was highest in the age groups of 0 – 4 years and 15 – 19 years, 28.7 and 12.8 per 100,000 inhabitants, respectively. Remarkably, in the age groups 10 – 14 and 15 – 19 years the incidence of serogroup C meningococcal disease was higher (4.0 and 7.0) than that of serogroup B meningococcal disease (3.6 and 5.7).

In 2001, the five predominant serotypes were 4 (37%; 257/262 group B), 2a (30%; 212/215 group C), 15 (8%; 55/56 serogroup B), 2b (6%; 37/40 group C) and 1 (5%; 36/38 group B). The number of meningococcal disease cases due to isolates with serotype 2a was three times higher in 2001 (215) than in 2000 (71).

The PorA epitope distribution also significantly changed in 2001. In 2001, the five predominant PorA epitope combinations of 717 isolates were 1.7-2,1.4 (18%), 1.5,1.2 (19%), 15-1,1.10-8 (12%), 1.7-2,1.13-2 (6%) and 1.5-2, 1.10 (4%). In 2000, of 539 isolates the same five PorA epitope combinations were predominant, but the distribution was different; 1.7-2,1.4 (32%), 1.5,1.2 (11%), 15-1,1.10-8 (5%), 1.7-2,1.13-2 (7%) and 1.5-2, 1.10 (7%).
Conclusions: In 2001 the incidence of meningococcal disease in the Netherlands showed an increase of 33%. This rise of meningococcal disease significantly changed the PorA epitope distribution, hence will affect the efficacy of PorA based vaccines. However, the rise of the incidence was exclusively due to serogroup C meningococci. An effective conjugate group C capsular polysaccharide vaccine is available. Nationwide vaccination of the population in the age group 0 – 19 year has been started in May 2002.

Antimicrobial susceptibilities of *Neisseria gonorrhoreae* in the United States

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Introduction: *Neisseria gonorrhoeae* is estimated to infect over 710,000 people in the United States each year. Over time, the successful treatment and control of gonorrhea transmission have been complicated by the ability of *N. gonorrhoeae* to readily acquire antimicrobial resistance to a variety of antimicrobials. Surveillance of gonococcal resistance in the United States is conducted through the Gonococcal Isolate Surveillance Project (GISP).
Methods: Since 1987, STD clinics in approximately 26 U.S. cities have provided the first 25 male urethral gonococcal isolates each month to GISP for antimicrobial susceptibility testing by the agar dilution method. Clinical information was abstracted from medical records.
Results: Approximately 5,000 isolates are collected through GISP annually; preliminary data on 4330 isolates for January-September 2001 are available. Ciprofloxacin-resistant (MIC≥1.0 µg/ml) isolates in GISP were first identified in 1991 when only one GISP site had a ciprofloxacin-resistant isolate. During the first 9 months of 2001, 24 such isolates were reported from six GISP sites including Honolulu, where 13/60 (21.7%) of isolates were ciprofloxacin-resistant, and San Francisco, where 6/274 (2.2%) were; the remaining 5 isolates were from 4 other West Coast sites. An isolate with decreased susceptibility to azithromycin (MIC≥1.0 µg/ml) was first identified through GISP in 1993; in the first 9 months of 2001, there were 10 such isolates from five GISP sites. Isolates with decreased susceptibility to cefixime (MIC≥0.5 µg/ml) and ceftriaxone (MIC≥0.5 µg/ml) remain rare; during 1992-2000, there were 41 isolates with decreased susceptibility to cefixime and during 1987-2000, there were four isolates with decreased susceptibility to ceftriaxone. In the first 9 months of 2001, there were three isolates with decreased susceptibility to cefixime from Honolulu; all three isolates were also resistant to ciprofloxacin, penicillin, and tetracycline.
Conclusions: Ciprofloxacin-resistant gonococci have become endemic in Hawaii and are increasing in prevalence on the West coast. Isolates with decreased susceptibility to azithromycin are also rare but are being identified more frequently and in more locations. Only sporadic isolates with decreased susceptibilities to cefixime and ceftriaxone have been identified through GISP. However, in 2001, isolates with resistance to penicillin, tetracycline, and ciprofloxacin as well as decreased susceptibility to cefixime were identified in Hawaii for the first time. Ongoing nationwide antimicrobial susceptibility monitoring is necessary to ensure appropriateness of gonorrhea treatment recommendations in the United States.

Fluoroquinolone resistance and clinical treatment failure in gonorrhoea: report of eight cases

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Background: Ciprofloxacin and ofloxacin have been recommended as first line therapy for uncomplicated gonorrhoea in China. However, the prevalence of *Neisseria gonorrhoeae* showing reduced susceptibility or resistance to ciprofloxacin has risen markedly.
Objective: To report 8 cases of fluoroquinolone treatment failure in gonorrhea.
Study Design: The identification of *Neisseria gonorrhoeae* isolates were made by oxidase test and a rapid carbohydrate utilization test. The sensitivity testing was performed by an agar dilution method.
Results: All the eight patients were male, presenting from October 1999 to June 2001. They had a history of taking ciprofloxacin or ofloxacin or levofloxacin before presentation and the symptom of urethritis did not improve. The diagnosis of gonorrhea was based on the examination of smear and culture. The patients were treated with either spectinomycin, 2.0 g intramuscularly or ceftrixone 0.25 g intramuscularly. All the eight isolates were identified as *Neisseria gonorrhoeae* strains, which gave a positive reaction in oxidase test and only utilized glucose in carbohydrate utilization test. All the isolates were resistant to ciprofloxacin, having MICs of ciprofloxacin of 2 and 8 μg/ml. Two of them were penicillinase-producing Neisseria gonorrhoeae (PPNG). All the strains were susceptible to spectinomycin and ceftrixone. Plasmid-mediated tetracycline resistant *Neisseria gonorrhoeae* (TRNG) was not detected among these strains.
Conclusion: Fluoroquinolone resistance in *Neisseria gonorrhoeae* resulted in clinical treatment failure of fluoroquinolone in gonorrhea.

Antibiotic resistance of *Neisseria gonorrhoeae* isolated in Scotland during 2001

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Introduction: After many years of declining incidence, gonorrhoea is increasing once more. Effective therapy is vital in controlling the spread of gonorrhoea but resistance to antibiotics used in first line therapies can compromise therapeutic success. Surveillance of the nature and level of antibiotic resistance is essential to formulate and review treatment policies. National surveillance, which includes testing of all isolates, is performed by the Scottish *Neisseria gonorrhoeae* Reference Laboratory (SNGRL). This report defines the level and nature of antibiotic resistance in gonococci isolated in Scotland in 2001 and describes the outcome when patients were treated inappropriately.

Materials and Methods: Gonococcal isolates from 817 episodes of infection were submitted to SNGRL for antibiotic susceptibility testing and phenotyping. Minimum inhibitory concentrations (MICs) for penicillin, ceftriaxone, tetracycline, ciprofloxacin and spectinomycin were determined by an agar dilution method. All isolates were tested for penicillinase production (PPNG) using an acidimetric method. Clinical resistance to the various antibiotics was defined by the following MICs: penicillin (> 1mg/l); tetracycline (>1 mg/l); spectinomycin (> 64 mg/l); ciprofloxacin (>0.05 mg/l); there is no resistance category for ceftriaxone.

Results and Discussion: Resistance occurred in a 19.5% (159/817) of all isolates. Penicillin resistance was present in 6% of isolates (5.8% were PPNG and 0.2% were chromosomally resistant); tetracycline resistance in 16.4% (5% were TRNG and 11.4% were chromosomally resistant; and ciprofloxacin resistance in 7.8% of isolates (4.2% had MICs in the range 1 to 32 mg/L). No isolates were resistant to spectinomycin and none had a ceftriaxone MIC > 0.25 mg/L. There was a strong correlation between plasmid mediated penicillin resistance and ciprofloxacin resistance: 66% (27/41) of PPNG and PPNG/TRNG were resistant to ciprofloxacin while 47% (27/58) of ciprofloxacin resistant isolates were PPNG and/or PPNG/TRNG.

Of 22 plasmid mediated penicillin resistant isolates without chromosomal resistance to ciprofloxacin, 14 infections were acquired abroad, and one patient was treated with a penicillin which resulted in treatment failure. Of 26 plasmid mediated penicillin resistant isolates with ciprofloxacin resistance, 14 infections were acquired abroad, 14 were treated with ciprofloxacin and when the outcome was known there was a 50% failure rate. Of 20 non plasmid mediated penicillin resistant isolates with chromosomal resistance to ciprofloxacin, only four infections were acquired abroad, 12 were treated with ciprofloxacin and when the outcome was known there was a 29% failure rate.

Conclusions: Continuous antibiotic resistance surveillance, epidemiological information, and treatment outcome data are essential in formulating appropriate treatment policies to ensure effective treatment for gonorrhoea and minimise the spread of resistant gonococci.

Reduced sensibility to penicillin of *Neisseria meningitidis* strains isolated in Cuba

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Introduction: The study of antimicrobial susceptibility in *Neisseria meningitidis* strains is carried out considering two well defined situations: antimicrobial drugs for therapeutic use and those used for prophylaxis of meningococcal disease. A systematic study of the isolated strains is necessary to study the behavior of this pathogen against the drugs used for these purposes and establish the correct selection of the antimicrobial drug to be used "*in vivo*", whether for therapeutic or prophylactic purposes. Reports of strains with decreased susceptibility and/or resistance are more frequent every day. In Cuba, for this reason, a systematic surveillance of the isolated strains is carried out to identify the phenotypic characteristics of the circulating strains and the appearance of resistant strains.
Materials and Methods: 124 strains isolated in Cuba from nasopharyngeal carriers during 1998-1999 were studied. All were identified by genus, species, serogroup according to the conventional methods. Sero/subtypes and immunotypes were identified by whole-cell ELISA using monoclonal antibodies. Antimicrobial susceptibility was determined by the agar dilution method. CMIs to the following drugs were determined: penicillin, ceftriaxone, chloramphenicol, rifampin, ciprofloxacin and sodium sulphadiazine.
Results: Strains NA:NT:P1.NST:L3,7,9 prevailed (22,8 %). The 82 % of the strains showed resistance to sulfonamides (CMI>25 μg/ml) and the 13 % showed reduced sensibility to penicillin (CMI=0.125 μg/ml). All the strains (100 %) were sensitive to the rest of drugs investigated.
Conclusions: The results obtained contribute important facts about the strains isolated during this period and warn us that the surveillance on the circulating strains should be maintained to point out any change in the epidemiological markers of this microorganism.

Immune response induced in mice by two meningococcal polysaccharide-protein conjugates and spacer influence

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Meningococcal infection is an important cause of morbidity and mortality worldwide. Group B and C serogroups *N. meningitidis* are responsible for most cases in the developed world. Polysaccharide (Ps) vaccines induce protective serum bactericidal antibodies in adults but are poorly immunogenic in young children and may induce tolerance. The conjugation can improve their immunogenicity. Therefore, we conjugated (carbodi-imide) PsC from group C *N. meningitidis* to tetanus toxoid (TT) or outer membrane vesicles (OMV) from group B *N. meningitidis*. In addition, the influence of the spacer arms [1,6- diaminohexane (AH), 1,8-diaminooctane (AO), 6-aminohexanoic acid (AA) and adipic acid dihydrazide (ADH)] with TT conjugated was determined. The anti-PsC and anti-OMV IgG, IgG1 and IgG2a responses were measured by ELISA. The anti-TT IgG response was also measured. In addition, bactericidal activity was determined. High titers of anti-PsC, anti-OMV and anti-TT IgG were found in all immunized groups. TT as carrier induced mainly anti-PsC IgG1, but OMV induced also anti-PsC IgG2a. The anti-OMV IgG and IgG subclasses were not affected by conjugation, neither the anti-TT IgG was affected. The different spacers increased the anti-PsC IgG response, but the highest titers were found for ADH. It also increased the IgG2a and bactericidal activity. In conclusion, the ADH spacer and OMV carrier induced more IgG2a than the other conjugates.

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