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P001

Functional analysis of PrmC of *Neisseria meningitidis*

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Objective
PrmC plays a key role in the accuracy of termination of translation in *Escherchia coli* K12 by methylation of glutamine of the GGQ domain of the protein release factors. So far, the function of only *E. coli* and *Chlamydia trachomatis* PrmC has been experimentally established, although PrmC homologues are well-conserved in nature. The objective of this study was to evaluate the genetic organization and functional characterization of prmC in *Neisseria meningitidis.*
Methods
The genome sequences of *N. meningitidis* strains MC58 and Z2491 were assessed for homologues of prmC of *E. coli.* Potential PrmC function of neisserial genes was assessed by complementation of the growth deficiency in the *E. coli* prmC knockout strain SC5. Transcription and translation of putative neisserial prmC was assessed by RT-PCR, Northern blotting and SDS-PAGE.
Results
In *N. meningitidis* Z2491 one open reading frame (ORF) (NMA0369) of 822 bp was found, encoding a 30 kDa putative PrmC (42% identity with E. coli PrmC). NMA368 (462 bp) upstream of NMA369 had been annotated as a putative integral membrane protein (PUTP). The stop codon of putp is located 5 nucleotides downstream of the start codon of prmC. Interestingly, the corresponding genome region of strain MC58 contains one large ORF (NMB2065) of 1272 bp, encoding a putative protein of 46 kDa. Here, deletion of one nucleotide near the junction of putp and prmC created a frame shift, resulting in loss of the stop codon of putp, thus creating an in-frame fusion between the putp and prmC. Homologues of putp in other bacterial species, or any other fusions between prmC and other genes were not found. NMA0369 as well as NMB2065 could trans-complement the growth defect in E. coli SC5, indicating functionality of both putative PrmCs. RT-PCR data indicated a 1300 bp long transcript from NMB2065. However, only a 30 kDa recombinant protein was detected by SDS-PAGE, suggesting that only a part of the transcript is being translated, or that the transcription starts at an internal promoter. Using prmC as a probe for Northern blotting of RNA isolated from SC5-NMB2065, two transcripts (˜1300 nt and ˜800 nt), of which the larger species is far less abundant, were detected. In RNA isolated from SC5-NMA369, a single transcript of ˜800 nt was detected.
Conclusion
In *N. meningitidis* MC58, PrmC is encoded by an abnormal large ORF. This ORF is completely transcribed into a large (1300 nt) transcript. Of interest, the second, much more abundant 800 nt transcript observed, suggests either processing of the larger transcript, or the presence of an internal transcription start. Translation into functional PrmC of normal size is most likely from an internal ribosomal binding site.

P002

LOS genotype signature of *N. meningitidis* strains belonging to 4 serogroup B epidemiological complexes

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Objective
Lipooligosaccharide (LOS) is a major virulence factor of *N. meningitidis* and is divided into 12 immunotypes.

Three genetic loci (*lgt*-1, *2*, *3*) were reported to encode the glycosylases responsible for biosynthesis of the LOS oligosaccharide chains. Meningococcus is able to decorate the inner-core of its LOS by *O*-acetylation of the *N*-acetylglucosamine (GlcNAc) and/or by adding phosphoethanolamine groups (PEA) or glucose on Heptose II (HepII). Genes responsible for addition of such decoration have been described (*lot-3* for addition of O-acetyl, *lpt-3* and *lpt-6* for addition of PEA respectively at position 3 and 6 of HepII and *lgtG* for glucose addition on position 3 of HepII). The objective of the study is to determine if an LOS-genotype signature could be found among strains belonging to the same clonal complexe.

Methods

In this study we analyzed 142 *N. meningitidis* strains belonging to 4 epidemiological complexes responsible for serogroup B disease (ST-32, ST-11, ST-41/44 and ST-8) for the presence of genes belonging to the *lgt-1* (*lgtA*, *lgtB*, *lgtC*, *lgtE*) and *lgt-3* loci (*lgtG* and *lpt-6*) and also for the presence of *lpt-3* and *lot-3* genes. By sequencing homopolymeric tracts of phase-variable genes (*lgtA*, *lgtC*, *lgtG* and *lot-3*) ON/OFF phase were determined.

Results

We found a genetic signature of *lgt-1* associated to ST-32 and ST-11-strains: 95% of ST-32 strains studied displayed the type VI *lgt-1* and 93% of ST-11 the type VII *lgt1* locus. For ST41/44 and ST-8 diversification occurred for this last locus as 2 or 3 different types of *lgt-1* were found among the strains tested belonging respectively to those two complexes. A strict signature of *lgt-3* locus was found for the 4 clonal complexes studied: ST-11 and ST-8 strains are all of type I (*lgtG* and an *lpt-6* genes are always present), ST-32 all of type II (*lgtG* is always present and *lpt-6* always absent), ST-41/43 are all of type IV (no *lgtG*, no *lpt-6* genes). *Lpt-3* gene and *lot-3* genes were present on all the strains studied independently of their clonal complex. In terms of phase status of gene responsible for inner-core decoration, phase ON was demonstrated for *lot-3* gene for all ST-11, ST-8 and ST41/44 strains while phase variation was detected among ST-32 strains. For *lgtG* gene, phase variation was seen for all the three complexes that displayed this gene (ST-8, ST-11 and ST-32).

Conclusion

Overall this study indicates that genes responsible for LOS biosynthesis are not randomly distributed among *N. meningitidis* strains and a clonal complex signature of LOS-genotype was found.

P003

Comparative genomics of *Neisseria meningitidis* carriage and disease isolates

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Neisseria meningitidis is a leading cause for infectious childhood mortality worldwide. Therefore, most research efforts have hitherto focused on disease isolates belonging to only few hyper-virulent clonal lineages. However, up to 10% of the healthy human population are temporarily colonised by genetically diverse strains mostly with little or no pathogenic potential. Currently, little is known about the biology of carriage strains and their evolutionary relationship with disease isolates, and the expression of a polysaccharide capsule by most disease isolates is the only trait that has been conformingly linked to pathogenic behaviour in *N. meningitidis*.

Methods

Therefore, to gain insight into the evolution of virulence traits in this species, whole-genome sequences of three meningococcal carriage isolates were obtained and compared to the genome sequences from three disease isolates already available in public databases as well as to the genome sequences from *N. gonorrhoeae* and *N. lactamica*, respectively.

Results

Based on intra-specific comparisons of the six meningococcal genomes, a number of surface located candidate virulence genes were found to display a differential chromosomal location possibly due to phage-mediated recombination events. This indicates that some lysogenic phages might act like

evolutionary catalysts by restructuring the host's genome and thus potentially facilitating phenotypic diversification like the modulation of virulence in *N. meningitidis*. In addition, the insertion sequence IS1655 was involved in large chromosomal rearrangements in *N. meningitidis* and by in silico genome comparisons and dot blot hybridization it was shown to be restricted only to *N. meningitidis*.

Conclusion

The low sequence diversity observed for IS1655 combined with a genome-based phylogenetic reconstruction suggest that *N. meningitidis* might have emerged as an un-encapsulated human commensal from a common ancestor with *N. gonorrhoea* and *N. lactamica* and acquired the genes responsible for capsule synthesis within evolutionary recent times.

P004

Impact of defects in DNA repair genes on genome instability and phase variation in *Neisseria meningitidis*

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Objectives

Neisseria meningitidis (the meningococcus, Mc), an important human pathogen, is known to be highly mutable yet different strains show various levels of mutation rates. Defects in DNA repair components are hypothesized to increase the mutational load and levels of phase variation. Phase variation is caused by the instability of polynucleotide tracts, which affects gene expression or on-off switching. However, the relative contribution of specific DNA repair defects on phase variation and mutagenicity in various Mc strain backgrounds has not been defined. In this study, we performed *in vivo* monitoring of phase variation as well as spontaneous and stress-induced mutagenicity, and observed the functional dependence in defined DNA repair mutants of a panel of representative Mc strains.

Methods

A system for measuring the frequency of the switching of a polynucleotide tract inside the spectinomycin (Spc) resistance gene allowing quantitative assessment of the switching rate was established. To promote efficient natural transformation, a 12-mer DNA uptake sequence (DUS) was inserted into plasmid pARR2107, a kind gift from H. L. Alexander (1), containing the Spc switching locus inside the hpuB gene, named pUD. This plasmid was used to transform a panel of representative Mc wildtype strains and DNA repair mutants, and the spontaneous switching rate was compared. The quantitative effects of Mc DNA repair null mutants including Δfpg, ΔmutY, ΔmutS and ΔmutL were assessed, allowing analysis of the impact of the different DNA repair components on phenotypic expression. The cumulative effects of strains deficient in multiple DNA repair genes are also investigated. Results from these experiments are compared with findings on spontaneous and stress-induced mutation rates. In this context, we are also performing Mc cellular fractionation and searching for DNA binding proteins in general. Ultimately, the phase variation system will also be utilized for monitoring the effects in the mutants corresponding to the DNA binding proteins identified by "fishing for DNA binding proteins"by 2D gel separation, solid phase overlay assay with DNA substrates and MS-MS analysis, providing a platform to study the *in vivo* effects of DNA binding proteins in general.

Results and conclusions

We find that both Δfpg and ΔmutS mutants in some strains significantly increased phase variation in comparison to wildtype strains, however, the highest increase was observed for ΔmutS mutants. The fpg null mutants increased the mutation load two fold, while ΔmutS caused a 30 fold increase, indicating that the mismatch repair protein MutS has a more pronounced effect on the stability of polynucleotide tracts in Mc than the base excision repair component Fpg. We also observed large strain-specific differences in the basic phase variation and mutation rates, some of which were very high and indeed indistinguishable from the corresponding DNA repair null mutants. These results suggest that large intraspecies differences exist, and we will correlate these differences to fitness for survival, adaptability and virulence.

Reference
1. Alexander HL, Richardson AR, Stojiljkovic I. Natural transformation and phase variation modulation in *Neisseria meningitidis*. Mol Microbiol. 52:771-83, 2004.

Figure 1: Switching rate in Mc DNA repair mutants

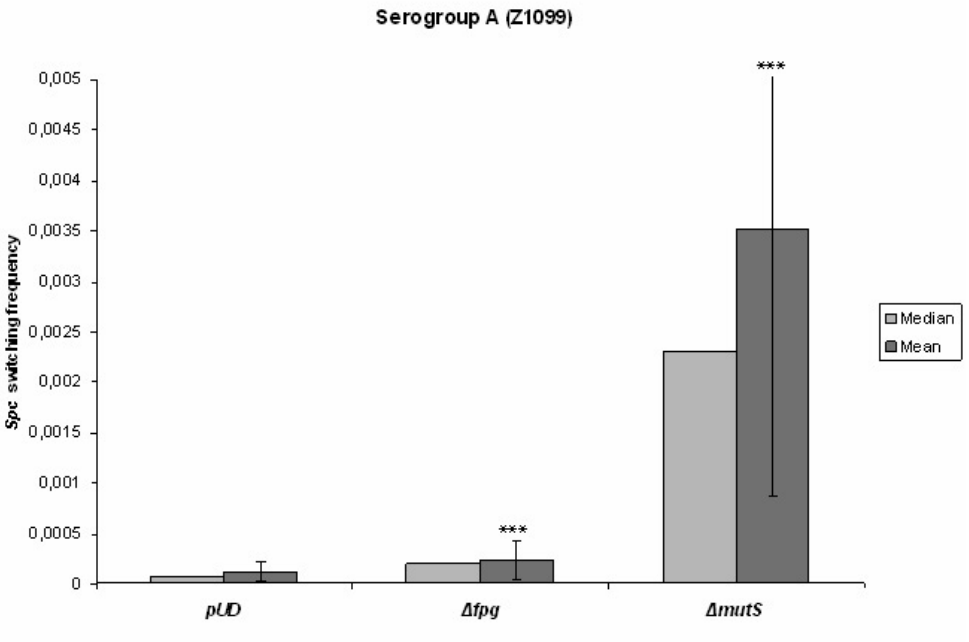

Figure 1. Assessment of meningococcal phase variation. Phase variation frequency for strains NmZ1099_pUD (control), NmZ1099_pUDΔ*fpg* and NmZ1099_pUDΔ*mutS*. Error bars represent standard deviation, both mean and median values are shown (N=34). ***p<=.001 calculated by the Wilcoxon signed-rank test. Phase variation frequency is more than 2-fold and 30-fold increased in the Δ*fpg* and Δ*mutS* background, respectively.

P005

The roles of gonococcal c-type cytochromes in electron transfer to nitrite and nitric oxide during oxygen-limited growth

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Pathogenic Neisseria can grow aerobically using oxygen as the terminal electron acceptor to support energy production and growth. When the supply of oxygen is severely limited, they induce the synthesis of enzymes for a truncated denitrification pathway in which nitrite is reduced via nitric oxide to nitrous oxide. Gonococci lack genes for nitrate reduction, and pseudogenes for a nitrous oxide reductase do not encode functional proteins. The inducible nitrite reductase, AniA, is a copper-containing protein homologous to NirK in other denitrifying bacteria. It is located on the outer bacterial membrane, but how electrons are transferred across

the periplasm from the quinol pool or the cytochrome bc1 complex in the cytoplasmic membrane to AniA is unknown. Also unknown are how electrons reach the outer membrane cytochrome c peroxidase, Ccp, or to the blue copper protein, Laz. The physiological role of Laz also remains controversial.
In an accompanying presentation, we have described the construction and properties of mutants defective in each of three c-type cytochromes, c2, c4 and c5. We have shown that mutations in genes for either cytochrome c4 or c5 result in loss of about 50% of the respiratory capacity of the parent. We now report that these mutants are also more sensitive than the parent strain, F62, to growth inhibition by nitrite, but they are still able to reduce nitrite. Although oxygen reduction is unaffected by the cytochrome c2 mutation, rates of nitrite reduction by this strain are far lower than those of the parent. This cytochrome is therefore implicated as one of the redox proteins that transfer electrons directly or indirectly to AniA. However, the residual rate of nitrite reduction in the cytochrome c2 mutant is significant, suggesting that there is an alternative electron transfer pathway to nitrite that does not involve cytochrome c2. Double mutants have been constructed in attempts to identify this alternative pathway, and to confirm the proposed electron transfer pathways to oxygen. Results of experiments with myxothiazole, which inhibits electron transfer through the cytochrome bc1 complex, and with the artificial electron donor ascorbate + TMPD will be presented that indicate how electrons are transferred to the neisserial outer membrane.

P006

The type IV DNA secretion system of *Neisseria gonorrhoeae*

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Approximately 80% of *N.gonorrhoeae* strains contain a horizontally acquired 57 kb genetic element, the Gonococcal Genetic Island (GGI). The GGI contains at least three operons. Two operons encode homologs of the F-plasmid type IV conjugation system of Escherichia coli. One smaller operon encodes homologs of a relaxase (TraI) and a coupling protein (TraD), while the larger operon encodes proteins with homology to the Mating Pair Formation (MPF) components of the F-plasmid system. The third large operon encodes proteins with putative functions in DNA processing. Based on similarities with the F-plasmid conjugation system, it was proposed that the GGI encodes a type IV DNA conjugation machinery. Conjugal DNA transport via type IV secretion systems is one of the main causes responsible for spreading antibiotic resistance in Neisseria, but the mechanism that governs conjugal DNA transport is still unknown.
We have set out to study the DNA secretion mechanism of N.gonorrhoeae, and we determined the minimal composition of the type IV DNA secretion machinery. To characterize the DNA transfer genes in the GGI, insertion-duplication mutagenesis was used to create polar and non-polar mutations in all genes of the GGI. The mutants were tested for their ability to secrete DNA using a fluorescence-based DNA secretion assay. Type IV secretion systems contain a pilin subunit, which is assembled in a pilus like structure involved in attachment to the acceptor cell. In several systems, the pilin subunit is processed by a peptidase which circularizes the pilin protein before it is assembled into the pilus. The GGI contains both a pilin homologue (TraA) and a peptidase (TrbI). Remarkably, neither deletion of TraA, nor of TrbI, has any effect on DNA secretion. Using advanced BLAST searches, we found that our laboratory strain MS11A strain contains a frameshift mutation, resulting in a truncation of 14 amino acids. To check whether the truncated TraA is also found within the population, clinical isolates were tested for the presence of the full length TraA. Approximately 17 % of the clinical isolates contained the truncated version. No relation was observed between any of the characteristics of the clinical isolates and the absence or presence of functional TraA. DNA secretion experiments demonstrated that the strain with full length TraA does not secrete any DNA. Deletion of TrbI in the strain with full length TraA (which abolishes correct assembly of TraA) shows similar DNA secretion levels as observed for the wild type strain. Remarkably, preliminary experiments with strains containing the full length TraA showed an increase of at least 104 in the conjugation frequency in filter plate assays. This demonstrated that full length TraA might be used for conjugation, while non-functional

TraA results in DNA secretion. Currently, we test the mobilization of gonoccocal βlactamase plasmids mediated by the GGI posessing full length TraA.

P007

A novel IS200/605-like insertion sequence in *Neisseria meningitidis* associated with clonal complexes cc18 and cc213

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Objectives
By serendipity we discovered an insertion sequence (IS) hitherto unknown to exist in the Neisseriae. IS elements are of relevance since they may contribute to the genetic variability of meningococci, enhancing virulence. The objectives of this study were: i) to determine the genetic structure of this element; ii) to evaluate the distribution of this IS among the meningococcal population and iii) to assess whether this IS element shows active transposition in meningococci and in *E. coli.*
Methods
Hundred seventy seven isolates from patients with meningococcal disease, representing 23 clonal complexes (cc), were obtained from the Netherlands Reference Laboratory for Bacterial Meningitis (AMC/RIVM) and were characterized by serogrouping and Multi Locus Sequence Typing (MLST) (1). DNA sequences were determined using BigDyeTerminator chemistry (applied Biosystems) according to the instructions of the manufacturer. The distribution of the IS element among meningococci was assessed by PCR and their copy number by Southern blotting.
Results
The novel IS element is 1.8 kb long and shows no similarity with sequences in any of the publicly available Neisseriae genome sequences. However, it shows high similarity with IS loci in the genomes of Dichelobacter nodosus VCS1703 (nucleotide 81% identity) and Haemophilus somnus 2336 (nucleotide 75% identity). The IS does not contain inverted repeats at it ends. It comprises two open reading frames (ORF) in divergent orientation, encoding an IS200-like transposase and an IS605-like transposase, respectively. The latter ORF contains a premature stop codon. Accordingly, we classified this novel meningococcal IS as IS200/605. The loci in *D. nodosus* and *H. somnus* have the same genetic organization. In addition, IS200/605 like IS are found, among other species, in *Polaromonas naphthalenivorans* and *Helicobacter pylori.* Of 118 meningococcal isolates initially tested for presence of the IS, 12 were positive; 5 of cc18, 5 of cc213, one of cc334 and one of cc376, indicating that IS200/605 is found in a limited number of cc. We then tested 56 cc213 and 3 cc18 isolates, which all, with the exception of one cc18 isolate, were positive for the IS200/605. All positive isolates carried only one copy of the IS, which was always inserted at the same position between NMB0493 and NMB0494 (MC58 annotation). The one cc18 isolate that tested negative for the IS in PCR did also not contain remnants of the IS200/605 element at that position.
Conclusions
The IS200 and IS605 elements are widely distributed among prokaryotes. Also IS200/605 like transposable elements are found in several species. Here we show for the first time the existence of an IS200/605 in meningococci. Its limited distribution in isolates of only 4 clonal complexes, suggests a recent acquisition. Currently, we are investigating whether the meningococcal IS200/605 is capable of transposition in *E. coli* and *N. meningitidis.* However, all IS200/605-positive meningococcal isolates carry only one copy inserted at the same position in their genome, indicating lack of active transposition in meningococci.
References
1. Neisseria Multi Locus Sequence Typing website (http://pubmlst.org/neisseria/) developed by Keith Jolley and Man-Suen Chan (Jolley et al. 2004, BMC Bioinformatics, 5:86).

P008

Stress response related sigma factors in *Neisseria meningitidis*

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Objectives
The meningococcoccus (*Neisseria meningitidis*) encounters several different environments during colonization and infection. These environments result in harsh growth conditions for the meningococcus to which it has to adapt by expressing different sets of genes under each condition. Alternative sigma factors are involved in the stress response of some bacteria. We investigated the role of sigma factor E (rpoE) and sigma factor H (rpoH) in the stress response of *N. meningitidis*.
Methods
Knockout mutants of rpoE and rpoH were constructed by replacement with a gene encoding erythromycine resitance (ermC) in *N. meningitidis* H44/76. In addition, rpoE and rpoH were inserted in vector pEN11 allowing IPTG inducible expression of each gene in *N. meningitidis* H44/76. Protein profiles of rpoE and rpoH mutants were compared with wild type when grown under varying iron concentrations (severely depleted or not) using SDS polyacrylamide gel electrophoresis. Differentially expressed proteins were identified by MALDI-TOF mass spectrometry.
Results
We did not succeed in constructing the rpoH knockout mutant, suggesting that, as found in gonococci, this mutant is not viable. The rpoE knockout mutant had a slightly reduced growth rate compared to the wt under iron replete conditions as well as under iron deplete conditions. In addition, the rpoH overexpressing and rpoE overexpressing *N. meningitidis* H44/76 also showed slightly reduced growth under both growth conditions. Protein profiles of rpoH overexpressing meningococci showed no detectable differences with those of wt meningococci. Protein profiles of rpoE knockout meningococci and wt meningococci were comparable. However, protein profiles of rpoE overexpressing meningococci grown under iron replete conditions showed a protein that was absent from the profiles of wt meningococci. Mass spectrometry revealed that this differentially expressed protein contained methionine sulfoxide reductase (Msr, NMB0044). In addition, under iron depletion conditons FrpB expression was induced in wt meningococci. Remarkably, overexpression of rpoE under iron limitation conditions resulted in downregulation of FrpB.
Conclusion
The rpoH knockout mutant in meningococci is probably not viable. Overexpression of rpoE resulted in the induction of Msr, as was found in gonococci [1]. Under iron limitation the expression of FrpB is reduced when rpoE expression is increased. Currently, we are investigating the mechanism by which the RpoE mediated repression of FrpB is obtained.
Reference
1. Gunesekere IC, Kahler CM, Ryan CS, Snyder LA, Saunders NJ, Rood JI, Davies JK. Ecf, an alternative sigma factor from Neisseria gonorrhoeae, controls expression of msrAB, which encodes methionine sulfoxide reductase. J Bacteriol. 2006;188:3463-9.

P009

Mutagenesis without insertion of antibiotics resistance genes to obtain multiple-gene knockout Neisseria mutants

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Objectives
Mutagenesis, such as gene disruption, is one of the important ways to analyze function of specific proteins of pathogenic bacteria. These bacteria normally express several essential virulence factors in order to establish infection. Pathogenic Neisseria have a large number of important virulence factors, such as Opa, Opc, LOS, pili. To clarify and confirm the detailed function of each factor more precisely, we aimed to establish a method for construction of mutants with disruption in multiple genes of Neisseria.
Methods
Both 5'- and 3'- flanking regions of a target gene (crgA: LysR-type transcriptional regulator) from wild-type *N. meningitidis* FAM20 were cloned into a suicide vector, pABB-CRS2, which has R6K ori, sacB gene, Gateway attR recombination sites, and ampicillin resistant gene, and is available for mutagenesis in enteropathogenic *E. coli* and Bordetella. After introduction of the suicide vector into the wild-type strain by natural transformation, the transformants were cultured on sucrose-containing GC-plates for counter selection. Genomic DNA was prepared from sucrose resistant colonies and analyzed by PCR to examine if the bacteria had the mutation in the target gene.
Results
A number of independent ampicillin resistant transformants, with the suicide vector sequence in their genomes, were obtained. Sucrose counter selection was available only when bacteria were grown at 30˚C. All of the examined sucrose resistant bacteria had deletion mutation in the target gene.
Conclusion
In the present study, the mutagenesis method was established. However, the appearance of transformants after the introduction of the suicide vector in Neisseria was still low. Optimizations of 1) lengths of flanking regions of target genes, 2) number and position of DNA uptake sequence (DUS) in cloned Neisseria DNA on the suicide vector, and 3) transformation methods, such as natural transformation or electroporation are currently in progress.

P010

Gonococcal resistance to reactive nitrogen species

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Objectives
Studies began examining the ability of *N. gonorrhoeae* to metabolize nitric oxide (NO) because we propose that gonococcal denitrification is partly involved in the immunosuppressive nature of the disease. NO has a multitude of functions in cell signaling, but also plays a pivotal role in the immune response to pathogens, where it can have cytotoxic properties. We investigated the resistance to reactive nitrogen species (RNS) in *N. gonorrhoeae*. Studies compared the effects of both NO and peroxynitrite (ONOO-) on the growth and viability of laboratory strain F62, a variety of clinical isolates, and *E. coli*. Resistance to RNS would offer the organism a strategy to evade host defense mechanisms.
Methods
General protocols for the genetic manipulation of gonococcal strains were utilized to create insertionally

inactivated genes that are known or thought to be involved in RNS defense within other bacterial species. These included ahpC (alkyl hydroperoxide reductase), dnrN (conserved hypothetical), msrA (peptide methionine sulfoxide reductase), and norB (nitric oxide reductase). An Apollo 4000 free radical analyzer was used with probes capable of real-time measurement of both NO and oxygen in order to examine the respiration status of a wild-type F62, along with a norB- mutant strain while under a challenge of high NO concentrations. Susceptibility to RNS was assayed by measuring growth and/or killing after the addition of either molecular generators of RNS or the direct addition of such species, followed by CFU plating.

Results

N. gonorrhoeae exposed to high concentrations of NO (2 mM) showed no defect in growth rate, while a mutation within nitric oxide reductase (norB-) gene caused NO to be bacteriostatic at this concentration. The role of NorB was examined under aerobic conditions while in the presence of a NO donor, DETA/NO. Both F62 and a norB- strain showed addition of high NO levels caused inhibition of cytochrome oxidase, leading to accumulation of oxygen for both strains. Therefore, under aerobic conditions norB does not function to detoxify NO, but instead is utilized to continue respiration as NO is used as an alternative electron acceptor. When challenged with ONOO-, *N. gonorrhoeae* F62 had a delayed growth rate while E. coli DH10B showed a 3-4 log peroxynitrite-mediated killing, as assayed by CFU plating. Resistance is either induced or ONOO- damage is repaired since growth after treatment resumes after an initial lag phase. To ensure the resistance of F62 was not unique, we tested eight other gonococcal strains for the effect of ONOO- on viability. All strains grew in the presence of peroxynitrite, although there was some reduction of growth in the presence of RNS. We next determined if gonococcal orthologs to known or putative RNS resistance genes functioned in *N. gonorrhoeae*. Mutations in ahpC, dnrN,and msrA, alone or in combination had little effect on peroxynitrite resitance.

Conclusion

N. gonorrhoeae has a remarkable resistance to RNS, including both NO and ONOO-. This is a novel mechanism that exists across clinical isolates which gives a better understanding of how the bacterium evades clearance by the human immune system.

P011

Biochemical analysis of gonococcal NsrR, a nitric oxide sensing Rrf2-type transcriptional repressor

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Objectives

The ability of *Neisseria gonorrhoeae* to reduce NO, a key signaling molecule involved in host defense, may have important immunnomodulatory effects during the course of infection. Several gonococcal genes, including the nitrite reductase, aniA, and the nitric oxide reductase, norB, have been shown to be regulated by the NO-sensing Rrf2-type transcriptional repressor NsrR. Previous work has focused on the genetic characterization of the NsrR regulon. Current research has focused on the biochemical analysis of NsrR interaction with its DNA binding site and with its ligand, NO.

Methods

An nsrR::FLAG fusion gene was constructed and used to knock out the wild-type nsrR gene in gonococcal strain F62. Chromatin immunoprecipitation was performed in the FLAG fusion strain to confirm *in vivo* NsrR binding. Purified NsrR::FLAG protein was prepared for *in vitro* use by overexpression of the nsrR::FLAG fusion gene in *E. coli* and subsequent collection with an anti-FLAG affinity resin. Gel shift assays were performed with purified NsrR::FLAG to characterize DNA binding. Modification and evaluation of the NsrR binding site was accomplished by site-directed mutagenesis of translational norB::lacZ promoter fusions. Site-directed mutagenesis was also used modify the nsrR gene for the purpose of identifying specific residues involved in protein function.

Results
Immunoprecipitation of formaldehyde crosslinked DNA/protein complexes with anti-FLAG antibody from a gonococcal strain expressing nsrR::FLAG showed enrichment of DNA in the norB/aniA intergenic region and in the dnrN upstream region. Recombinant NsrR::FLAG protein was isolated from *E. coli* to greater than 90% purity. Gel shift analysis showed direct binding of NsrR::FLAG to biotin-labeled norB upstream region in vitro. Addition of 100X concentration of unlabeled target DNA to the binding reaction abrogated the apparent shift, while addition of anti-FLAG antibody to the binding reaction shifted biotin-labeled norB to very high molecular weight. Addition of a long half-life NO donor to the binding reaction also inhibited the shift of biotinylated norB. Gel shift assays were used to estimate the dissociation constant (Kd) of NsrR::FLAG/operator binding in the norB, aniA, and nsrR upstream regions, which were found to be 7nM, 19nM, and 34nM respectively. Single amino acid substitutions of C90A, C97A, or C103A, as well as various amino acid substitutions in the putative DNA binding helix of the NsrR protein greatly inhibited repression of norB::lacZ in a gonococcal strain.
Conclusions
Binding of NsrR to its operator was confirmed both *in vivo* and *in vitro*. The NsrR binding site in norB, with the best match to an inverted repeat sequence, displayed the highest level of repression and had the lowest dissociation constant compared to the binding sites in other genes of the NsrR regulon, which displayed weaker binding as the inverted repeat sequence became less perfect. In the presence of NO, NsrR/DNA binding was abrogated. NsrR contains three cysteines presumed to coordinate an iron-sulfur cluster involved in NO-sensing. Substitution of any of these cysteines greatly inhibited the ability of NsrR to bind DNA. Amino acid substiutions in the predicted DNA-binding helix of the NsrR protein also affected the ability of NsrR to bind.

P012

sodC as a species identifier for Real-time PCR detection of *N. meningitidis*

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Objectives
Real-time PCR is the molecular method used widely for species identification during meningococcal carriage studies, for characterization of routine bacterial meningitis surveillance isolates, and for detection of *N. meningitidis* in CSF or other specimens. Current real-time PCR diagnostics for *N. meningitidis* target the capsule transport gene, *ctrA* [1]. However, over 16% of meningococcal carriage isolates lack the genes for capsule biosynthesis and transport [2, 3], including *ctrA*, rendering this target gene ineffective at species identification in this population of meningococcal isolates. Other nongroupable meningococci sustain recombination events at the capsule locus that also may involve *ctrA* [2]. The Cu-Zn superoxide dismutase gene, *sodC*, is found in *N. meningitidis* but not in any other *Neisseria* spp. [4]. To identify all *N. meningitidis*, regardless of capsule genotype or expression status, *sodC* was evaluated as a target gene for real-time PCR.
Methods
sodC was sequenced from 14 geographically and temporally dispersed groupable and nongroupable meningococci. A consensus sequence was generated using the Lasergene v. 7 program SeqMan (DNASTAR, Inc., Madison, WI). Primer Express v. 3.0 (Applied Biosystems, Foster City, CA) was used to design primers and probes based on this consensus sequence. Using an Mx3005P real-time PCR machine (Stratagene, La Jolla, CA), primer concentrations were optimized and dissociation curves were generated, followed by probe concentration optimization and lower limit of detection (LLD) determination.

The optimized primer and probe concentrations were tested for sensitivity and specificity. Cell lysates from 145 *N. meningitidis* isolates were tested to determine the sensitivity of this assay. The serogroup breakdown of the sensitivity panel was as follows: 64 nongroupable, 11 serogroup A, and 10 each of serogroups B, C, Y, W135, Z, X, and 29E. Cell lysates from 36 non-meningococcal *Neisseria* spp. and other genera whose clinical presentation or laboratory identification might be confused with *N. meningitidis* were tested to determine the specificity of this assay. C_t values ≤35 were considered positive; C_ts in the range of 36-40 equivocal; and C_t values ›40 negative.

Results

A standard curve was generated using 7 10-fold serial dilutions of a known quantity of *N. meningitidis* serogroup B QIAamp-prepared DNA. The slope was found to be -3.347 (99.0% amplification efficiency) with an R^2 value of 0.995 (indicating reproducibility and consistency of the replicates). The assay detected 10.6 copies of *sodC* with a C_t value of 37; 106 copies were detected with a C_t value of 34.

Using this assay, 98.6% (143/145) of *N. meningitidis* isolates tested were *sodC* positive, with a range of C_t values from 16.39 to 29.86. All 36 (100%) members of the specificity panel were negative for *sodC* using this assay.

Conclusions

The *sodC* real-time PCR assay is a highly efficient, sensitive, and specific method for identification or detection of *Neisseria meningitidis*, especially during carriage studies in which over 16% of meningococcal isolates may lack capsule genes.

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P013

Genetic modifications of three meningococcal group B multivalent vaccine strains

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Objectives

Native outer membrane vesicles (NOMV) of *Neisseria meningitidis* are highly immunogenic, but their high endotoxin content has prevented their use as a vaccine against group B meningococci. Furthermore, first generation of OMV vaccines prepared with deoxycholate provided relatively narrow mostly subtype-specific immunogenicity. To overcome these obstacles we have developed a multivalent NOMV Group B vaccine by using an approach in which we: 1) use three antigenically diverse group B strains; 2) introduce mutations into genomic DNA to reduce NOMV toxicity; 3) establish expression of additional PorA proteins of high prevalence in North America; 4) provide increased expression of several minor conserved proteins with protective potential; 5) stabilize expression of truncated LOS structures which lack lacto-N-neotetraose by inactivation of the lgtA gene.

Methods

Three *N. meningitidis* strains, B1 [parental strain H44/76(B:15:P1.7,17:L3,7)], B2 [parental strain 8570(B:4:P1.19,15:L3,7v)] and B3 [parental strain B16B6(B:2a:P1.5,2:L2)] were selected. Two types of plasmids were constructed for the mutagenesis of the vaccine strains. The first type contained a disruptive antibiotic resistance insert to knock out undesirable genes. The second type contained functional genes encoding proteins of interest in order to enhance the native presentation of these OMP antigens. Each plasmid contained flanking sequences, homologous to the appropriate loci of bacterial genomic DNA for

recombination and also included the gonococcal DNA uptake sequence that increased transformation efficiency. The plasmids were introduced into the genome via homologous recombination followed by antibiotic or monoclonal antibody selection of the transformants.

Results

Each vaccine strain was genetically modified to carry the following deletions: ΔlpxL1 (to reduce LOS-associated toxicity); ΔsynX (to achieve capsule-negative, non-sialylated LOS phenotype, thereby further increasing safety); ΔlgtA (to eliminate phase-variable expression of lacto-N-neotetraose, and to stabilize expression of the short-chain LOS immunotypes, L8-3, L8-5, and L8-2 LOS in strains B1, B2, and B3, respectively). Each strain was also modified to express a second, heterologous PorA (to increase the antigenic assortment). Expression of minor conserved outer membrane proteins was increased in each of the three strains to achieve broader antigenic cross-reactivity. Specifically, strain B1 expressed NadA under the control of the homologous PorA promoter; and strains B2 and B3 expressed homologous variants 1 and 2 of GNA1870 (fHbp), respectively, both under the control of the IPTG-inducible orthologous Ptac E. coli promoter. All genetic modifications demonstrated stability during at least 6 observed passages.

Conclusion

The constructed NOMV vaccine strains were found to produce NOMV that were non-pyrogenic at a level consistent with safety in humans and demonstrated viability for cultivation despite multiple genetic modifications. Furthermore, NOMV prepared from the strains exhibited good cross-reactive immunogenicity. These results suggest that the vaccine is suitable for further evaluation in clinical trials.

P014

The periplasmic PilB protein from *Neisseria meningitidis*: Regeneration mechanism of the methionine sulfoxide reductase activities

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Neisseria meningitidis is a Gram-negative pathogen that remains a leading cause of bacterial meningitis and septicaemia world-wide. Its pathogenicity is linked to its ability to resist reactive nitrogen and oxygen species, which are produced by phagocytes in response to the infection of host. *N. meningitidis* have different antioxidants systems including the PilB protein. The PilB protein is described to be specific towards human obligatory pathogenic bacteria of Neisseria genus and to be implicated, *in vivo*, in survival in the presence of hydrogen peroxide.

PilB is a periplasmic three-domain protein: the central and C-terminal part displays a methionine sulfoxide reductase activity of class A (MsrA) and B (MsrB) activity, respectively, while the N-terminal domain displays a disulfide oxido-reductase activity. Msrs are antioxidant repair enzymes that catalyse the thioredoxin(Trx)-dependent reduction of methionine sulfoxide (MetSO) back to methionine. MsrA and MsrB are two structurally-unrelated classes of Msrs, with opposite stereoselectivity towards the S and R isomers of the sulfoxide function of MetSO, respectively. Recent *in vitro* studies, on the N-terminal domain produced as isolated folded entity, have shown that 1) the N-terminal domain possesses a disulfide redox active site with a redox potential in the range of that of Trx, 2) the N-terminal domain recycles efficiently only the oxidized forms of the MsrB domain of PilB from *N. meningitidis*, as an isolated form, and 3) this domain presents a Trx-fold, with more exactly a DsbE topology. The DsbE family is a typical membrane anchored periplasmic thiol/disulfide oxidoreductase family involved in electron transfer during the maturation of c-type cytochromes.

So far, studies have been performed only on isolated domains of the PilB protein. Therefore, the question arises whether the N-terminal domain is operative in reducing the downstream MsrA and MsrB domains in the PilB context, and the nature of the periplasmic disulfide oxidoreductase responsible for the N-terminal domain recycling. That's why the full-length PilB protein was overproduced and purified, as well as the N-terminal domain of the putative DsbD protein from *N. meningitidis*, which is a good candidate as periplasmic N-terminal domain reductant. A structure-function relationship study has been carried out

using site-directed mutagenesis, stopped-flow and fluorescence approaches. Data will be presented which illustrate the exact mechanism of the Msr activity of PilB in the periplasm.

P015

A cis-Acting DNA element upstream of the pilin expression locus is required for pilin antigenic variation

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To help avoid immune surveillance, *Neisseria gonorrohoeae* promotes high frequency gene conversion events between many silent pilin loci and the single expressed pilin locus, pilE; resulting in the production of variant pilin proteins. Transposon insertions have been isolated in the non-coding region upstream of pilE that blocks pilin antigenic variation (Av) [1, 3]. To define which DNA sequences in the non-coding region upstream of pilE are required for pilin Av, this region was randomly mutagenized using error prone PCR, and the mutations were introduced into the Gc chromosome by DNA transformation. 103 transformants unable to undergo pilin Av were selected by screening for a stable, piliated colony morphology, and DNA sequence analysis revealed that each mutant carried a point mutation in a specific region upstream of pilE. Further characterization of this DNA element by site directed mutagenesis revealed that mutating any one of 12 specific bases in this region blocks pilin Av. In addition, a point mutation in this region rescues the Holliday junction processing mutant shown in previous studies to be synthetically lethal during pilin Av [2]. We conclude that these 12 base pairs in the non-coding region upstream of the pilus expression locus defines a cis-acting DNA element required for directing recombination from the silent loci to the expressed pilin gene and may define the initiation site for pilin Av.

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P016

Identification of genetic elements differing between disease- and carriage-associated meningococcal Isolates

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Objective

Neisseria meningitidis, or meningococcus, is a Gram-negative bacterial pathogen that is a leading infectious cause of death worldwide. While it can cause a rapid and devastating invasive form of disease, it is carried asymptomatically by 8-25% of the general population. It is unknown why some highly virulent strains emerge, causing widespread outbreaks or epidemics, while many other strains are associated with asymptomatic carriage, or sporadic disease. Our goal was to identify some of the genetic elements underpinning these differences, by performing subtractive hybridisation comparisons between selected disease-associated strains and carriage-associated strains.

Methods
We selected several strains from our collection to begin these studies. Preliminary experiments were performed to compare an isolate of the serogroup B New Zealand epidemic strain (NZ98-254) with a related serogroup B strain (NZ357-1) that has been isolated repeatedly from asymptomatic carriers, but which has not caused disease in New Zealand. Initial results will be expanded with additional strains derived from international epidemics, and carriage-associated strains of the same serogroup. All strains have been previously characterised by multi-locus sequence typing (MLST) and serological studies. We isolated genomic DNA from our strains of interest, and performed subtractive hybridisation using the PCR-Select Bacterial Genome Subtraction Kit (Clontech), according to the manufacturer's instructions. This is a technique used to identify genetic regions present on one strain (the "tester" strain) but absent in a second strain (the "driver" strain). Resulting subtracted DNA fragments were cloned into the TOPO TA Cloning Kit (Invitrogen), transformed, and sequenced. Sequence data was analysed and open reading frames identified by BLASTn and BLASTx. Future experiments will include performing blotting techniques to look for association of a particular genetic element with a panel of disease- or carriage-associated strains.
Results
Preliminary experiments were performed using NZ98-254 (the NZ epidemic strain type) as the tester strain, and NZ357-1 (a carriage-associated isolate) as the driver. Approximately 25 genes were identified as being present in NZ98-254 and absent in NZ357-1. Of these genes, about two thirds were identified as hypothetical proteins, all of which were present in MC58, the sequenced serogroup B strain. Additional genes which have been previously described as strain variable elements, e.g., putative phage-associated and restriction modification systems, were identified, supporting the validity of our approach. In addition, several possible virulence factors were identified, including putative autotransporters (implicated in adhesion and host cell interaction with other pathogens) and a gene encoding a putative DNA-binding regulatory protein.
Conclusions
There is little overlap between the genes we identified by subtractive hybridisation and those identified in published studies; this is likely due to the strains chosen for analysis. We intend to proceed by expanding on subtractive hybridisation studies, to assess the prevalence of specific genes in a panel of disease- and carriage-associated meningococci, and to conduct functional studies by targeted mutagenesis in disease-associated strains.

P017

Structural and biochemical characterisation of the oxidoreductase NmDsbA3 from *Neisseria meningitidis*

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Objectives
Oxidoreductases (DsbA) are responsible for the addition of disulphide bonds into unfolded polypeptides in the periplasm of gram-negative bacteria. *Escherichia coli* possess a single DsbA which has a broad substrate range. *N. meningitidis* contains three DsbA proteins, *Nm*DsbA1, *Nm*DsbA2 and *Nm*DsbA3, which demonstrate substrate specificity. *Nm*DsbA1 and *Nm*DsbA2 show overlapping substrate specificity towards Type IV pilin, PilE, whilst *Nm*DsbA3 does not recognize this protein substrate. To understand the basis of broad substrate and narrow substrate recognition by oxidoreductases, we have solved the crystal structure of the *Nm*DsbA3 and compared this with *Ec*DsbA.

Method
N-terminal His tagged *Nm*DsbA3 was over-expressed in *E. coli* from a pET45(+) system and purified using Fast Protein Liquid Chromatography (FPLC). *Nm*DsbA3 was then concentrated in 10mM Hepes, 150mM $NaCl_2$, 1mM EDTA pH6.8 buffer to 20mg/ml for crystallography studies. Selenomethionine (^{Se}Met)-labelled *Nm*DsbA3 was expressed from *E. coli* B384 in minimal media containing ^{Se}Met. Diffraction patterns were collected at the Monash Center for Synchrotron Science.
Results
The structure of reduced NmDsbA3 was determined to a resolution of 2.3 A. NmDsbA3 and EcDsbA were superimposed with an r.m.s.d. of 1.9 Å over 115 Cα atoms and the following differences in structure were noted. In EcDsbA, the active site is positioned at the N-terminus of helix α1, where the CXXC forms the first turn of the helix. In *Nm*DsbA3, Cys^{34} and Cys^{37} of the CXXC motif are present on an extended loop that precedes helix α1. A second critical difference lies in the conformation of the loop which connects strand β3 in the thioredoxin domain to helix α2 in the helical domain. A two-residue deletion in *Nm*DsbA3 results in a significant change in the conformation of this loop. There is also a difference in the conformation of the loop which connects helix α6 to strand β4 of the thioredoxin domain and contains the conserved *cis*-Pro residue. These three regions of DsbA form a contiguous surface and each contributes to the substrate binding site that has recently been identified from the structure of a covalent complex between *Ec*DsbA and a substrate peptide. In addition to the differences in the regions which form the substrate binding surface of DsbA, the loop linking strand β5 to helix α7 of the thioredoxin domain of *Nm*DsbA3 is four residues shorter than in *Ec*DsbA, which results in helix α7 having one less turn of helix in *Nm*DsbA3. This helix forms part of the hydrophobic groove below the active site in DsbA enzymes that forms the binding site for DsbB. However, *in vitro* assays indicated that the rate of *Nm*DsbA3 oxidation by DsbB was the same as that for *Ec*DsbA.
Conclusion
Critical differences between the structures of *Nm*DsbA3 and *Ec*DsbA, principally in the loops that define the substrate-binding surface, may provide a rationale for the limited substrate specificity that has been reported for meningococcal DsbA enzymes. One of these loops contains the conserved *cis*-Pro residue that is common to all thioredoxin-like proteins and has previously been shown to regulate substrate specificity in the disulfide isomerases DsbC and DsbG.

P018

An insight into the basis of substrate specificity of meningococcal oxidoreductases

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Objectives
Most Gram-negative bacteria, including *E. coli*, contain a single periplasmic DsbA enzyme, which introduce disulphide bonds into a wide range of protein substrates. In contrast, *Neisseria meningitidis* contains three DsbA enzymes, two of which (*Nm*DsbA1 and *Nm*DsbA2) share 73% amino acid sequence identity. Both enzymes have been shown to be necessary for the correct functioning of Type IV pili in *N. meningitidis*. However, heterologous expression in *E. coli* suggests that these enzymes have separate substrate recognition profiles, in that only *Nm*DsbA1 can recognize the substrate, FlgI, and restore motility to an *E. coli* DsbA mutant. The least conserved region in these proteins is located between residues V^{61}-V^{150} which comprise the alpha (α)-domain and the flexible hinge region that links this to the conserved thioredoxin domain containing the active site motif, $C^{30}PHC^{33}$. To examine the role of hinge flexibility and α-domain conformation in substrate specificity of oxidoreductases, we created chimeric DsbA enzymes in which the

residues between V^{61}-V^{150} of the broad substrate *Ec*DsbA were replaced with the corresponding regions from *Nm*DsbA1 and *Nm*DsbA2. The effects of these changes on enzyme functionality were examined *in vivo* by complementation of an *E. coli* DsbA mutant.

Methods

Chimeric *dsbA* genes consisting of *NmdsbA1* and *NmdsbA2* α-domains spliced into the *EcdsbA* thioredoxin domain were created by Three Way Splice Overlap Extension Polymerase Chain Reaction (SOE PCR). These genes were cloned into the low copy expression vector pHSG576 and transformed into the *E. coli dsbA* mutant strain JCB571. Chimeric DsbA proteins were then assessed in their ability to restore motility to JCB571 by stabilization of FlgI and resistance to the reducing agent, dithiothreitol (DTT).

Results

Replacement of the *E. coli* α-domain and hinge region (residues V^{61}-V^{150}) with that from *Nm*DsbA2 or *Nm*DsbA1 did not interfere with the ability of the chimeric enzymes, *Ec*TDN*m*DsbA2α and *Ec*TDN*m*DsbA1α, respectively, to act as oxidoreductases capable of restoring JCB571 resistance to DTT. However, *Ec*TDN*m*DsbA2α but not *Ec*TDN*m*DsbA1α, retained the ability to recognize FlgI. Specific alteration of the hinge region of *Ec*TDN*m*DsbA1α, from meningococcal DsbA1 $I^{147}SGT^{150}$ to *Ec*DsbA $L^{147}RGV^{150}$, restored ability of the enzyme to oxidize FlgI. In addition, insertion of an extra valine residue before L^{147} in the *E. coli* hinge region of *Ec*TDN*m*DsbA1α ablated the recognition of FlgI although the enzyme was a functional oxidoreductase. Similar modifications to the hinge region of *Ec*TDN*m*DsbA2α ($I^{147}DGT^{150}$) had no observable effect on the ability of this chimeric enzyme to act as an oxidoreductase or to recognize FlgI. However, a random mutation in the α-domain of *Ec*TDN*m*DsbA2α converting the alanine residue at position 76 to a threonine residue ablated the ability of the chimera to recognize FlgI without affecting oxidoreductase function.

Conclusion

We have observed that model peptides bind the interface of the two domains of DsbA, specifically interacting with the peptide loop between α6 and α4 ($L^{147}RGV^{150}$) and the type IV β turn between β3 and β2 ($F^{63}M^{64}$). Using FlgI oxidation as a model *in vivo* substrate, we have confirmed the biological relevance of these residues in determining the substrate recognition profile of these enzymes.

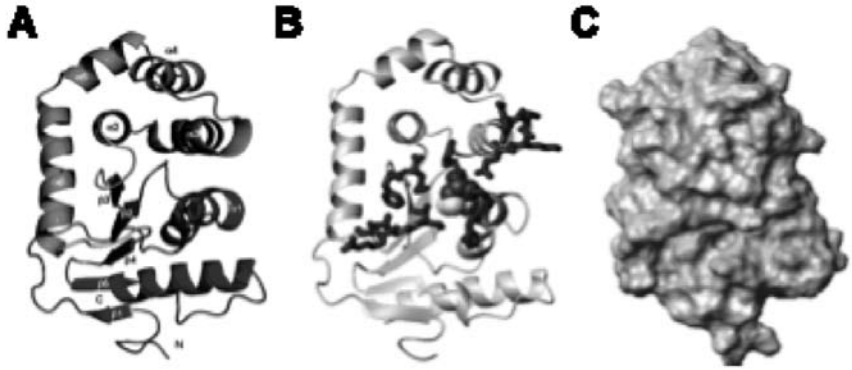

Figure 1 A Ribbon diagram of *Ec*DsbA showing the thioredoxin (blue) and α-helical (magenta) domains and the substrate binding regions (orange). Elements of secondary structure are numbered sequentially from the N-terminus. **B** Residues of the *Ec*DsbA, which make contact with the substrate, are highlighted and the active site shown in CPK **C** Surface of the *Ec*DsbA with residues that form the hydrophobic groove shaded in green. There is no overlap between the residues in the hydrophobic groove shown in (C) and the residues, which make contact with the substrate in the crystal structure (B)

P019

Proteomic and microarray expression analyses of manganese regulation in *N. gonorrhoeae*

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Objectives

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection, gonorrhea, is a host-adapted pathogen that has evolved complex and novel mechanisms to cope with oxidative stress. Manganese (Mn) and the Mn(II) uptake system, MntABC, play a key role in protection against reactive oxygen species in *N. gonorrhoeae* independently of enzymatic superoxide or catalase. MntABC is also regulated by the peroxide responsive regulator, PerR.

To provide a more comprehensive view of the regulatory network and the molecular mechanism of Mn protection, we performed a systematic expression profile analysis at the transcriptional and proteomic level to investigate the global expression changes that take place in a high Mn environment, which may contribute to the oxidative stress resistance phenotype observed.

Methods

N. gonorrhoeae cells were grown in the presence and absence of Mn(II). These samples were analysed by using parallel proteomic (one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE) coupled with 1D liquid chromatography - tandem mass spectrometry (1D LC-MS/MS) and the quantitative method, i.e., isotope coded affinity tag (ICAT)) and genomic (microarray transcriptome profiling) techniques. Also, the polyphosphate kinase (ppk), exopolyphosphatase (ppx) and pyrophosphatase (ppa) mutants were generated and their phenotypes were examined by oxidative killing assays.

Results

The proteomic results revealed that 98 proteins were differentially regulated at the post-transcriptional level under conditions of increased or decreased Mn. The Mn-regulated proteins have a broad range of functions including oxidative stress defence (i.e. superoxide dismutase (SodB), azurin, bacterioferritin), transport, cellular metabolism (i.e. Ppa), protein synthesis, RNA processing, cell division, pilin and the proteins involved in the pilus assembly, such as PilC1 and PilQ.

The ppk and ppa mutant strains showed increased resistance to oxidative stress relative to the wild-type strain, whereas the ppx mutant strain was more sensitive to oxidative stress. Mn provided protection against exogenous superoxide and hydrogen peroxide in the wild-type as well as in the ppk, ppx and ppa mutant strains, indicating that Mn does not require polyphosphate (polyP) for its antioxidant action. The ppk, ppx and ppa mutants had altered fitness in their ability to invade and to survive within an *ex vivo* primary human cervical epithelial cell model, indicating a key role for polyP and pyrophosphate (PPi) in intracellular survival.

Conclusion

The proteomic data has revealed that expression of pili was downregulated when cells were grown in the Mn supplement, and that pilin regulation along with all other expression changes observed were controlled by a post-translational mechanism. Also, these data indicate that increased orthophosphate (Pi) and PPi levels effect Mn dependent resistance to oxidative stress, suggesting that PPi may form complexes with Mn(II).

P020

Insertion of IS1301 Increases the expression of capsule biosynthesis genes in *Neisseria meningitidis*

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Objective
Neisseria meningitidis is the leading cause of bacterial meningitis and septicaemia in children and young adults. Conjugate vaccines against serogroup C *N. meningitidis* raise antibodies against the polysaccharide capsule, a major virulence factor for the bacterium consisting of polysialic acid. Screening of 109 clinical isolates obtained in Spain between 1999 and 2003 has identified three strains which are resistant to killing by serum from immunised individuals as determined by serum bactericidal assays. Genetic analysis of the capsule biosynthesis (cps) locus revealed that these strains contain an insertion sequence, IS1301 in the same position and orientation within the intergenic region (IGR) between the capsule biosynthesis (sia) and capsule transport (ctr) operons. Expression of these operons is controlled by overlapping divergent promoters within the IGR. Our objectives were to investigate how the presence of IS1301 within the IGR confers resistance to anticapsular antibodies, and the mechanism by which it does this.
Methods
Gene expression levels were investigated using RT-PCR, FACS, western blotting and reporter gene constructs in *E. coli.* Transcription profiles of different strains were investigated using *in vitro* transcription assays. The role of IS1301 in the IGR in *N. meningitidis* was investigated using reporter fusions.
Results
Real time RT-PCR demonstrated increased levels of siaA and ctrA transcripts in the presence of IS1301 in the IGR. FACS analysis showed that the amount of capsule is increased on strains with IS1301 in the IGR when compared to those without it. Reporter fusions were made with IGRs with and without IS1301 fused between lacZ and xylE. Enzyme activity for both gene products showed increased expression of LacZ (reporting SiaA) and XylE (reporting CtrA) in the presence of IS1301 when compared to the absence of IS1301. *In vitro* transcription assays showed that promoters with and without IS1301 give rise to different transcription profiles. To address the mechanistic role of IS1301, reporter constructs have been made in *N. meningitidis*, where the native promoter architecture is maintained.
Conclusion
The presence of IS1301 within the IGR between the sia and ctr operons leads to serum resistance. The presence of IS1301 in the IGR increases both the amount of transcript and the amount of protein produced by both operons. *In vitro* transcription assays indicate that IS1301 introduces novel transcriptional start sites. Further work will indicate if the changes mediated by IS1301 are due to specific sequences within IS1301 or the interruption of promoters already present within the IGR.

P021

HrpA Regulation and Role in *N. meningitidis* pathogenesis

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Objective
Neisseria meningitidis is the etiologic agent of meningococcal meningitis and a common isolate of the human respiratory tract. Effort is underway to develop an acellular protein based vaccine that will recognize

all serogroups of *N. meningitidis*. One potential vaccine candidiate is HrpA, a homologue of the FHA adhesin of *Bordetella pertussis* that is currently a component of the acellular whooping cough vaccine. *N. meningitidis* strain NMB has two copies of the hrpA gene: NMB0497 and NMB1779. This study set out to determine the expression patterns of these genes and to find a functional role for the encoded proteins.

Methods

Double mutants of the NMB0497 and NMB1779 genes were constructed in both wild- type and siaA-D backgrounds. Real time TaqMan RT-PCR was performed by probing regions of each gene that were unique and used the rmpM transcript as a standard. Results were reported from delta-delta CT calculations. Rabbit polyclonal antibodies were produced to a 300 amino acid recombinant fragment of HrpA and used to probe for protein in western blots. Biofilms were run in novel once flow through cambers designed to hold a monolayer of 16HBE14 tissue culture cells and analyzed by z-series on a confocal microscope and Comstat analysis of biomass and average height were calculated. Significance was determined by Student's t-test.

Results

These two genes are regulated with both genes displaying 2x down-regulation in the presence of 10 μM ferric nitrate and 5-6x up-regulation after 2 hours in the presence of an anaerobic environment. However, NMB0497 is 3.6x up-regulated in planktonic bacteria detaching from biofilms grown over 16HBE14 cells and NMB0497 is 4x up-regulated after 5 hours of cell association with the same cell line. Western blots demonstrate production of protein from these transcripts as two distinct bands at approximately 220 kDa and 180 kDa, with the smaller possibly being a processed form of the protein. We have demonstrated a significant decrease in biofilm biomass and average height in the double hrpA mutant in both encapsulated and unencapsulated wild-type backgrounds.

Conclusions

Interestingly, biofilms are commonly thought to have oxygen barriers that create oxygen deprived environments in the deeper recesses of the biofilm. This would correlate well with previous data showing HrpA involved in attachment to tissue where the deep part of the biofilm touching the tissue is likely oxygen limited. An unknown factor, independent of oxygen concentration, further upregulates NMB0497 after association with the 16HBE14 line.

P022

Factors affecting excision of the gonococcal genetic island from the chromosome

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The gonococcal genetic island (GGI) is a 57 kb region of the *N. gonorrhoeae* chromosome that shows sequence characteristics of horizontally-acquired DNA. The region encodes a type IV secretion system that acts in the export of DNA, thereby providing DNA for natural transformation. The GGI is flanked by a direct repeat. One copy of the repeat matches the recognition sequence for the site-specific recombinase XerCD (dif), while the other copy has 4 mismatches to the dif consensus sequence. The GGI is present in 80% of gonococcal strains and some *N. meningitidis* strains. The absence of the GGI from some *N. gonorrhoeae* strains and its presence in some *N. meningitidis* strains as well as the similarity of the flanking DNA to recombination sites suggest that the GGI may be a mobile element that can excise and integrate in the chromosomes of these two species.

We examined the recombination that results in loss of the GGI by measuring the frequency of loss of a marker located in the GGI and by using PCR to detect the junction created in the chromosome by excision of the GGI.

We replaced the imperfect dif with a perfect dif site in a derivative of *N. gonorrhoeae* strain MS11. In this double-dif strain the GGI was lost in a significant portion of the population after 20h of growth as reflected

by loss of a chloramphenicol resistance marker. Strains that were CmS were found to lack GGI genes. These results indicate that the dif sequence affects GGI excision and that the GGI is not stably maintained as an extrachromosomal element. Mutation of recA did not decrease the frequency of GGI loss, suggesting that homologous recombination is not involved in excision. However, mutation of xerD diminished GGI loss, suggesting that the GGI is excised by XerCD.
To further examine the role of dif in GGI excision, we created a model island by integrating a 2kb plasmid carrying a perfect dif site and an erythromycin resistance marker into the chromosome. The process created a strain carrying this "Erm island"flanked by two perfect dif sites. The Erm island was lost from the chromosome at high frequency. Introduction of the 4 mismatches or 2 mismatches in the dif sequence diminished Erm island loss. The sequence flanking dif also influenced recombination frequency. Inclusion of 90 bp of the GGI sequence in the Erm island plasmid enhanced integration of the construct into the chromosome as compared to a construct containing dif. Flanking sequence also influenced loss of the entire GGI. A survey of low-passage isolates identified strains that gave strong products for both the excised GGI and the integrated GGI. DNA sequencing of the dif region from one of these strains, IN522, revealed the presence of a 16bp deletion near dif. Incorporation of this deletion into the Erm island strain increased the frequency of island loss. Overall these studies demonstrate that site-specific recombination can excise the GGI and that mutations in the imperfect dif are necessary for stable maintenance of the GGI.

P023

Molecular- genetic analysis of abcZ and fumC housekeeping genes of *Neisseria meningitidis*, isolated from patients

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Objectives
The aim of the research was to perform sequencing of 2 housekeeping genes of *N. meningitidis* (abcZ and fumC), isolated from patients, and to set phylogenetic relationships between investigated strains.
Methods
We used 11 isolates of *N. meningitidis* (serogroup A- 1 isolate, serogroup B- 8 isolates, and serogroup C- 2 isolates). Detection of housekeeping genes as markers of phylogenetic relationship was carried out with the help of PCR. Sequencing reaction was performed based on Senger method. Amplification and sequencing protocols, allele information for multilocus sequence typing (MLST) were taken over the web-site http://pubmlst.org/neisseria/. Nucleotide sequences of abcZ and fumC genes of isolates analyzed were compared with the data in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and Neisseria MLST database (http://pubmlst.org/neisseria).
Results
Sequence analysis of abcZ gene of 11 *N. meningitidis* strains, isolated from patients, showed their west-european origin, and sequences of some strains (1, 308 and 325), admittedly, have indigenous origin- they differ greatly from alleles in the Internet database and while constructing evolutional dendrogramm perform separate cluster. Moreover, after these sequences were compared with those in database, similarity in the major nucleotide positions was found, but in general search no similar alleles were found. Strains 1, 308 and 325, isolated from patients, appear to be more old, and the rest isolates associated with each other according to the extend of sequences homology (more than 70% of homology) of analyzed fragments. While analyzing fumC gene of 7 *N. meningitidis* isolates, sequence analysis showed similarity to west-european alleles and to east allele (strain 275). Two fumC sequences (83 and 308 strains) fundamentally differ from other analyzed sequences, perform separate cluster and underlie the constructed phylogenetic tree, what suggests their earlier appearance (probably indigenous origin). So, both general and rare alleles fundamentally import the intraspecific allelic formation.

Conclusions
Meningococcal population in the republic has heterogeneous structure. According to the analysis of abcZ and fumC genes sequences received one can say, that the population of meningococci in the country has both indigenous and west- european and east origin, which may be determinated by migration and genetic exchange between strains. High similarity (70% and more) of the received sequences to alleles in the Internet database suggests the occurrence of genetic replacement and mutations in abcZ and fumC genes, that are not studied yet in details and are not represented in the database. And similarity in 90% and more, probably, supposes that well- known alleles of *Neisseria meningitidis* acquire mutations.

P024

Regulation of pilE transcription in *Neisseria gonorrhoeae*: a role for integration host factor in promoting transcript stability

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Gonococci coordinate the expression of several surface antigens during the infectious process. The expression of some surface antigens changes stochastically (e.g., Opa protein expression), whereas the expression of other surface proteins appears to be regulated (e.g., pilE transcription). The pilE gene encodes for the antigenically-variable PilE polypeptide that is the major protein component of the pilus organelle. pilE transcription turns-off towards the beginning of the stationary growth phase. Previous work has shown that integration host factor (IHF) binds upstream of the pilE promoter, with binding required for optimal transcription. An *in vitro* transcription-translation assay confirms these previous observations. In gonococcal mutants where the pilE promoter IHF binding site is deleted, transcription decreases approximately ten-fold, with this decrease in transcription being accompanied by an accumulation of smaller RNA species that appear to be derived from the primary pilE transcript. Primer extension analysis, in conjunction with 5′ RACE analysis, identified multiple 5′ endpoints of the small RNA molecules. Even though the primary pilE transcript sequences are variable, each small RNA species contained a unique constant region sequence, that, when assessed using RNA algorithms, is predicted to bind to the 5′ end of the pilE transcript. Moreover, the small RNA molecules also accumulate when the pilE promoter structure is disrupted through gene insertions. Surprisingly, what little full-length transcript that is present in these various mutants, the full-length message remains untranslated. Accordingly, an attenuation mechanism is presented to account for the regulation of PilE expression.

P025

Role of cell death and autolysis for DNA release and biofilm development in *Neisseria meningitidis*

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Objectives
Biofilm formation by *Neisseria meningitidis* is a general trait of un-encapsulated meningococcal variants which renders meningococci resistant to penicillin. Microcolony formation but not biofilm formation is dependent on twitching motility (Lappann et al., Mol. Microbiol., 2006).
Extracellular DNA (eDNA) is very important for the development of Pseudomonas aeruginosa biofilms

(Whitchurch et al., Science, 2002). Autolysis and cell death are crucial for *Staphylococcus aureus* biofilm formation (Rice et al., PNAS, 2007).
We now investigated the role and the mechanism of eDNA in meningococcal biofilm development.
Methods
Unencapsulated derivatives (siaD-) of strains MC58 (SgB, ST-32 cc), 2120 (SgC, ST-11), and 2594 (SgA, ST-5) were included in this study. Meningococcal biofilms were grown in a standardized flow-chamber biofilm model using minimal growth media (see Lappann et al., Mol. Microbiol., 2006). Treatment with the endonuclease DNase I of planktonic cells for biofilm initiation as wells as of biofilms at different time points was carried out. Biofilm formation experiments including the addition of meningococcal DNA to either DNase-treated or non-treated planktonic cells were performed. eDNA and dead cells within biofilms were visualized using different DNA-specific fluorescence probes. The extent of DNA release of planktonic cells and of culture supernatants was determined. The proportion of lysed cells in biofilms or planktonic cultures, respectively, was measured using ß-galactosidase release assays. Mechanical stability of meningococcal biofilms was tested by increasing shear forces within the flow-chamber system.
Results
Treatment with DNase I in the early, but not in the late stages of meningococcal biofilm development abrogated biofilm formation of strains MC58siaD- and 2594siaD-. Interestingly, biofilm formation of strain 2120siaD- was not significantly affected by added DNase I. Addition of pure DNA and crude cell extracts to eDNA-free meningococcal cells widely restored biofilm formation of MC58siaD-, suggesting that the DNase I effect was a consequence of removal of eDNA. A large proportion of cells in the early biofilms of MC58siaD- carried eDNA compared to only a minor population of cells in 2120siaD- biofilms. Dead cells were abundantly present in mature MC58siaD- biofilms associated with high shear stress tolerance, whereas the opposite was true for 2120siaD- biofilms composed of mostly vital cells. This finding suggests that eDNA release by avital cells might contribute to biofilm stability. To address the source of DNA genes involved in autolysis and cell wall biogenesis were generated and implied that cell death and autolysis result in eDNA release, and subsequent stabilization of biofilm formation in ST-32 cc and ST-5 meningococci.
Conclusions
Extracellular DNA plays a key role for initial meningococcal biofilm formation, but not in all strains. In some lineages, cell death and lysis via release of eDNA renders mature biofilms resistant to shear stress, which affects biofilm morphology. It is yet unclear, whether molecular mechanisms of *in vitro* biofilm models hold true for nasopharyngeal carriage. However, it might nevertheless be speculated that the low prevalence of ST-11 carriage (Claus et al., JID, 2005; Maiden et al., JID, 2008) is at least partially a result of reduced shear stress tolerance.

P026

Functional reconstitution of the O-linked protein glycosylation system of *Neisseria gonorrhoeae* in *E. coli*

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Objectives
Neisseria gonorrhoeae (Ngo) expresses an O-linked protein glycosylation pathway that targets PilE, the protein subunit of type IV pili as well as multiple other periplasmic proteins. Genetic analysis has defined a set of genes required for protein glycosylation (pgl). Although corresponding products are essential for protein glycosylation, it remains unclear if they alone are sufficient. Likewise, their functions have been inferred and not directly demonstrated. Here, we describe the functional transfer of components of the Ngo pgl system into *E. coli*, and demonstrate how this system can be used to identify novel Ngo protein targets.
Methods
The Ngo pglFBCD locus was amplified by PCR and cloned into pACYC184 and pglO was amplified by PCR and cloned into the broad-host-range expression vector pJB658. Plasmids containing the *C. jejuni* (Cje) pgl locus

(cloned in pACYC184) were provided by B. Wren (ICL). Rabbit antiserum recognizing the *Cje* heptasaccharide was provided by D. Newell (VLA). Rabbit antiserum recognizing the DATDH glycan was generated by immunization with purified Ngo pili bearing DATDH. Immunoblotting was used to detect glycosylation.

Results

Ngo PglO (equivalent to *N. meningitidis* (Nme) PglL) has been modelled as an oligosaccharyltransferase (OTase) utilizing lipid-linked glycan precursors. To test this hypothesis, we expressed PglO in *E. coli* with the genes encoding the lipid-linked heptasaccharide of *Campylobacter jejuni* (Cje) and PilE. Immunoblotting showed an extra PilE species of reduced electrophoretic mobility that reacted with heptasaccharide - specific antibodies. This reactivity was dependent on the expression of both PglO and the Cje genes and was abolished in a background expressing a PilE S63 missense mutant. These results confirmed findings made using Nme PglL. We then replaced the *Cje* genes with the Ngo pglFBCD gene cluster proposed to be required for the synthesis of a lipid-linked DATDH precursor. Using polyclonal antibodies recognizing the DATDH sugar, we found that PilE and other Ngo protein substrates were glycosylated in *E. coli* in a PglO - dependent fashion. Using bioinformatics we were able to express potential Ngo glycoproteins in *E. coli* and show that they were glycosylated.

Conclusion

These findings allow us to conclude that Ngo PglB, C, D, and F are sufficient for the synthesis of the lipid-linked DATDH sugar and that PglO acting as an OTase is sufficient for the transfer of the glycan from the lipid-linked precursor to proteins. Furthermore, we show that PglO has relaxed specificity both with regard to its glycan donors and protein substrates. These properties may be advantageous in developing novel glycoengineering strategies. The ability to modify and manipulate the Ngo pgl system in *E. coli* should also facilitate characterization of the structural features of the protein substrates involved in dictating glycan acceptor site selection.

References

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P027

Life in a sessile community: how protein expression changes during meningococcal biofilm formation

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Objectives

Neisseria meningitidis is a human pathogen that causes meningitis and sepsis. Nevertheless, asymptomatic nasopharyngeal carriage of the bacterium is crucial for the transmission of the pathogen and interaction with the host. Previous data led to the assumption that meningococci persist asymptomatically in tonsillar tissue in a biofilm-like stage (Sim *et al.*, 2000). We therefore established an *in vitro* biofilm model to study bacterial mechanisms relevant to the carrier state (Lappann *et al.*, 2006). In this study the alteration of protein expression during biofilm growth in a flow system employing a chemically defined minimal medium was investigated.

Methods

The whole cell proteome of *N. meningitidis* #3671 biofilms (capsule null locus isolate, ST-845), grown for 24 and 48 h, respectively, was compared with the proteome of an exponentially grown planktonic culture, both cultivated at 37ºC in modified Neisseria defined medium (NDM; Archibald and DeVoe, 1978, Lappann *et al.*, 2006). Biofilms were cultivated in a flow-tube system. Preparation of whole cell protein was done as previously described (Bernardini *et al.*, 2004). For 2D-gelelectrophoresis 400 μg of protein were adsorbed

onto an immobilized pH gradient IPG gel strip (17 cm, pH 3-10 linear pH gradient range). The isoelectric focusing (IEF) was performed in a PROTEAN IEF cell (Bio-Rad, Munich, Germany). In the second dimension proteins were separated on linear 12% polyacrylamide SDS gels using the Protean II xi Cell from Bio-Rad and gels were stained with Coomassie Brilliant Blue G-250. At least 3 different gels from more than 6 biological replicates were prepared for each experimental condition. Gels were scanned and analysed using the PDQuest‹H›TM‹/H› Advanced software (version 8.0, Bio-Rad). Differentially expressed proteins were identified by mass spectrometry.

Results

Associated with biofilm growth, 17 proteins changed more than 2 fold in their up or down expression. Most of the proteome changes were observed already after 24 h. 14 proteins could be identified by mass spectrometry. The four down-regulated proteins were involved in energy metabolism, protein synthesis and transcription. Up-regulated proteins were assigned to various functions, e.g. energy metabolism, biosynthesis, and cell envelope. One of the up-regulated proteins, GalM (aldose 1-epimerase), was recently shown to be controlled by FNR, a global regulatory protein involved in adaption to oxygen restricted conditions (Bartolini *et al.*, 2006). The up-regulated proteins MntC and Cu-Zn-superoxide dismutase might be involved in stress response elicited by biofilm growth.

Conclusions

Meningococci adapt to biofilm growth by alteration of protein expression. The observed pattern requires confirmation by independent methods. Furthermore, the possibility of topological differences in the relatively complex biofilm structure will be taken into account. Changes of biofilm morphology by mutation of regulated genes will be studied. The results will contribute to our understanding of metabolic alterations in meningococcal micro-colonies, which might be relevant for the understanding of nasopharyngeal colonization.

P028

Expression of the *E. coli* Tol-Pal complex in *Neisseria meningitidis*: Effects on membrane stability and vesicle production

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Objectives

The Tol-Pal complex consists of six proteins and is well conserved in gram negative bacteria, however it is absent from many high vesiculating species including *Neisseria.*

In *E. coli* deletion of the Tol-Pal complex has been shown to produce a high vesiculating mutant with poor membrane stability and defects in cell division.

This study aims to express the Tol-Pal complex in *Neisseria meningitidis*, to test the potential use of the Tol-Pal complex to regulate vesicle production.

Methods

Expression of Tol Pal complex: The two operons including the native promoters of the Tol Pal complex were amplified from *E. coli* K12 strain and inserted into the Neisserial shuttle vector pMIDG201 to create pMIDGTolPal. Transformation of *N. meningitidis* H44/76 with pMIDGTolPal was confirmed with Kanamycin selection and gene expression was confirmed by RT-PCR.

Slot blotting: Outer membrane vesicles (OMV) were prepared by ultracentrifugation and FPLC. Blots were detected with a monoclonal antibodies against PorA (NIBSC 01/514 and 01/538). Polymyxin B survival assay: *N. meningitidis* cultures were spun down and resuspended media containing Polymyxin B (10μg/ml) and control media without antibiotic. Cultures were incubated for two hours before plating out.

Results

pMIDGTolPal was created and a H44/76 strain was transformed. Transformation was confirmed by antibiotic selection and colony PCR. Upon recovery of the plasmid it was discovered that recombination

had resulted in the maintenance of antibiotic resistance but loss of the Tol-Pal complex. Transformation of *N. meningitidis* in which the *GNA33* gene had been deleted tolerated the pMIDGTolPal plasmid unmodified and Tol-Pal gene expression was confirmed.
Addition of 0.5% sodium chloride to the media restores growth of ΔGNA33 to that of the wild type. No difference in growth rate was observed between the ΔGNA33 mutant and ΔGNA33::Tol-Pal strain. Vesicles were prepared and the ubiquitous OMV protein PorA was used as a surrogate for vesicle quantification. Levels of PorA were increased above wild type in the GNA33 mutants. Expression of the Tol-Pal complex further increased PorA levels. Polymyxin B induces outer membrane stress by increasing membrane permeability. Incubation with Polymyxin B reduced survival of the wild type to 25% of the untreated control. Survival of the ΔGNA33 was significantly decreased compared to the wild type ($p<0.05$). Survival of the ΔGNA33::Tol-Pal strain was further decreased compared to ΔGNA33 ($p<0.05$).
Conclusions
Expression of the Tol-Pal complex in *N. meningitidis* is not tolerated and the expression cassette is rapidly lost. However, this can be overcome by disruption of GNA33, a protein that influences the structure of the outer membrane and vesiculation. Expression of the Tol-Pal complex does not induce changes in growth rate although it does reduce the stability of the outer membrane and increase vesiculation, in contrast to the effect in *E. coli*. A reduction in membrane stability is not lethal in the GNA33 mutant and is unlikely to account for the negative selection in the wild type. Both GNA33 and Tol-Pal are known to have roles in cell separation, therefore synergistic effects of Tol-Pal and GNA33 on cell division may provide an alternative explanation.

P030

Hfq, a key modulator in the riboregulated network of *Neisseria meningitidis*

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Objectives
The conserved protein Hfq has emerged as the key modulator of riboregulation, i.e. the regulation of translation by small RNA (sRNA). The Hfq protein binds directly to both sRNAs and target mRNAs and acts as an RNA chaperone, facilitating basepairing between sRNA and target mRNA. We assessed the role of Hfq in *Neisseria meningitidis*.
Methods
An hfq knockout was contructed by gene replacement with a gene encoding for erythromycine resistance (ermC) in H44/76. The effect of Hfq on key phenotypic characteristics was compared between wildtype and Δhfq strain. Growth kinetics in both iron replete and deplete GC + 1% vitox broth were recorded spectrometrically. Furthermore, resistance to a range of antibiotics and oxidative stress factors was tested using E-tests and Kirby-Bauer discs. All tests were simultaneously performed with a H44/76 Δhfq pEN11-hfq complemented strain. Proteins in whole cell lysates and in separated cell fractions of both the wildtype and Δhfq strain grown in iron replete and deplete environments were separated using 1D and 2D SDS-PAGE. Protein spots differentially expressed between wt and Δhfq strain were excised and analyzed by MALDI-TOF mass spectrometry.
Results
The H44/76Δhfq strain shows a markedly reduced growth rate evident in a prolonged lag period and a belated stationary phase compared to the wildtype and hfq complemented Δhfq strain. The Δhfq strain was extremely sensitive to ultraviolet light as compared to wt H44/76 meningococci and hfq complemented Δhfq strain. Likewise, a significant reduction in MIC was seen for components inducing oxidative stress and for all antibiotics except the macrolides.
Comparison of protein profiles of Δhfq H44/76 cells and wt H44/76 cells revealed that 24 unique proteins were differently expressed in H44/76Δhfq. Most of these proteins are involved in metabolism and all except one are downregulated. Four proteins involved in transcription/translation are upregulated.

Notably, the outer membrane protein PilF, concerned with pilus assembly, seems to be upregulated. In contrast, protein profiles of outer membrane fractions indicated reduced expression of PilE in the external milieu of the Δhfq H44/76 cells. In addition, proteins involved in oxidative and heat shock stress are upregulated in Δhfq H44/76 cells, while the major ferric iron binding protein is down regulated.

Conclusion

We have shown that the Hfq protein plays an essential role in cell metabolism, growth, and survival in *Neisseria meningitidis*. This strongly suggests that Hfq plays an essential role in the Neisserial riboregulated network.

P031

The HmbR hemoglobin receptor of *Neisseria meningitidis* is regulated by the MisR/S two-component signal transduction system

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Objectives

Outer membrane receptors for iron acquisition are among the major bacterial surface entities critical for meningococcal pathogenesis. Two phase-variable receptors, HpuA/B and HmbR, are involved in hemoglobin utilization in *N. meningitidis* and are regulated by iron concentration. Microarray analysis identified that the MisR/S two-component signal transduction system regulates transcription of hmbR and the objective of this study is to understand this regulatory mechanism.

Methods

Nonpolar misR, misS and misRS double mutants were generated in the wild type, the hpuB::erm/hmbRoff and hpuB::erm/hmbRon genetic backgrounds, by replacing an internal coding sequence with a nonpolar aphA-3 cassette. DNA sequencing analysis, Southern blots and Western blots confirmed these mutations in different parental strains. The utilization of hemoglobin and other iron sources was evaluated by disk diffusion assays under iron-restricted growth conditions and the levels of hemoglobin binding to the bacterial surfaces was investigated using whole cell hemoglobin binding assays. Electrophoresis mobility shift assays (EMSA) and DNase I protection assays were used to study the interaction of MisR with the hmbR promoter region. The relationship between the MisR and Fur regulation was investigated using site-directed mutagenesis of the Fur box and lacZ reporter assay.

Results

No differences in free iron utilization were noted for all three mis mutants using disk diffusion assays. However, significant growth defects were detected in all the mutants when hemoglobin was the sole iron source. This phenotype was observed in both the hpuB+ and hpuB- backgrounds indicating that the MisR/S system positively regulates the expression of the other hemoglobin receptor, hmbR. A reduction of hmbR expression in these mis mutants was confirmed by real time PCR and β-gal assays in both iron-replete and iron-limiting conditions. Consistently, whole cell hemoglobin binding assays showed reduced levels of hemoglobin bound to the cells of the mis mutants, suggesting fewer HmbR receptors being expressed on the bacterial surface. In addition, EMSA and DNase I protection assays demonstrated that MisR directly interacts with the hmbR sequence upstream of the Fur box, which overlaps with the promoter elements. Fur box mutations that eliminated iron regulation significantly diminished the negative effect of misS mutation, suggesting that the MisR/S system regulates hmbR expression by antagonizing the Fur repression.

Conclusion

The MisR/MisS two-component system regulates the hemoglobin utilization by directly modulating expression of the hemoglobin receptor, HmbR. The regulatory mechanism of the MisR/S two-component system appears to function through relieving the repression by Fur at the hmbR promoter.

P032

Fur mediated sRNA regulation in the pathogenic Neisseria is independent of the RNA chaperone protein Hfq

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Iron is an essential nutrient for a number of bacteria, yet at high concentrations it can catalyze the formation of reactive oxygen species that are toxic to a bacterium. Thus iron homeostasis is a tightly regulated process. In the pathogenic Neisseria, iron regulation is mediated primarily by an iron-responsive transcriptional repressor termed the Ferric Uptake Regulator (Fur). Previous work in our laboratory identified a novel small RNA (sRNA) molecule, in both *Neisseria meningitidis* MC58 and *N. gonorrhoeae* FA1090, which is repressed by Fur in a high-iron environment and transcribed in a low-iron environment. This sRNA was shown to repress expression of components of the Tricarboxylic Acid Cycle, including genes encoding succinate dehydrogenase (sdhCDAB), under iron-deplete conditions, and was therefore termed Neisserial regulatory RNA involved with iron (Fe) (NrrF). NrrF was predicted to basepair with the sdhA and sdhC mRNAs, suggesting a direct interaction *in vivo*. Since no other small RNA molecules have been identified in the pathogenic Neisseria to date, we wished to further investigate the mechanism of NrrF-mediated regulation. Our initial efforts focused on the RNA-binding protein Hfq, which has been shown to be an essential cofactor for small RNA-mediated regulation in many other bacterial species and often affects sRNA stability. Because NrrF has a potential Hfq-binding site, we hypothesized that Hfq would play a role in NrrF-mediated regulation in *N. meningitidis*. To test this hypothesis, an hfq- deletion mutant and a complemented hfq- strain were constructed, and the regulation of genes of interest was examined by quantitative RT-PCR. Additionally, NrrF stability was examined in all strains. Surprisingly the hfq- mutation had no effect on NrrF-mediated regulation of either the sdhA or sdhC genes, and NrrF half-life did not differ between strains. Interestingly, however, the ¬hfq- mutation did significantly attenuate growth in iron-deplete CDM media when measured by optical density and colony forming units and this defect was largely rescued by the addition of an iron source to the media. Furthermore, regulation of another iron-responsive gene, sodB, was defective in the hfq- mutant. Collectively our results indicate that Hfq is not an essential cofactor in NrrF mediated regulation in *N. meningitidis* and suggest that the Neisseria Hfq protein may not serve the same global role in sRNA regulation as observed in other pathogens. Furthermore, although Hfq was not required for NrrF-mediated regulation in *N. meningitidis* it may play a role in cellular physiology and the response to iron starvation.

P033

Transcriptional profiling of *Neisseria gonorrhoeae* biofilm indicates that biofilms grow using anaerobic or microaerophilic metabolism

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Objectives

Many illnesses and infections in humans are exacerbated and/or caused by biofilms. Recently, it has been postulated that nearly all chronic bacterial infections persist as biofilms.

N. gonorrhoeae, the etiologic agent of gonorrhea, is frequently asymptomatic in infected women, often leading to prolonged or chronic infections. Prolonged infection with gonorrhea can result in pelvic inflammatory disease, tubo-ovarian abscesses, infertility, and ectopic pregnancy. This prompted our laboratory to investigate the ability of *N. gonorrhoeae* to form biofilms. We have recently demonstrated

that *N. gonorrhoeae* forms biofilms over glass and primary and immortalized cervical cells (TCX). We have evidence from patient biopsies that also strongly suggests that biofilm occurs during natural cervical infection. However, little is currently known about the mechanism of biofilm formation by *N. gonorrhoeae* or the signals that regulate biofilm production, architecture, and dispersal. Thus, we elected to compare the transcriptional profiles of *N. gonorrhoeae* biofilm to planktonic modes of growth in order to identify genetic pathways involved in biofilm formation and maintenance.

Methods

Biofilm RNA was extracted from *N. gonorrhoeae* 1291 grown for 48 hours in continuous flow chambers over glass. Planktonic RNA was extracted from the biofilm runoff, which was filtered through a glass wool column to exclude detached biofilm flocs. RNA samples were run in triplicate and hybridized to custom Affymetrix® arrays. Data was analyzed in ArrayAssist® and normalized using the RMA algorithm. Genes with a fold change ≥ 2.0 and a p-value ≤ 0.05 were identified as differentially regulated. These results were confirmed with quantitative RT-PCR, and we examined the ability of relevant knockout mutants to form biofilm.

Results

When biofilm was compared to planktonic growth, 3.8 % of the genome was differentially regulated. Genes highly up-regulated in biofilm included aniA, norB, and ccp, which play critical roles in anaerobic metabolism and oxidative stress tolerance. Down-regulated genes included the nuo gene cluster (NADH dehydrogenase) and the cytochrome bcI complex, which are involved in aerobic respiration and are thought to contribute to endogenous oxidative stress. We determined that aniA, ccp, and norB knockout mutants are attenuated for biofilm formation over glass and TCX. Remarkably, the norB mutant exhibited the most dramatic biofilm-deficient phenotype. This was unexpected, because AniA and NorB are members of the same pathway where AniA reduces nitrite to nitric oxide (NO) and NorB reduces NO to nitrous oxide. However, it is plausible that NO could build up in the norB mutant, which has a functional copy of aniA, and it has been reported that sublethal concentrations of NO can prevent biofilm formation or elicit dispersal of established biofilm in *Pseudomonas aeruginosa*. Thus, we examined the effect of NO on *N. gonorrhoeae* biofilm. We subsequently confirmed that sublethal concentrations of NO both prevent biofilm formation and cause biofilms to disperse in *N. gonorrhoeae*.

Conclusions

Altogether, this data suggests that biofilm formation could minimize oxidative stress during natural cervical infection and allow *N. gonorrhoeae* to maintain a NO steady state that is non- or even anti-inflammatory. Furthermore, NO may be an important biofilm signaling molecule.

P034

Characterization of a *Neisseria gonorrhoeae* Hfq mutant

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Objective

Hfq is a RNA chaperon, which functions as a pleiotropic regulator for RNA metabolism in bacteria [1, 2].
It has been shown for several pathogenic bacteria that Hfq regulates indirectly virulence by binding to riboregulators that modulate the stability or translation efficiency of RNA transcripts.
The deduced protein of open reading frame NGO0326 shows high similarities (75-90%) to Hfq proteins from other pathogens such as *Salmonella enterica*, *Haemophilus influenzae*, *Yersinia pestis*, and *Bordetella pertussis*. In order to elucidate the role of NGO0326 (hfq) in *Neisseria gonorrhoeae*, we generated a mutant with a deletion in hfq.

Methods & results

Infectivity and transcriptional differences induced by the hfq mutation in *N. gonorrhoeae* strain MS11 were analyzed. The hfq mutation leads to pleiotropic effects in *N. gonorrhoeae* affecting mildly growth rate and adherence to epithelial cells.

Transcriptional analysis revealed that more than 300 open reading frames (ORFs) were differentially regulated in the hfq mutant compared to the Neisseria wild type strain. Microarray experiments were carried out as two-color hybridizations. To compensate specific Cy-dye effects and to ensure statistically relevant data, a technical replicate as color-swap dye reversal was performed. Regulated genes were identified with a stringent cut off of 1.75 fold and a p-value ‹0.001. Among the differentially regulated ORFs were dnaG and rpoD, affecting replication and transcription, several genes encoding chaperons, as well as genes of the pgl (pilin glycosylation) cluster whose regulation might affect adhesion properties. Alterations in virulence regulation and gene transcription in hfq mutant background are currently studied in more detail.

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P035

Role of type IV pilus retraction in *Neisseria gonorrhoeae* gene expression

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Objectives

Neisseria gonorrhoeae expresses type IV pili (Tfp) which play an important role in a number of functions, e.g. DNA uptake by natural transformation, twitching motility and attachment to host cells. An important feature of Tfp is their ability to retract, a process that is dependent on the ATP binding protein PilT. Recently, it has been shown that retraction of a single gonococcal pilus can exert forces of 50-100 pN on its substrate [1]. By the formation of Tfp bundles the achieved force is even 10 times higher [2]. The mechanical forces associated with the binding of retractile Tfp to epithelial cells have been shown to influence host cell gene expression through stimulation of mechanosensitive pathways [3]. However, nothing is known about the impact of Tfp retraction on the bacteria themselves. As the formation of microcolonies - organized structures of up to 100 diplococci - relies on the interaction of several diplococci with each other Tfp retraction could represent an important means by which single bacteria communicate within a microcolony. Therefore, we examine the role of Tfp retraction in *N. gonorrhoeae* gene expression.

Methods

To address the question whether pilus retraction alters neisserial gene expression we performed DNA microarrays with *N. gonorrhoeae* wild type strain MS11, a nonretractile pilT mutant, and nonpiliated mutants grown on GCB agar plates.

Results

Microarray analysis comparing the transcription profile of *N. gonorrhoeae* wild type MS11 and the retraction-deficient pilT mutant revealed 63 pilT-responsive genes. Most interestingly, three regulators were downregulated in the pilT mutant, which suggests that PilT affects the transcription of a subset of regulated genes through these regulators [4]. PilT may bring about these transcriptional changes by its role in Tfp retraction or by an unknown activity not related to the pilus system. Therefore, we additionally analyzed transcriptional changes in nonpiliated *N. gonorrhoeae* pil mutants. We found a number of similar regulated genes in both, nonretractile and nonpiliated mutants.

Conclusion

The identification of a subset of similar regulated genes in nonpiliated and nonretractile *N. gonorrhoeae* mutants compared to the wildtype suggests that *N. gonorrhoeae* most likely responds to a dysfunction in Tfp with a regulation of these genes. In order to identify genes exclusively regulated in response to retractile force we currently investigate the influence of artificially exerted mechanical forces on neisserial gene expression. For this purpose a cone-and-plate rheological system is used to generate a

homogeneous laminar flow on *N. gonorrhoeae* cells. Genes regulated due to this shear stress in MS11 and the pilT mutant will be compared to the previously identified regulated genes in the different pil mutant strains. We anticipate to find an overlapping set of regulated genes. This could provide a hint for signalling functions of pilus retraction in *Neisseria gonorrhoeae* interbacterial communication.

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P036

A complex promoter architecture directs transcription of the nadA gene of *Neisseria meningitidis*

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Objectives

NadA is a member of the trimeric autotransporter family of adhesins and is an important vaccine candidate of *Neisseria meningitidis*. It is currently known that NadA is expressed in different strains at different levels although the mechanisms of regulation of expression of this antigen are not fully understood. The expression of meningococcal vaccine antigens within the *Neisseria meningitidis* population has fundamental implications for the coverage of the recombinant 5CMVB vaccine. We are currently investigating both cis- and trans- acting elements which may play a role in varying gene expression.

Methods

We have generated a range of nadA promoter fusions to the gfp reporter gene which allows the investigation of single copy fusion variants in *N. meningitidis* and also plasmid borne fusion variants in the *E. coli* heterologous system in an attempt to identify the cis-acting regulatory elements within the promoter sequence. These include a phase-variable tetra-nucleotide repeat upstream of the promoter and a cis-acting region involved in growth phase regulation. In addition, *in vitro* binding experiments have identified the binding sites of a number of regulatory proteins involved in transcriptional control of nadA.

Results

NadA expression is affected at multiple levels. Transcription of nadA responds to growth phase and the encoded protein accumulates maximally in stationary phase. Deletion analysis of the promoter identifies a region distally upstream which is necessary for growth-phase regulation (GPR). Transcription of the PnadA promoter is induced in stationary phase and the cis-acting GPR element is necessary for repression of the promoter during log-phase. Furthermore, cell extracts of MC58 strain reveal a protein which binds to the GPR and currently attempts are being made to identify the binding protein through proteomics.

The tetra-nucleotide repeat directly upstream of the PnadA is involved in phase variation of promoter strength. Different numbers of repeats from 4 to 13 copies result in quasi-periodic differential transcription levels from the PnadA promoter however all phase variant promoters are growth phase regulated.

This region of the promoter is bound by the IHF regulatory protein and also the alpha-subunit of RNA polymerase. Differential binding of these proteins is not observed between the different phase variant promoters. These data suggests that alteration of the spacial orientation of a complex promoter architecture due to differential number of tetra-nucleotide repeats may result in the phase-variable promoter strength.

Conclusion

Our current hypothesis is that IHF binds to the promoter at the distal junction of the tetra-nucleotide repeats and causes a bending or looping of the DNA thereby differentially altering the relative positions of the distal upstream GPR element and also of two UP-like elements with respect to the promoter. Depending on the number of the repeats, this may result in the optimal or suboptimal interaction with

the UP-like elements of the alpha subunits of RNA-polymerase at the promoter and also of an as yet unidentified factor, a trans-acting GPR protein bound to the distal GPR element.

P037

Phase variable type III restriction modification systems of *Neisseria meningitidis*

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Objectives
Several phase variable type III DNA restriction-modification (R-M) systems are present in *Neisseria meningitidis.* Phase variation, the high frequency reversible switching of gene expression, is a common feature of host-adapted bacterial pathogens. R-M systems are traditionally thought of as conferring protection against foreign DNA, and several roles have been proposed for phase variable R-M systems based on DNA restriction function, including regulation of the frequency of lateral DNA transfers within and between bacterial populations. In addition, a class of epigenetic regulators of gene expression has evolved from phase variable type III R-M systems, and these 'phasevarions' (phase variable regulons) control expression of multiple genes via differential methylation of the genome. Phase variable R-M systems of *N. meningitidis* have been investigated in order to determine their role in DNA restriction and/or gene regulation in meningococci.
Methods
Available *N. meningitidis* genome sequences have been screened for the presence of type III R-M systems, and the distribution and features of these genes in a large panel of strains was determined by PCR and sequence analysis. *N. meningitidis* mutant strains have been constructed which lack the methylase (mod) genes of the type III R-M systems. The frequency of lateral DNA transfer has been studied using DNA transformation assays, while gene expression has been studied using microarray, real-time PCR and Western blot analysis.
Results
Two phase variable methylases of type III R-M systems have recently been described in *N. meningitidis* that act as 'phasevarions' (Fox et al., 2007). A third novel phase variable type III R-M system has now been identified that is primarily found in lineage III strains of *N. meningitidis.* Three separate alleles of this gene exist, based on differences in the central DNA binding domain of the gene. The methylase gene of this R-M system contains tandem 5'-CCGAA-3' repeat units that vary in number both between strains and within a single strain during growth, indicating that this repetitive tract of DNA mediates phase variable expression of mod. The mod knockout strains of all three types of type III R-M systems have been analysed to determine if they influence regulation via an epigenetic mechanism of differential methylation of the genome, with a particular focus on expression of candidate antigens for a serogroup B meningococcal vaccine.
Conclusions
The phase variable nature of the R-M systems studied, and their variable distribution within *N. meningitidis* strains, resulting in different patterns of DNA restriction and gene expression, may have an impact on the evolution of meningococcal strains and the rise of hyper-virulent clonal groups. In addition, the modulation of gene expression through the random on/off switching of a phase variable regulator, could have important implications for vaccine development.

P38

Characterization of ngo1427: A peroxide-Induced, transcriptional regulator of *Neisseria gonorrhoeae*

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Symptomatic gonococcal infection is characterized by a purulent exudate which consists of viable and nonviable *Neisseria gonorrhoeae* (*Gc*) associated with neutrophils. This association of *Gc* and neutrophils in the exudate suggests that *Gc* survives the anti-microbial actions of neutrophils, including oxidative killing mechanisms. To determine how *Gc* coordinates its activities against oxidative killing, a transcriptome profile of *Gc* following hydrogen peroxide treatment was performed [1]. 75 gene products were found to be upregulated greater than 2.5 fold, including a putative transcriptional regulator, ngo1427, which was upregulated 6.3 fold. ngo1427 is predicted to encode a protein that belongs to the class of S24 autoproteases. This class includes the SOS system regulator LexA and the lambda CI regulator. To test whether ngo1427 has autoproteolytic activity consistent with the S24 family, NGO1427 was purified and shown to require the S24 peptidase active site to undergo autoproteolysis. Although ngo1427 is upregulated by hydrogen peroxide, a ngo1427 mutant did not show altered resistance to redox-disruption, hydrogen peroxide killing, neutrophil-mediated killing or UV irradiation. Microarray analysis showed that NGO1427 represses the expression of three genes: ngo1427, an adjacent, divergently transcribed gene ngo1428, and recN. ngo1428 is predicted to encode a Neisseria-specific putative integral membrane protein and recN encodes a protein important for DNA repair that is required for survival against reactive oxygen species in *Gc*. Examination of the role of RecA in NGO1427 function, as well as the role the conserved S24 active sites in regards to NGO1427 activity, is currently underway.

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P039

Induction and regulation of gonococcal resistance to a human cationic antimicrobial peptide LL-37

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Objectives

Antimicrobial peptides (APs) are conserved and highly effective components of host defense and are produced by all classes of life including humans. APs frequently act as broad-spectrum antibiotics and may be used in the future as a novel therapeutic agent. However, there is a concern that bacteria could develop mechanisms of resistance to APs during the infection. Gonococci typically infect epithelial cells or interact with phagocytic cells that produce a number of antimicrobial peptides (APs) during the innate host defensive response against invading pathogens. We hypothesized that *in vivo*, secreted APs are often at a concentration below antimicrobial levels. We thought that gonococci can respond to this as a stress and can exhibit inducible AP-resistance. In order to determine how gonococci respond to human APs, we used the human cathelicidin LL-37 as a model AP to test this hypothesis.

Methods

Transcriptional profiling was performed by microarray analysis that employed total RNA extracted from strain FA19 grown in the presence or absence of LL-37 (0.5 μg/ml) for 3 hours. Real-time quantitative RT-PCR and beta-galactosidase fusion reporter assays were used to confirm gene regulation results. The

susceptibility of gonococci to purified LL-37 was performed using growth inhibition assays described previously by Shafer et al (1998). Insertional-inactivation of target genes (misS, ccp, or nadC) in strain FA19 and complementation of the misS mutant were performed using standard procedures.

Results

We found that growth of gonococci in a sub-lethal concentration of human LL-37 resulted in the differential expression of over 90 genes (increased or decreased expression) and observed that this could induce gonococcal resistance to this AP. The differentially expressed genes included regulators of gene expression (a two-component regulatory system termed MisR-MisS that resembles PhoP-PhoQ) and genes encoding an inner membrane protein similar to the NadC sodium symporter and the periplasmic cytochrome c peroxidase. Growth of gonococci in the presence of LL-37 stimulated expression of misR-misS, but repressed expression of nadC and ccp. Genetic inactivation of misS, nadC or ccp contributes to constitutive or inducible levels of LL-37 resistance expressed by gonococci. Loss of misS abrogated inducible LL-37 resistance but had no impact on constitutive resistance levels. Loss of ccp or nadC resulted in increased constitutive resistance to LL-37. However, loss of ccp abrogated inducible resistance while loss of nadC insignificantly influenced on inducible resistance. The MisR-MisS two component regulatory system was found to repress nadC and ccp.

Conclusions

Gonococcal growth in a sub-lethal level of LL-37 can induce resistance to this AP and this may influence gonococcal susceptibility to innate host defenses during infection. The two-component MisR-MisS system plays a crucial role in this induction by down-regulating nadC and ccp expression. We hypothesize that the decreased membrane potential due to loss of nadC or ccp impacts LL-37 activity at the inner membrane level or compromises its ability to cross this membrane to reach intracellular targets or both.

P040

National case-control study on risk for death and *Neisseria meningitidis* sequence types in Denmark, 2000-2007

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Objectives

In the past decades efforts to further diminish the case-fatality rate from meningococcal disease has proven challenging due to the often rapid progression of the disease in patients. In this study our objective was to characterise a subset of *N. meningitidis* isolates to establish which sequence types were present in Denmark in the period 2000-2007 and whether any clonal complexes were associated with increased risk of death.

Methods

Isolates and information on disease outcome were obtained from the integrated national laboratory and epidemiological surveillance system. We designed a matched case-control study with 50 isolates from fatal case-patients and 50 isolates from convalescent control-patients. Serogrouping, serotyping, serosubtyping and multilocus sequence typing (MLST) were performed on the hundred isolates.

Results

The clonal complex ST-32/ET-5 was found in 36% of the isolates, followed by ST-11/ET-37 complex (14%) and ST-41/44 complex/Lineage 3 (14%). Eight new sequence types were found. We did not uncover any of the clonal complexes to be significantly associated with a fatal disease outcome. When including both the genotype and the phenotype in a multivariate logistic regression model, we found for ST-32/B:15:P1.7,16 that the phenotype tend to have a greater association to a fatal outcome than the genotype, table 1.

Conclusion

Although the numbers were low, the present study indicates that phenotyping may be a better predictor of mortality than MLST, which suggest that each typing method has its advantages and disadvantages. If this notion can be confirmed by other studies, it may stimulate additional research regarding pathogenesis of severe illness, e.g., if certain surface molecules trigger a cytokine storm more than others.

Table 1: Test for association between N. meningitidis sequence type, phenotype and death

Clonal complex	McNemars exact test OR (95% CI)	Multivariate conditional logistic regression*** OR (95% CI)	Likelihood-ratio test****
ST-32/ET-5 complex	1.25 (0.62-2.58)	0.77 (0.24-2.47)	0.66
B:15:P1.7,16*	2.14 (0.82-6.21)	3.61 (0.88-14.73)	0.06
ST-11/ET-37 complex	1.33 (0.41-4.66)	Not Applicable	Not Applicable
C:2a:P1.2,5**	2.33 (0.53-13.98)	Not Applicable	Not Applicable
ST-41/44 complex/Lineage 3	0.56 (0.15-1.85)	Not Applicable	Not Applicable

* B:15:P1.7,16; B:15:P1.7; B:15:P1.16

** C:2a:P1.2,5; C:2a:P1.2; C:2a:P1.5

*** Both the clonal complex and the phenotype included as independent risk factors.

**** When excluding one independent risk factor in the conditional logistic regression model.

P041

Phasevarion mediated differentiation of *Neisseria gonorrhoeae*

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Many host-adapted bacterial pathogens contain DNA methyltransferases (mod genes) associated with type III Restriction-Modification (R-M) systems that are subject to phase variable expression (high frequency reversible ON/OFF switching of gene expression). In *Haemophilus influenzae* the random switching of the modA gene controls expression of a phase variable regulon of genes (a"phasevarion"), via differential methylation of the genome in the modA ON and OFF states (Srikhanta et al. PNAS 12(15) 5547-5551). Phase variable mod genes are also present in *Neisseria gonorrhoeae* and *Neisseria meningitidis* suggesting that phasevarions may occur in these important human pathogens (Fox et al. Mol Micro 65(6) 1375-1379).

Objectives

N. gonorrhoeae FA1090 contains two phase variable type III R-M systems (modA13 and modB1). The aim of the first part of this study was to investigate whether further alleles of modA and modB are present in *N. gonorrhoeae* and to look at the distribution of mod alleles and the repeat sequence type and number. The second part of this study aimed to determine if the phase variable type III R-M system of *N. gonorrhoeae* FA1090 (modA13) played a role in gene regulation and virulence. The final objective was to determine the biological significance of modA13 phase variation.

Methods

Phylogenetic studies of the mod alleles were performed using sequence analysis on a diverse collection of *N. gonorrhoeae* isolates. To determine whether phase variable expression of *N. gonorrhoeae* FA1090 modA13 gene regulates gene expression, a mutant strain lacking the modA13 gene was made and its phenotype analyzed by microarray analysis using *N. gonorrhoeae/meningitidis* genome microarrays (TIGR). In order to characterize the phenotype of our modA13 knockout mutants, we performed biofilms assays under continuous flow conditions. Quantitative association, invasion, and survival assays were also performed using pex cells.

Results

Phylogenetic studies on phase variable mod genes associated with *N. gonorrhoeae* revealed that this organism has two distinct mod genes - modA and modB. There are also distinct modA alleles that differ

only in their DNA recognition domain. The *N. gonorrhoeae* strains contained either the modA12 or modA13 allele, and only the modB1 allele. Microarray analysis revealed that FA1090 modA13 controls a phasevarion of 17 genes that were either up- or down-regulated in the FA1090modA13::kan mutant strain, some of which were virulence associated. Functional studies using *N. gonorrhoeae* FA1090 confirmed that modA13 ON and OFF strains have distinct phenotypes in biofilm formation and in a primary human cervical epithelial cell model of infection.

Conclusion

This study, in conjunction with our previous work in *H. influenzae*, indicates that this may be a common strategy used by host-adapted bacterial pathogens to randomly switch between "differentiated"cell types.

P042

Distribution of modA and modB in *Neisseria meningitidis*

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Several host-adapted bacterial pathogens contain DNA methyltransferases (mod genes) associated with type III Restriction-Modification (R-M) systems that are subject to phase variable expression. In *Haemophilus influenzae* the random switching of the modA gene controls expression of a phase variable regulon of genes (a"phasevarion"), via differential methylation of the genome in the modA ON and OFF states (Srikhanta et al. PNAS 12, 5547-51, 2005). Additionally, our recent survey of H. influenzae strains indicated significant sequence heterogeneity in the central, variable region of the mod gene associated with target site recognition (Fox et al. Nucleic Acids Res 35, 5242-52, 2007). Multiple phase variable mod genes (modA and modB) are also present in *Neisseria meningitidis* suggesting that *N. meningitidis* may also display distinct mod alleles and that phasevarions may occur in this important human pathogen (Fox et al. Mol Micro 65,1375-79).

Objectives

N. meningitidis contains two phase variable type III R-M systems (modA and modB). The aim of this study was to investigate whether further alleles of modA and modB are present in *N. meningitidis* and to look at the distribution of mod alleles and the repeat sequence type and number.

Methods

Phylogenetic studies of the mod alleles were performed using sequence analysis on a collection of 107 *N. meningitidis* isolates. The modA nucleotide sequences were assembled using the Staden sequence analysis package and all sequences aligned in the Seqlab alignment program (Genetics Computer Group, Madison, Wis.). Phylogenetic analysis was undertaken using the software package ClonalFrame version 1.1 Annotation was then undertaken by importing the tree into the Molecular Evolutionary Genetics Analysis software package (MEGA ver 4.0).

Results

Phylogenetic studies on phase variable mod genes associated with type III R-M systems revealed that *N. meningitidis* has two distinct mod genes - modA and modB. The complete 107 strain MLST modA survey revealed that the majority of *N. meningitidis* strains had either the modA11 or modA12 allele, with modA15 found in two strains and modA4 and modA18 found in one isolate each. The most notable associations were in capsule type, where 100% of serogroup A strains and 92% of serogroup C strains contained the modA12 allele. Some association with clonal complex was also observed with meningococci belonging to the ST-32 clonal complex predominantly harbouring the modA11 allele. Further clustering could be seen among ST-41/44 and ST-8 clonal complexes. Two distinct modB alleles, modB1 and modB2, distinguished by differences in their DNA recognition domain, were also observed.

Conclusion

Our phylogenetic studies on the mod genes of 107 meningococcal isolate collection reveal that

differences in the DNA recognition domain within the mod gene results in distinct mod alleles. Based on these differences two major modA alleles (modA11 and modA12) and two distinct modB alleles (modB1 and modB2) were found. This suggests the possibility that multiple phasevarions exist within *N. meningitidis*, each regulating a different set of genes.

P043

The recognition sequence of M.NgoAXII, a phase variable type III methyltransferase of *Neisseria gonorrhoeae*

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Phase variable type III Restriction-Modification (R-M) systems have been described in a number of pathogenic bacteria (Fox et al. Mol Micro 65(6) 1375-1379). It has been shown that the phase variation of these R-M systems correspond to differences in the expression of multiple genes in both *Haemophilus influenzae* (Srikhanta et al. PNAS 12 (15) 5547-5551) and Neisseria species. Although the expression state of the methyltransferase (Mod) has been shown to regulate the expression of a wide array of other genes in these organisms, the molecular mechanisms by which this occurs at individual promoters has not been characterized. In order to determine how differential methylation impacts global gene expression, the DNA sequence recognized, and therefore modified, by the methyltransferase must be identified.

Objectives

Neisseria gonorrhoeae FA1090 has two phase variable type III R-M systems, each shown to regulate a distinct group of genes (see Srikhanta et al Poster). This study aims to identify the methylation target sequence of one of these methyltransferases (ModA13).

Methods

The methylation target sequence was elucidated by differential digestion by ApoI of plasmid pGFP extracted from mod On and mod::kan cells. Differences in restriction pattern between methylated and unmethylated plasmid were used to map the putative methylation target. This was confirmed by digesting chromosome extracted from mod On and mod::kan cells with ApoI and probing across a region where the ApoI restriction site overlapped with the putative methylation target site. The functionality of the Res associated with this system was investigated by transforming mod On (and therefore res ON) cells with mod methylated and mod unmethylated plasmids.

Results

The recognition sequence of the M.NgoAXII was identified to be AGAAA, with the methyl group being added to the final adenosine in this sequence.

Conclusion

Now that the methylation target for M.NgoAXII is known, this site can be mapped onto the FA1090 chromosome, as well as other strains that carry the modA13 allele. This information can now be used to investigate the molecular mechanism by which phase variable DNA methylation can regulate a diverse array of genes, some of which are known virulence factors and vaccine candidates.

P044

Annotation of a *Neisseria lactamica* genome sequence and comparison with genomes from pathogenic Neisseria

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Objectives

1. To fully annotate the completed genome sequence of *N. lactamica*.
2. To directly compare the *N. lactamica* genome sequence to the genome sequences of three *Neisseria meningitidis* isolates (Z2491, MC58, FAM18) and the *Neisseria gonorrhoeae* isolate FA1090, in order to reveal similarities and differences among the species.
3. To provide data that can be used in the identification of genetic and functional differences, mechanisms of pathogenicity, novel vaccine components, and for studies of the evolution of virulence.

Methods

The *N. lactamica* isolate 020-06, with the sequence type ST-640 (the central genotype of the ST-640 clonal complex), was chosen for genome sequencing. This was carried out by standard methods at the Wellcome Trust Sanger Institute, Cambridge. The genome sequence is searchable using the Blast server available at: http://www.sanger.ac.uk/cgi-bin/blast/submitblast/n_lactamica.

The complete *N. lactamica* genome sequence was downloaded from the FTP site at the Wellcome Trust Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/nl/) and an automatic gene prediction program (Glimmer3) was used to identify coding regions. These were then annotated manually using the genome viewer Artemis. The genome sequences from *N. lactamica*, *N. meningitidis* and *N. gonorrhoeae* were compared using the Artemis comparison tool (ACT).

Results

The *N. lactamica* genome consists of a circular chromosome of 2,217,455 bp length, with a G+C content of 52.27%, and a plasmid of 3,151 bp length. Approximately 2060 coding sequences have been identified, which were compared to known and hypothetical genes in the pathogenic species. Comparisons have revealed several *N. lactamica*-specific loci including prophage insertions and a number of loci encoding probable large surface proteins. There is evidence that some *N. lactamica*-specific loci are the result of horizontal genetic transfer from unrelated species. Although a number of *N. lactamica* specific genes have been identified, the majority of genes in *N. lactamica* were also present in the genomes of the pathogenic Neisseria examined, including many described as virulence associated.

Conclusions

Sequencing and annotating the complete genome of *N. lactamica* has provided a large amount of data useful for determining relationships among the pathogenic Neisseria and this commensal. The high degree of similarity among their genomes is remarkable, considering that *N. lactamica* is a harmless commensal while *N. meningitidis* and *N. gonorrhoeae* have the potential to cause serious disease. Further experimental work is required to determine whether any of the similarities or differences identified are present in other *N. lactamica* isolates.

P045

The Role of IHF and NMB0398 in *Neisseria meningitidis* gene regulation

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Objectives
Integration host factor (IHF) mediates a variety of genetic events in Gram-negative bacteria, including transcriptional regulation of specific genes. In *Neisseria gonorrhoeae*, IHF has been implicated in expression of at least one of the major virulence determinants, the pilin subunit PilE. Although much effort has been focussed upon understanding the role of IHF in Neisserial gene regulation, the generation of non-lethal ihf mutants has not been reported in either *N. gonorrhoeae* or *Neisseria meningitidis*. We have previously described the isolation of an ihfA mutant and a complemented mutant in *N. meningitidis* NMB. The objectives of this study were to determine the role of IHF (and subsequently, the IHF regulated gene NMB0398) in gene regulation in *N. meningitidis*.
Methods
Using the otherwise isogenic *N. meningitidis* wildtype, ihfA mutant and complemented mutant strains, we have employed microarray analysis to profile gene expression, and assess the role of IHF in gene regulation.
Results
We have identified over 400 genes that are differentially regulated 1.5 fold up or down in the presence or absence of IHF ($p<0.05$). Amongst these are genes encoding components of the translation machinery and NMB0398, a putative ArsR-like transcriptional regulator.
As NMB0398 is differentially expressed in the presence of IHF, it is possible that some genes identified as potentially IHF-regulated may in fact be regulated by NMB0398. Thus, we have also constructed an NMB0398 mutant and complemented mutant in the *N. meningitidis* NMB background and carried out microarray analysis of these strains.
Conclusions
The results suggest that some genes identified as being potentially IHF-regulated are actually NMB0398-regulated, and also indicate that NMB0398 may play a role in regulation of a subset of *N. meningitidis* genes involved in metal ion homeostasis.

P046

In silico identification of new potential vaccine antigens within the *Neisseria meningitidis* genome sequence

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Objectives
Neisseria meningitidis serogroup B infections are a serious health threat to developed and developing countries. In the past, several outer membrane proteins have been identified through bioinformatic analysis of the genome and they have been further characterized as potential vaccine candidates. In this study we have identified, using an alternative in silico approach, a number of novel proteins with a potential outer membrane localization that could also be exploited as vaccine components. The ability of some of the identified proteins to induce a protective response is also reported.

Methods
A pipeline using free internet servers was structured to predict putative outer membrane proteins with vaccine potential. We exploired only those genes that codify for proteins with unknown function. For signal peptide detection in gene products we employed SignalP and LipoP programs. PsortB was used to predict cellular localization of the gene products. Homology searches were performed using Blast program and also COG database. The MBGD database was used to study the neighbourhood of the genes of interest. The analysis of conservation, as the most important criteria, was done exploiting all the published neisserial genomes and sequencing targeted genes in a panel of representative strains. A selection of target proteins was cloned, expressed, purified and assayed in immunization schedules in Balb/C mice. Sera from immunized mice were tested for their ability to recognize the meningococcal surface and/or mediate complement dependent bacteria lysis.
Results
All the genome of the *Neisseria meningitidis* serogroup B strain MC58 was revisited in a search for surface-exposed proteins. Initially, from 2155 genes, we selected 757 that have unknown or hypothetical function assigned, 230 of these codify for products with putative signal peptides. In the next step, 101 proteins were selected based in a potential outer membrane cellular localization and all were carefully examined in terms of homology, conservation degree and genomic localization. Some proteins that did not fulfill these criteria were also included based on their genome location and homology results. Then, a set of 49 candidate genes was finally selected, from which 7 proteins were experimentally tested. These antigens gave positive results in surface localization assays, and some of them also induced bactericidal titers after immunization.
Conclusions
The ability of bioinformatics to characterize genomic sequences from pathogenic bacteria by predicting genes that may encode vaccine antigens, e.g. surface localized proteins, has been corroborated. Our study demonstrated that utilization of genome sequences by applying bioinformatics is still possible to expedite the vaccine discovery process in *N. meningitidis* rapidly providing a set of uncharacterised candidates for further testing.

P047

Wide-scale screening for the SBA resistance-associated with *IS*1301

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Objective
Recently it has been identified serogroup C *Neisseria meningitidis* strains showing resistance to the serum bactericidal activity (SBA) in vaccinees sera. This particular characteristic is the result of an insertion sequence, *IS*1301, in the intergenic region between the sia and ctr operons, leading to an increase in the transcript levels of surrounding genes, increasing the amount of capsule. It has been proposed that it provides a generic mechanism to protect the microorganisms against bactericidal antibodies (not only capsular).
The emergence of strains resistant to bactericidal antibodies is a potential threat to the success of vaccines, either polysaccharide but also protein vaccines. The analysis of the presence and future spread of the described mechanisms seems very important in this context. The objective of this study was to analyze how frequent the mechanisms might be present in a wide panel of recent meningococcal invasive strains isolated from invasive cases in France and Spain representing different serogroups and clonal complexes.
Material & methods
We have screened a wide panel of meningococcal strains for the presence of the *IS*1301 just amplifying by PCR one region of the capsule biosynthesis locus (cps) producing a significantly larger product in those strains harbouring the *IS*1301 (≈1.1 kb) compared with wild type isolates (≈300 kb). One hundred and seventy five group B, 68 group C, 16 group Y, 14 group W135 and 3 showing other serogroups, all of them isolated in France in 2008 were analyzed at the Institute Pasteur. Two hundred and six group B and 34

group C strains (all serogroup C meningococci isolated from conjugate vaccine failure cases) all of them isolated in Spain (group B strains from 2005-2006 and group C meningococci from 2003-2007) were tested at the Institute of Health Carlos III. In addition, 62 serogroup Y strains isolated in Latin American countries were included among the strains analyzed at the Spanish Institute.

Results & discussion

Among strains isolated in France, 15 serogroup B (8.6%) and 1 serogroup C (2.9%) showed a 1.1 kb fragment after amplification, suggesting presence of the *IS*1301 at the cps. Three group B (1.4%) and 2 serogroup C (5.9%) showed the largest fragment of 1.1kb among the Spanish isolates; in addition, 2 serogroup Y (3.2%) showed a result associated with the IS1301 insertion. The IS+ isolates showed different phenotypes (serotype/serosubtype antigenic combinations) and genotypes (STs) suggesting a wide spread of the IS between the sia and ctr operons. With these data, we can conclude that the insertion of the *IS*1301 associated with SBA resistance is not a rare event. Nevertheless, the mechanisms for SBA resistance might only explain 2 of 34 vaccine failure cases and there were not differences when this group was compared with the other groups of strains. Whether the IS+ meningococcal strains found in this study confer resistance to serum remains to be determined as it impacts on the development and the use of current or future meningococcal vaccines.

P048

Putative TonB dependent receptor NMB0964 of *Neisseria meningitidis*: gene expression and variability

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Objectives

The outer membrane protein NMB0964 of *N. meningitidis* is a putative TonB dependent receptor. In this study, we analysed expression of NMB0964 under serum stress, its sequence variability, and the protein expression patterns in wildtype strains.

Methods

Oligonucleotide microarrays were used to screen selected meningococcal genes for alteration of gene expression. Reporter gene expression assays were performed using translational fusions to the beta-galactosidase gene, which was inserted into NMB1877. Sequence variability was assessed using a strain collection comprising type strains and other invasive strains representing 10 sequence types. Variation of protein expression was analysed using 72 mostly invasive strains.

Results

NMB0964 was up-regulated upon non-lethal treatment with normal human serum in comparison to heat inactivated (HI) serum, as already suggested by Dove et al 2003. Sequences between position 474 and 111 bp upstream of the start codon of NMB0964 were required for the effect as suggested by reporter gene assays. Although complement activation on the surface of the bacteria was tightly controlled for, up-regulation of NMB0964 might have been biased by non-physiological effects of heat inactivation of the control serum. Therefore, classical pathway (CP) activation was consecutively induced by antibodies, whereas serum treatment in the presence of non-activating antibodies served as a control. Protein expression, as monitored by NMB0964 specific antibodies, appeared to be enhanced if the CP was activated. Using C3 deficient sera, we are currently investigating whether NMB0964 expression under this condition required C3. Sequence analysis: the overall mean distance of amino acid sequences derived from 14 strains (10 STs) was 0.02. There were 51 variable positions among 764 amino acids. Clusters of variable sites were observed in the N-terminus, and between positions 300 and 430. One strain, i.e. ST-23 strain DE8427, did not express the protein because of a point mutation resulting in a pre-mature stop

codon. Protein expression analysis using a polyclonal antiserum after growth of the bacteria on Columbia blood agar revealed expression in all but one of 72 strains. Protein expression levels varied considerably, and there was no association of expression levels to clonal complexes.

Conclusion

NMB0964 gene expression was associated, directly or indirectly, to complement activation. Amino acid alignments showed enhanced variation between amino acids 300 and 430; peptide scanning analysis is on the way to determine antibody binding to this region. With one exception all strains expressed the protein, however, to a varying degree.

P049

Regulation of the expression of integration host factor in *Neisseria gonorrhoeae*

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Background

Integration host factor (IHF) is a small nucleoid protein complex that influences, amongst other processes, gene expression. IHF comprises of an α and β subunit, encoded by *ihfA* and *ihfB* respectively. IHF can regulate the expression of genes either directly (in the absence of another regulatory protein) or indirectly (through an additional regulatory protein) to result in activation or repression of gene expression. Preliminary data has shown that in *Neisseria* IHF can be regarded as a global regulator of transcription. It has previously been shown that IHF controls transcription of *pilE*, encoding the type 4 pilin subunit. The IHF regulon therefore contains a gene that is central to virulence, and our knowledge of infectious process will depend upon understanding how this regulon is controlled.

Objectives

Given the importance of IHF in the global regulation of gene expression in *Neisseria gonorrhoeae* (Ng), we aimed to investigate the regulation of expression of *ihfA* and *ihfB* by performing a transcriptional analysis involving mapping the relevant promoters and other functional sites, as well as confirming whether co-transcription with neighbouring genes occurs. We have also investigated the effect of growth phase on expression, given that transcription decreases in stationary phase.

Methods

We used reverse transcriptase PCR to confirm co-transcription with neighbouring genes and primer extensions to map transcription start sites. As we have not been able to isolate an Ng IHF mutant, wild type and mutant promoter regions were cloned into a CAT reporter system and the level of promoter activity was assessed in an *Escherichia coli* (Ec) wild type and *ihfA* mutant background, to determine the effect of removing IHF on levels of expression from the cloned promoters. Experiments in wild type Ng will also be performed once suitable constructs have been made (in progress) that can be introduced into the chromosome of Ng. Western blotting was used to measure the level of IHF expressed by Ng.

Results

We have shown that *ihfA* can be transcribed from a promoter immediately upstream, or another upstream of *pheT*, which is located 5′ of *ihfA*. In addition to the previously identified IHF binding sites directly upstream of *ihfA* there are other sequences required for maximal transcription, but the exact role of these is not yet known. There does not seem to be a promoter immediately 5′ of *ihfB*, and the promoter used is upstream of *rpsA*, the gene upstream of *ihfB*. From studies performed in Ec, differences can be seen between mid exponential and stationary phase samples for each promoter, and each seems to be regulated by IHF. As observed for *ihf* transcripts, the level of IHF protein decreases in stationary phase.

Conclusion

The transcriptional regulation of IHF production in Ng is complex, but appears to be auto-regulated, and influenced by growth phase.

P050

Antimicrobial susceptibility of *Neisseria gonorrhoeae* strains in Miami-Florida, 2007

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Objectives
Evaluation of antimicrobial susceptibilities of *Neisseria gonorrhoeae* strains prospectively isolated from a public health clinic in 2007 in Miami, Florida.
Methods
Males and females attending the Miami-Dade County Health Department Downtown Sexual Transmitted Diseases (STD) Clinic were asked to participate in a study to development a new diagnostic test for Gonorrhea and Chlamydia. One of the assessments included the collection of urethral and cervical samples and *N. gonorrheae* cultures of the collected specimens. The first samples collected from the urethra and cervix of all participants were used for inoculation of the plates containing the modified Thayer-Martin medium and placed in a CO2 enriched environment. The plates were shipped to a single central commercial laboratory for further identification and susceptibility testing. The susceptibility of *N. gonorrheae* isolates was tested against the following antibiotics: penicillin, tetracycline, cephalosporins (cefepime, cefixime, cefpodoxime, ceftazidime, ceftriaxone and cefuroxime), spectinomycin and quinolones (ciprofloxacin and ofloxacin) using the disk diffusion method. Clinical and laboratory Standards Institute susceptibilities breakpoints were used for interpretation of the inhibition zone diameters. We used simple descriptive statistics to analyze the results. This study was approved by the Institution Ethics Committee.
Results
A total of 69 *Neisseria gonorrhoeae* isolates were collected in the reference laboratory from May 1st to December 16th 2007. Characteristics of the patients and susceptibility results are detailed in table 1. Overall, there were only two isolates (3%) fully susceptible to all tested antibiotics. Additionally, 41 (60%) of the isolates had decreased susceptibility to two classes of antibiotics, 6 (9%) had decreased susceptibility to three classes of antibiotics, and one had resistance to all four classes of antibiotics. All isolates were susceptible to cefalosporins. The 10 patients with quinolone resistant isolates were of black race, predominantly African - American and heterosexual (9 patients for both) and 6 of them had history of treated gonorrhea.
Conclusions
Strains with decreased susceptibility to multiple antibiotics is a growing problem in our clinic. The need for rapid and easily available methods to detect resistance in *N. gonorrhea* is a priority in STD research.

Table 1: Characteristics of patients with gonorrhea

Number of patients	69
Gender	
Male	66 (95.7%)
Female	3 (4.3%)
Age (median, range)	20 (18 – 60)
Education	
Primary	9 (13%)
Secondary	50 (72.5%)
College	10 (14.5%)
Race	
Black	64 (92.7%)
White	5 (7.3%)
Ethnicity	
Hispanic	6 (8.7%)
African - American	63 (91.3%)
Sexual preference	
Opposite	67 (97.1%)
Same	2 (2.9%)
History of prior STD	
Yes	10 (14.5%)
No	59 (85.5%)
Coinfection with Chlamydia	12 (17%)
Susceptibility testing	
Penicillin	
Susceptible	5 (7.3%)
Intermediate or Resistant	64 (92.7%)
Tetracycline	
Susceptible	23 (33%)
Intermediate or Resistant	46 (67%)
Quinolones	
Susceptible	59 (85.5%)
Intermediate or Resistant	10 (14.5%)
Spectinomycin	
Susceptible	67 (97.1)
Intermediate or Resistant	2 (2.9%)

P051

Ciprofloxacin resistance in *Neisseria meningitidis* in France

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Objectives
Ciprofloxacin is frequently used in adults for chemoprophylaxis of meningococcal disease. Resistance to this antibiotic in *N. meningitidis* is rare and until recently was reported only from sporadic cases. However, recent reports described for the first time several ciprofloxacin resistant (Cip-R) serogroup A meningococci from two outbreaks in Delhi and cluster of three serogroup B meningococci in USA. Therefore we aimed to test meningococcal isolates for ciprofloxacin resistance, to determine the mechanism responsible for that phenotype and check the clonal expansion of Cip-R isolates.
Methods
All clinical *N. meningitidis* isolates received from 1999 to 2007 at the French Reference Centre for Meningococci were screened for ciprofloxacin resistance using Etest method. There were 4900 isolates from France and 246 from African countries. DNA fragments of the gyrA, parC and parE genes of Cip-R isolates were amplified and sequenced. Cip-R isolates were also characterized by serotyping and MLST analysis.
Results
Only 3 isolates tested were resistant to ciprofloxacin with MIC values of 0.19 mg/L, and all of them were

from invasive disease cases that occurred in France. Two serogroup A, serotype 4, serosubtype P1.9 Cip-R isolates belonged to different sequences types, although, to the same clonal complex, ST-5/subgroup III. The first isolate (Cip-R1) was isolated in 2004 from the blood culture of a 7 year-old girl and is most likely to be imported from Africa. The second one (Cip-R2) was from the CSF of a 77 year-old man who had arrived in 2006 to France from India. Its ST (ST-4789) is the same as ST of isolates from outbreak in Bangladesh and similar to Indian outbreak isolates. The Cip-R3 isolate, of serogroup W-135, nontypeable, subtype P1.5, cultured from blood and CSF samples of an 82 year old women in 2006, belonged to a new ST (ST-6361).
The sequencing of fragments of the gyrA, parC and parE genes revealed a mutation in the gyrA gene in the three Cip-R isolates resulting in Thr91Ile substitution. The Cip-R1 isolate had also additional alterations of Asn103Asp, Ile111Val and Val120Ile. The sequences of parC and parE genes were the same as of a ciprofloxacin susceptible isolate used. The association of the Cip-R phenotype with mutations in gyrA gene was confirmed by transformation into the susceptible isolate using appropriate PCR products.
Conclusions
Results of the study revealed that ciprofloxacin resistance was exclusively associated with gyrA mutations and suggested lack of clonal expansion of ciprofloxacin-resistant meningococci. The first report of the invasive case caused by W-135 ciprofloxacin resistant meningococci should raise caution when treating respiratory infections in elderly by quinolones, since this age group is the most affected by invasive meningococcal pneumonia.

P052

Distribution of a cephalosporin-resistant sequence type of *Neisseria gonorrhoeae* with a mosaic PBP2 associated with treatment failure in the Asia-Pacific region

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Objective
Reduced susceptibility of *N. gonorrhoeae* (NG) to third generation cephalosporins was detected first in Japan, and then more widely in the Asia-Pacific region, from 2000 onwards. This resistance was due primarily to alteration in PBP2, and more than 20 sequence changes have been detected in the penA gene. Particular attention was focussed on the effects of a mosaic PBP2 on cephalosporin susceptibility. Treatment failures occurred when oral third generation cephalosporins were used to treat infections due to NG with this particular alteration. This study determined the sequence type (ST) of NG with mosaic PBP2 in NG from Japan, Hong Kong and Australia to assess the similarities and differences between NG with mosaic PBP2 and the extent of spread of closely related STs.
Methods
Twenty-seven NG from Japan and 15 from Australia isolated between 2001 and 2005, and 11 NG from Hong Kong isolated in 2006, all with raised MICs for ceftriaxone (range 0.03 - 0.5 mg/l) and shown to possess the mosaic PBP2 on penA sequencing were genotyped using the NG multi-antigen sequence typing (NG MAST) methodology. MICs for ceftriaxone were determined by standard methods.
Results
Twenty-three distinct STs were present in the 27 NG from Japan and none of these were found in the seven STs in the 15 NG from Australia or the two STs in the 11 NG from Hong Kong. Ten of the 11 NG from Hong Kong were ST 835 and the other was a closely related ST, 2469. Four of the 15 Australian NG were ST 835 indistinguishable from those found in Hong Kong and another 7 were the closely related STs 1414,

1424 and 1677. The remaining 4 NG were of three distinct STs not represented elsewhere.

Conclusions

There were close similarities between the NG containing the mosaic PBP2 isolated in Hong Kong and Australia suggesting a wide distribution of NG of ST 835 or closely related STs. Additionally, the sensitive typing technique used indicates that there are multiple other and distinct STs with this characteristic in Australia and, notably, in Japan. Gonococcal populations are known to be highly diverse and to have the potential for rapid change and these data are consistent with this phenomenon. The Hong Kong isolates were all from 2006, and probably represent a clustering in time of this ST. Those from the other centres were small samples obtained over a longer time frame so that a larger study may well reveal linkages between the different STs detected. The study indicates that there are multiple subtypes of NG containing resistance determinants that compromise treatment with oral third generation cephalosporins and that these STs can spread widely and rapidly.

P053

Mutation of *Neisseria gonorrhoeae* isolates with mosaic penA genes to higher levels of cephalosporin resistance

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Objectives

To investigate the potential of penicillin resistant isolates of *N. gonorrhoeae* with mosaic-type penA genes to mutate to higher levels of cephalosporin resistance.

Methods

Mutations in the penA and ponA genes of twelve isolates of *N. gonorrhoeae* that exhibited elevated MIC values to penicillin or penicillin and cephalosporins were determined by DNA sequencing of PCR products. MIC values were determined by the agar plate dilution method. Initially, mutants were selected by plating bacteria onto supplemented GC base agar that contained a gradient concentration of either cefpodoxime or ceftriaxone. Additional mutants were selected and frequencies of mutation were determined on media that contained single antibiotic concentrations.

Results

All four of the isolates that exhibited elevated MIC values to penicillin (4-8 ug/ml) and cephalosporins (2 ug/ml cefpodoxime) possessed mosaic-type penA genes. Seven strains with higher values to only penicillin showed only the insertion of a single aspartic acid residue and three addition amino acid substitutions in penA. All of the isolates showed a consistent proline substitution in PBP1 (ponA). MIC values for azithromycin (0.5 ug/ml) and the ability to grow in the presence of Triton X100 (950 ug/ml) or erythromycin (2.5 ug/ml) indicated the presence of mutations in mtrR. When plated on gradient plates that contained cefpodoxime, all four of the strains with mosaic penA genes readily gave rise to mutants that exhibited elevated resistance to cefpodoxime. Only one of the strains produced mutants when plated on media with a gradient of ceftriaxone. MIC values for the selected mutants were ›8 ug/ml for cefpodoxime, 0.25-0.5 ug/ml for ceftriaxone, and 0.5-1.0 ug/ml of cefixime. The experimentally determined frequency of mutation to elevated resistance to cefpodoxime was 1.3 x 10-10 mutants/cfu. Identical experiments done with strains without mosaic-type penA genes yielded no such mutants.

Conclusion

There is potential for mutation to still higher levels of *in vitro* resistance to cephalosporins in strains of *N. gonorrhoeae* that possess the mosaic type penA gene. This appeared to be especially true for cefpodoxime. However, selected resistance to cefpodoxime led to 2-4 fold increases in MIC values to two other important cephalosporins. Work to determine the gene(s) involved is ongoing.

P054

Genetics of intermediate resistance to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*

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Objective
The recent emergence of intermediate resistance to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae* sounds a warning bell for the future effectiveness of these antibiotics in treating gonococcal infections. The hallmark of intermediate cephalosporin-resistant (cephI) strains is the presence of mosaic penA alleles encoding altered forms of penicillin-binding protein 2 (PBP 2) with 55-60 amino acid differences compared to PBP 2 from penicillin-susceptible strains. It remains unclear, however, what role other chromosomal determinants play in increasing resistance to ceftriaxone and cefixime. To address this, we undertook a systematic study to define the role of penicillin-resistance determinants in cephI resistance.
Materials & methods
The mosaic penA allele (penA35) from 35/02 (Lindberg et al. 2007 AAC 51:2117), a cephI strain with confirmed MICs of 0.12 μg/ml and 0.28 μg/ml for ceftriaxone and cefixime, respectively, was introduced into FA19 (penicillin- and cephalosporin-susceptible), FA6140 (penicillin-resistant, cephalosporin-susceptible), and FA19 containing one or more of the known chromosomally mediated penicillin-resistance determinants (mtrR, penB, and ponA1), and MICs of ceftriaxone and cefixime for the resulting transformants were determined.
Results: Introduction of penA35 into FA19 increased the MICs of ceftriaxone and cefixime from 0.0006 to 0.012 μg/ml (20-fold) and 0.0012 to 0.12 μg/ml (100-fold) respectively. Thus, penA35 confers the majority of donor cefixime resistance in cephI strains, but much less so for ceftriaxone resistance. Transfer of penA35 into FA6140, a CMRNG strain, increased the MICs of ceftriaxone and cefixime to 0.25 and 0.5 μg/ml, both of which are higher than the donor strain, 35/02. These data demonstrate that FA6140 contains all of the resistance determinants necessary for cephI resistance. Transfer of both mtrR and penB into FA19 penA35 increased ceftriaxone resistance 3-fold (to 0.04 μg/ml), whereas these determinants had no effect on cefixime resistance. Consistent with these data, inactivation of the MtrC-MtrD-MtrE efflux pump in 35/02 or FA6140 penA35 markedly decreased ceftriaxone resistance, while showing only modest decreases in cefixime resistance. Addition of the ponA1 allele from FA6140 into FA19 penA35 mtrR penB did not increase resistance, and reversion of the ponA1 allele to wild type in FA6140 penA35 did not cause a decrease in resistance to either ceftriaxone or cefixime, indicating that ponA is not involved in cephI resistance.
Conclusion
Taken together, these results indicate that the mosaic penA35 allele confers increased resistance to expanded-spectrum cephalosporins, but that other determinants are also needed to increase to the level of cephI clinical isolates. The majority of cefixime resistance is conferred by the penA35 allele, with less contribution from other determinants, whereas ceftriaxone resistance is nearly equally dependent on both. The gene(s) required to reach donor levels of cephI resistance appear to be the same as those that confer high-level penicillin resistance in CMRNG strains, but the molecular identities of these genes remain unknown. Our data also suggest that cephI resistant strains arose from a single transformation event in which penA alleles from commensal Neisseria species recombined into a penicillin-resistant strain harbouring mtrR, penB, ponA, and the unknown gene(s) involved in high-level penicillin resistance.

P055

Drugs resistance in gonococci in Italy: emergence of a multi resistant cluster circulating in a heterosexual network

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Objectives
The aim of the present work was to analyze 588 *Neisseria gonorrhoeae* isolates collected during the period 2003-2007 with or without concurrent HIV infection. In particular, we report the baseline study carried out in order to assess: i) the serological and antimicrobial susceptibility phenotype to five antimicrobial drugs; ii) the dissemination of drug or multidrug resistance and the molecular changes responsible; iii) *N. gonorrhoeae* Multi Antigen Sequence Typing (NG-MAST) to identify transmission network.
Methods
Strains were cultured on Thayer-Martin medium with 1% IsoVitaleX at 37ºC in 5% CO2, and stored at -80ºC. All isolates were accompanied by a patient form seeking the following information: nationality, residence, sexual orientation, previous antibiotic use, recent travel abroad, sexual partner, immune status and HIV infection. Serotyping of all isolates was performed with the Phadebact GC serovar test (Boule Diagnostics, Sweden). Antimicrobial susceptibility testing was performed by E-test and by microdilution method for tetracycline. MICs of penicillin, ceftriaxone, ciprofloxacin, spectinomycin, tetracycline were determined according to susceptibility interpretation standards proposed by CLSI. Sequencing of parC and gyrA genes for ciprofloxacin resistant strains and of mtrR, porB and rpsJ genes for CMRNG tetracycline resistant, multiplex PCR for the detection of (Penicillin Plasmid Producing N.gonorrhoeae) PPNG and (Tetracycline resistance *N.gonorrhoeae*)TRNG were also performed.
Results
Gonorrhoea infection occurred most frequently in men with heterosexual behaviour; the median age was 31 years. Approximately 8.3% of all infected patients were also HIV-1 positive. Serovar IB was the most frequently encountered. Ciprofloxacin, penicillin and tetracycline resistance phenotypes were detected as well as multi drug resistant strains. The percentage of resistant strains was statistically different if the patients were previously treated (P=0.02), and this is particularly evident (P=0) for those strains resistant to two or three antibiotics. In particular, the rate of ciprofloxacin resistance was 46% with a further 8.2% of strains with reduced susceptibility. 20.52% of isolates were resistant and 56.75% showed reduced susceptibility to penicillin, leaving only 22.73% of gonococci fully susceptible. Tetracycline resistant isolates represent 28% of those analyzed. 37% and 4.9% were resistant to two and three agents, respectively. Amino acid changes in parC and gyrA genes were found in CMRNG (Chromosomal Mediated resistance *N.gonorrhoeae*); TRNG genotypes also found among tetracycline resistance. All the penicillin resistant strains were PPNG. Despite the presence of different Sequence Types (STs), NG-MAST showed the persistence of ST661 in a specific geographic area. All these strains showed identical profiles in Pulsed Field Gel Electrophoresis (PFGE), were serotype IB, resistant to penicillin, beta-lactamase producing, resistant to ciprofloxacin and to tetracycline.
Conclusions
The significant percentage of not susceptible gonococci circulating in the country is worthy of note since Italy is considered as having a low incidence of gonorrhoea infection. The presence of multi resistance isolates provides a challenge for therapy even if all the examined strains were still fully susceptible to ceftriaxone and spectinomycin.
Interestingly, a correlation between a specific Sequence Type and antimicrobial resistance profiles was detected in a heterosexual network in a precise geographic area of the country.

P056

Molecular analysis of tetracycline resistance gonococci: rapid detection of resistant genotypes using a real-time PCR assay

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Objectives
A retrospective analysis for tetracycline susceptibility was performed on 289 gonococci isolated in Italy from 2003 to 2005. Molecular mechanisms of resistance were investigated by both sequence analyses of the three main genes associated to chromosomally mediated resistance (mtrR, penB and rpsJ genes) and by the identification of plasmids carrying the tetM determinant by PCR (American or Dutch type plasmids) associated to plasmid mediated resistance. Genetic relatedness of non susceptible strains was evaluated by Pulsed Field Gel Electrophoresis (PFGE). The aim of this study was to examine tetracycline resistant gonococci and to set up a real-time PCR method to identify, in the same assay, both the chromosomally and plasmid-mediated tetracycline resistance genotypes.
Methods
A total of 289 *N. gonorrhoeae* strains, isolated in Italy from April 2003 to June 2005 were tested for susceptibility to tetracycline by E-test and agar dilution method. Sequence analyses of mtrR, penB, rpSJ genes were performed. American and Dutch plasmids carrying the tetM determinant, were detected by PCR. The PFGE banding patterns were analyzed with the Diversity Database Fingerprinting Software (version 2, Bio-Rad Laboratory).
The rpsJ probes, labelled with the fluorophore LC-Red 640 and fluorescein, were complementary to the rpsJ mutated sequence; the probe used for the tetM determinant includes an upstream fluorescein probe complementary to both the American and the Dutch plasmids, and a downstream hybridization probe labelled with the fluorophore LC-Red705 complementary to the Dutch type plasmid sequence, harbouring a mismatch at second position compared to the American type plasmid sequence.
Results
Among the strains analyzed, 65 (22,5%) were resistant (MIC≥2 mg/L), 143 (49,5%) intermediate (0.5≤MIC≤1 mg/L) and 81 (28%) susceptible to tetracycline. Coexistence of chromosomally and plasmid mediated resistance to tetracycline was observed in the majority of resistant isolates. No clonal structure was highlighted by analysis of PFGE pattern profiles.
Real-time PCR assay was performed on DNAs of 58 gonococci strains of which 39 resistant, 8 susceptible and 11 intermediate to tetracycline. Different melting curves were obtained corresponding to the different genotypes. The results were in agreement with the molecular characterization of antibiotic determinants and with tetracycline susceptibility analyses. All the strains were run in triplicate and the results were always reproducible.
Conclusions
The rate of tetracycline resistance among Italian gonococci was of 22,5 percent in the study period. Italian gonococci with higher MIC values, showed both the chromosomal and plasmid mediated resistances The real-time PCR based method, which was set up designing primers and probes to rapidly identify chromosomal and/or plasmid associated resistance genotypes, also proved to be specific for N. gonorrhoeae DNA suggesting a future application directly on clinical samples since the specificity of the test. The melting curves analysis showed a good correlation with the plasmid and/or chromosomal tetracycline resistant genotypes.

P057

The TolC outer membrane protein of *Neisseria meningitidis* can be a functional replacement for an *E.coli* tolC deficient strain

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Objectives
In *E. coli*, TolC serves as the outer membrane protein (OMP) for many tripartite efflux pumps that export antimicrobials (e.g. AcrA-AcrB, EmrA-EmrB, MacA-MacB) or toxins (e.g. HlyB-HlyD), such as the RTX (repeat in toxin) toxin HlyA. There are two tolC homologs in *Neisseria meningitidis*, mtrE and tolC. In both *N. meningitidis* and *N. gonorrhoeae*, MtrE is the OMP channel used by the mtrCDE and farAB encoded drug efflux pumps, which export structurally diverse hydrophobic antimicrobials, including those of host origin involved in innate defenses (e.g antimicrobial peptides and long chain fatty acids). In contrast, the TolC-like protein is present only in *N. meningitidis.* A tolC null mutant of *N. meningitidis* strain M7 is not impaired in susceptibility to antimicrobials; however, it is deficient in exporting the RTX toxin FrpC (Kamal and Shafer, 2007). Since sequence analysis revealed that the meningococcal TolC of strain M7 and E. coli TolC are 22% identical over the entire amino acid sequence, we asked whether the meningococcal TolC could functionally replace TolC in *E. coli.*
Methods
We cloned the meningococcal tolC behind an IPTG-inducible lacZ promoter in pGCC4. The plasmid construct was introduced into isogenic *E. coli* strains MC4100 (wild-type) and RAM 1129, a tolC deletion mutant. MIC analysis was performed using antimicrobials (Triton-X 100, novobiocin, ethidium bromide, crystal violet, and rifampicin) recognized by the AcrA-AcrB-TolC efflux pump system. We also examined extracellular HlyA production, which is mediated by the HlyB-HlyD-TolC export system by detecting alpha-hemolysis around colonies of *E. coli* strains grown on sheep blood agar plates.
Results
We report that expression of the meningococcal tolC gene in a tolC null mutant of *E. coli* can restore wild-type levels of antimicrobial resistance and alpha-hemolysin production.
Conclusions
Our results indicate that the meningococcal TolC-like protein can function as an OMP channel for both a drug efflux pump (e.g. AcrA-AcrB-TolC) and a toxin secretion pump (HlyB-HlyD-TolC) when expressed in *E. coli.* Accordingly, we conclude that regions of functional importance of the *E. coli* TolC OMP are conserved in the meningococcal TolC-like protein.

P058

Neisseria meningitidis with reduced susceptibility to penicillin of serogroups other than C: analysis of penA alleles as an additional tool to improve typing of meningococci

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Objectives
In Italy, a high incidence of *Neisseria meningitidis* strains with reduced susceptibility to penicillin (PenI) is reported. This trend was observed since 2002 among serogroup C strains belonging to few serotypes and electrophoresis types (ET). In particular, PenI strains emerged and spread in 2002 within the C:2b:P1.5 ST-8 complex (cpx)/A4 cluster and in 2004 in the C:2b:P1.5,2 ST-8 cpx/A4 cluster. After the decrease of those

clones, meningococcal isolates showing susceptibility to penicillin and belonging to other serogroups and ETs began to increase up to a percentage of 45.5 in 2007.
The aim of this study was to analyze the phenotypic and genotypic characteristics of 155 PenI meningococci isolated from 2002 to 2007 searching for patterns of penA gene alterations associated with specific serogroups and sequence types.

Methods

Serogroup and sero/subtype were determined for all the examined strains. Susceptibilities to penicillin G and ampicillin were evaluated using the E-test method. The penA gene of PenI strains was sequenced and each sequence compared with those deposited in Genbank. The Multilocus Sequence Typing was used to assign the isolates to different clonal lineages.

Results

The percentage of PenI strains increased from 24% in 2002 to 45.5 % in 2007. In particular, after the emergence in 2002 of the C:2b:P1.5 ST-8 cpx/A4 and in 2004 of the C:2b:P1.5,2 ST-8 cpx/A4, as predominant clones of PenI strains, during the last three years we observed their gradual decrease in parallel with the increase of serogroup B, W135 and Y Pen I strains belonging to different clonal complexes. The penA sequence analysis showed the presence of 18 variants when analyzing the entire gene and of 14 variants considering only an internal 402 bp region corresponding to the most variable part of the gene. Besides the penA12, detected in all strains C2b:P1.5/P1.5,2 ST-8 cpx /A4, the major alleles found were penA14 (mostly among B, ST-269), penA9 (mostly among C, ST-11 cpx/ET-37) and penA20 (exclusively found among Y, ST-23 cpx/A3). A total of 17 PenI strains belonging to different serogroups and STs were characterized by the lack of mosaicism in the penA gene: interestingly, the MIC values for ampicillin were ‹0.064 μg/ml whereas for penicillin were ›0.094 μg/ml.

Conclusion

Invasive disease due to PenI meningococci increased twofold between 2002 and 2007 in our country. Molecular analyses defined the different serogroups and clonal lineages that replaced the C2b:P1.5/P1.5,2 ST-8 cpx/A4 during the last three years. They represent the majority of PenI strains recently circulating and are characterized by the presence of specific penA alleles predominant in the current meningococcal population. PenA sequencing could be considered useful not only to identify isolates with reduced susceptibility to penicillin but also as an additional target gene for molecular tracing of meningococci over time.

P059

The global transcriptional regulator MtrR plays a role in regulating genes involved in cellular metabolism

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Objectives

Neisseria gonorrhoeae can resist antimicrobial agents through the expression of the inducible, energy-dependent Mtr (multiple transferable resistance) efflux pump whose structural proteins are encoded by the mtrCDE operon. This operon is negatively regulated by the transcriptional regulator MtrR and positively regulated by MtrA. Elevated expression of the mtrCDE locus can occur through mutations that impact mtrR expression (Veal et al, 2002) or function, or by the presence of mtrA (Rouquette et al, 1999). Enhanced levels of the MtrCDE efflux pump can increase gonococcal resistance to hydrophobic agents (HAs), including mediators of innate immunity systems that function on mucosal surfaces. Since this efflux pump system is important for gonococcal survival *in vivo* (Jerse et al, 2003) and loss of MtrR production can impact *in vivo* fitness (Warner et al, 2007), we sought to determine the number and type of genes under MtrR control.

Methods

For the purpose of identifying the MtrR regulon, we conducted a microarray analysis that employed total

RNA extracted from isogenic wild type (strain FA19) and MtrR-deficient gonococci. Once the regulon was defined by microarray analysis, regulation of selected genes by MtrR was confirmed by qRT-PCR and translational lacZ fusion assays. An MtrR-regulated gene (glnA encoding glutamine synthetase) was studied in detail and a non-polar mutation was constructed in strain FA19. The mutation was complemented by establishing glnA at a second site on the chromosome. These isogenic strains were studied in functional assays that included evaluation of growth and susceptibility to select HAs. The ability of MtrR and an additional transcriptional regulator (FarR) to bind to the DNA sequence upstream of glnA was evaluated by electrophoretic mobility shift assays (EMSA).

Results

Microarray analysis revealed that MtrR represses or activates, directly or indirectly, at least 67 genes. An interesting subset of these genes was similar to other bacterial genes involved in glutamine transport or biosynthesis. For instance, glnA was found to be subject to MtrR-repression. However, DNA-binding studies revealed that MtrR does not bind this DNA sequence. In contrast, FarR, which negatively regulates the farAB efflux pump-encoding operon by an MtrR-dependent mechanism (Lee and Shafer, 2003), was able to bind the DNA sequence upstream of glnA. Evidence that both FarR and MtrR control glnA was obtained by beta-galactosidase fusion assays that employed isogenic strains bearing mutations in mtrR or farR or both. Loss of glnA in gonococci negatively impacted bacterial growth but enhanced bacterial resistance to HAs recognized by the MtrC-MtrD-MtrE efflux pump.

Conclusions

Based on its capacity to directly or indirectly regulate a number of genes that are dispersed on the gonococcal chromosome, we conclude that MtrR is a global regulatory protein. The capacity of MtrR to regulate expression of glnA through a FarR-dependent mechanism in gonococci is of possible biologic significance since GlnA influences Salmonella fitness in vivo. Moreover, our results emphasize that loss of glnA can significantly impact gonococcal growth and levels of susceptibility to certain antimicrobials.

P060

Detection of penicillin intermediate susceptible Meningococci in Austria from 1987-1989 and 1995-2006: Comparison of E-test and penA PCR

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Objective

Antibiotic resistance testing of Meningococci has always been a controversial topic. Differences in used media and applied breakpoints made it impossible to compare data among different laboratories and different countries. The European Monitoring Group on Meningococci (EMGM) recommended in 2003 [1] the use of Mueller Hinton with sheep blood and E-test for reference laboratories but no consensus was found concerning the breakpoints. The emergence of penA genes with altered sequences leading to production of penicillin-binding proteins (PBP's) with a lower affinity to ß-lactam antibiotics added a further variable to the discussion. Should we use molecular biological methods to detect Penicillin intermediate strains?

At the Austrian National Reference Centre, Meningococci have been tested since 1995 with E-test on Mueller Hinton with sheep blood, using the breakpoint ≥ 0.125 mgL^{-1} to predict intermediate susceptibility. There are however reports that borderline MIC's (0.064 mgL^{-1}) show penA alterations. We decided to analyze all meningococci strains with a breakpoint of ≥0.064 mgL^{-1} by comparing the results of MIC determination using E-test and mutations in the penA gene using real-time PCR.

Methods

The MIC's were determined with E-test on Mueller-Hinton agar supplemented with sheep blood. The plates were incubated for 24 hours at 35ºC in a CO_2 incubator.

The molecular detection of mutations in the penA gene was done by real-time PCR with Lightcycler 2.0

from Roche according to the protocol described by P.Stefanelli et al.[2].
372 Strains were tested. The Meningococcal isolates chosen for the examination were taken from the years 1987 to 1989 (144 strains) and 1995 to 2006 (228 strains).

Results

According to the E-test results, 46 strains revealed a MIC of ≥0.064 mgL^{-1} and 17 strains had a MIC of ≥ 0.125 mgL^{-1}. PCR analyses revealed penA mutations in 24 strains. All of the 17 strains with a MIC of ≥ 0.125 mgL^{-1} also harboured mutations in the penA gene. Of 15 strains with a MIC of 0.064 mgL^{-1}, only four strains showed penA mutations and of 9 strains with a MIC of 0.094 mgL^{-1} only three strains revealed penA mutations.
All strains with a MIC of ‹0.064 mgL^{-1}, were also negative in the PCR.

Conclusions

As the MIC breakpoint between 0.064 and 0.125 mgL^{-1} remains controversial, it is recommended to combine the conventional MIC determination with a PCR based resistance mutation detection in the penA gene.

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P061

Polyamine uptake in *Neisseria gonorrhoeae* modifies growth and antibiotic resistance

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Objectives

The polyamines putrescine, spermidine and spermine are small cationic compounds that are synthesized de novo in prokaryotes and eukaryotes. Polyamines are modulators of key cellular functions (e.g., DNA synthesis, transcription, translation and cell signaling) and can be involved in mechanisms of antibiotic resistance and pathogenesis. We recently determined that a transcriptional repressor (MtrR) of the mtrCDE-encoded efflux pump operon can directly or indirectly activate transcription of genes encoding components of a polyamine transporter. Since the role of polyamines in modifying key cellular functions are not clearly understood in bacteria and have not been studied to any great extent in *Neisseria gonorrhoeae*, we asked if the MtrR-regulated potGHI genes are important in gonococci.

Methods

We disrupted the MtrR-regulated potH and potI genes, encoding the polyamine permeases, with a non-polar kanamycin resistance cassette in FA19. We compared the growth rate of the mutant and the wild-type strains and their susceptibility to different antibiotics in media supplemented with different concentrations of polyamines. We used PCR and DNA sequencing to characterize the potGHI operon in gonococcal isolates.

Results

The potGHI region in gonococci was characterized by PCR and DNA sequence analysis. A heretofore undescribed 159 bp repeat element (potentially encoding a 47 amino acid cationic peptide) was found in variable copies between potG and potH in gonococcal strains FA19, F62 and FA1090. Interestingly, this sequence was not repeated at this region in several fresh clinical isolates obtained from males with gonococcal uncomplicated infections or bloodstream isolates from patients with disseminated gonococcal infections (DGI). We found that insertional inactivation of the potHI genes in strain FA19 potHI::kan had two significant impacts on growth of gonococci in GCB broth: an increased lag phase and an impaired growth rate during the exponential phase of growth. Moreover, loss of PotH and PotI increased the susceptibility

of gonococci to the cationic antimicrobial peptide polymyxin B in media supplemented with increasing concentrations of spermidine.

Conclusion

We have identified a 159 bp repeat sequence within the potGHI operon in commonly used *N. gonorrhoeae* strains FA19, F62 and FA1090 but the significance of this observation is unclear since the sequence is not repeated in fresh clinical isolates or strains causing DGI. The potHI-encoded products appear to be important since their loss due to an insertional mutation significantly impacted growth of gonococci in broth. This finding suggests that the ability of gonococci to use a polyamine uptake system is important for maximal bacterial growth at sites or in fluids rich in polyamines. Moreover, in the presence of spermidine, the expression of the potHI genes may influence the ability of gonococci to resist the action of cationic peptides.

P062

MpeR is a global transcriptional regulator in gonococci that modulates levels of resistance to the human antimicrobial peptide LL-37 and potassium tellurite

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Objectives

MpeR is a transcriptional regulator of mtrF, which encodes an inner membrane protein that functions with the MtrC-MtrD-MtrE efflux pump when gonococci express high levels of resistance to structurally diverse hydrophobic agents that have anti-gonococcal activity (Folster and Shafer, 2005). MpeR is a member of the AraC family of transcriptional regulators in bacteria, but the capacity of MpeR to regulate genes other than mtrF was not known prior to this investigation. Accordingly, we sought to define the MpeR regulon to determine its mechanism of gene control, and learn the function of MpeR-regulated genes.

Methods

We defined the MpeR regulon by microarray analysis that used total RNA extracted from wild-type strain FA19 and an isogenic mpeR null mutant (FA19 mpeR::Kn). An MpeR-regulated gene (terC) potentially involved in resistance to tellurium was inactivated by insertion of the nonpolar kanamycin cassette aphA-3 within the coding sequence. Strains FA19, FA19 mpeR::kn, and FA19 terC::kn were tested for susceptibility to selected antimicrobials (potassium tellurite, silver nitrate and the human antimicrobial peptide LL-37) and MIC values were determined. Complementation analysis was performed using the Neisserial Insertional Complementation System that employed pGCC3 (Skaar et. al., 2002).

Results

We found that MpeR can regulate at least 46 gonococcal genes which are dispersed throughout the chromosome. These genes gave evidence of being MpeR-activated (16 genes) or MpeR-repressed (30 genes). The transcriptional profiling results confirmed that expression of mtrF was subject to MpeR repression and this result was confirmed using lacZ translational fusions in an mpeR complemented derivative of FA19 mpeR::kn. We found an MpeR-activated gene (2.17 X) that is potentially involved in gonococcal resistance to certain antimicrobials. This gene (NG1059) has been provisionally annotated as terC and is potentially phase-variable due to an 8 homopolymeric nucleotide repeat within the coding sequence; strains FA19, FA1090 and F62 were found to have a phase-on sequence. We found that a nonpolar mutation in mpeR and terC resulted in enhanced susceptibility of gonococci to potassium tellurite but not silver nitrate. The mpeR null mutant but not the terC mutant displayed increased susceptibility to the human cathelicidin antimicrobial peptide LL-37.

Conclusions

Our results suggest that MpeR is a global transcriptional regulator that can directly or indirectly control expression of several gonococcal genes. The capacity of MpeR to control levels of gonococcal susceptibility

to the human cathelicidin LL-37 has significance for the ability of gonococci to subvert innate host defenses during infection. Above and beyond its role in regulating mtrF, the ability of MpeR to control terC expression may have significance for determining levels of gonococcal susceptibility to certain antimicrobial metal compounds (e.g., potassium tellurite).

P063

Identification of novel mtrR and penB mutations in *N. gonorrhoeae* isolates from New Caledonia

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Objective
mtrR and penB are two important determinants in chromosomally mediated penicillin and tetracycline resistance. The mtrR determinant, which causes overexpression of the MtrC-MtrD-MtrE efflux pump, is due to mutations either in the promoter or coding region of mtrR. The penB determinant encodes mutations in the major outer membrane PorB1B porin. The most common mutation in mtrR is deletion of a single nucleotide in a 13-bp inverted repeat in the mtrR promoter (-A), while the critical mutations in penB are located at two positions in the constriction loop within the pore. The two mutations in penB associated with penicillin resistance are the double mutation G101D/A102D and the single mutation G101K. Identification of additional mutations in these two determinants is critical for comprehensive epidemiological monitoring of antibiotic resistance trends in *N. gonorrhoeae*. In a genotyping assay to track the existence of known resistance alleles in gonococcal isolates in New Caledonia, we previously identified strains with increased penicillin resistance but lacking the common mutations in mtrR and penB. This study aimed to identify whether novel mtrR and penB mutations were present in these strains.
Materials and methods
The mtrR promoter and coding regions were amplified from genomic DNA of four strains (307, 309, 319, and 361) with MICs of penicillin ranging from 0.13 to 0.38 μg/ml and used to transform FA19 to increased Triton X-100 resistance. The region encompassing the penB gene from these same isolates was amplified and used to transform FA19 penA mtrR (the mtrR determinant is the single nucleotide deletion) to increased penicillin resistance.
Results
The mtrR sequences from the four strains were identical and contained two different nucleotide insertions: a T insertion in the 13bp inverted repeat and a C insertion in the 5'UT region 90 bp upstream of the mtrC gene (+T/+C). When transferred into FA19, these mutations increased the MICs of Triton X-100 (from 0.125 to ›16,000 μg/ml), erythromycin (from 0.25 to 1 μg/ml) and crystal violet (0.63 to 2.5 μg/ml) to nearly the same levels as the -A deletion. A single +T insertion has been described previously but shown not to increase resistance to macrolides (Ng et al. 2002 AAC 46:3020), suggesting that the +C insertion upstream of mtrC has the greatest impact on resistance. The penB sequences from the four strains are identical and have an A102S mutation, but also contain nearly 30 other changes compared to PorB1B from FA1090. Transfer of these penB genes into FA19 penA mtrR increases the MIC of penicillin (from 0.12 to 0.75 μg/ml) and tetracycline (0.25 to 1.0 μg/ml).
Conclusions
The mtrR and penB alleles from four New Caledonia isolates harbour novel mutations that have not been described previously. Our transformation results demonstrate that these novel alleles confer increased resistance to penicillin and tetracycline, thus expanding the repertoire of mtrR and penB mutations that are known to confer antibiotic resistance in *N. gonorrhoeae*. These data pave the way for mechanistic studies to elucidate the molecular basis of these novel mutations.

P064

The *in vitro* susceptibility to 7 antibiotics of *Neisseria meningitidis* strains isolated last years in Romania

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Objective
To provide data regarding the actual *in vitro* susceptibility of meningococci to the antibiotics used frequently in Romania.
Methods
31 Strains of *N. meningitidis* coming from CSF, between January 2007- March 2008, were collected at the National Reference Center for Meningococci. The strains were identified to the species level by standards methods, were serogrouped and analysed for the susceptibility to 7 antibiotics: penicillin (Pc), erythromycin (Em), ceftriaxon (Cro), cefotaxim (Ctx), ciprofloxacin (Cip), rifampicin (Rif), sulfonamides (Sm), by agar dilution method according to CLSI 2006.
Results
The data were analysed according to EMGM working group and revealed the following aspects: 100 % susceptibility to Cro and Ctx, 96.7% susceptibility to Rif (MIC 50 and MIC 90=0.03 mg/l) and 96.7% susceptibility to Cip (MIC 50 and MIC 90=0.0015 mg/l).Concerning Pc it was revealed 58% susceptible strains, 38.7% intermediate and 3.3% resistant strains with MIC 50=0.06 mg/l amd MIC 90=0.12 mg/l.A high proportion (100%) of isolates were fully resistant to Sm, while 22.6 of strains were resistant to Em.Serogroup B accounted to the highest number of strains (51.7%), followed by serogroup C (35.5%), serogroup W135 (6.4%) and serogroup X (6.4%).
Conclusions
This study revealed that Cro,Ctx, Cip and Rif were the most efficient drugs.Sulfonamides must not be used neither for treatment nor for prophylaxis, as they would be ineffective.Serogroups B and C were predominant in this period in Romania. There is an urgent need for a stronger partnership between clinical medicine and public health for the surveillance of the antimicrobial usage. The need for new antimicrobial agents and vaccines is being felt in today's society.

P065

Nalidixic Acid for laboratory detection of ciprofloxacin resistance in *Neisseria meningitidis*

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Objective
Disk diffusion testing is one of the preferred methods to define susceptibility level either because limited resources or pressure for a quick response to the clinicians. Only recently, CLSI has incorporated the disk diffusion method and breakpoints for several antibiotics, suggesting that disk diffusion breakpoints derived for nalidixic acid (NA) can serve as a surrogate marker for gyrase A mutations associated with diminished fluoroquinolone susceptibility. The objective of this study is checking the breakpoints suggested in a panel of meningococci including all, ciprofloxacin susceptible (CipS) (MIC ≤ 0.03), intermediate (CipI) (MIC = 0.06) and resistant (CipR) (MIC ≥ 0.12) strains.

Material and methods

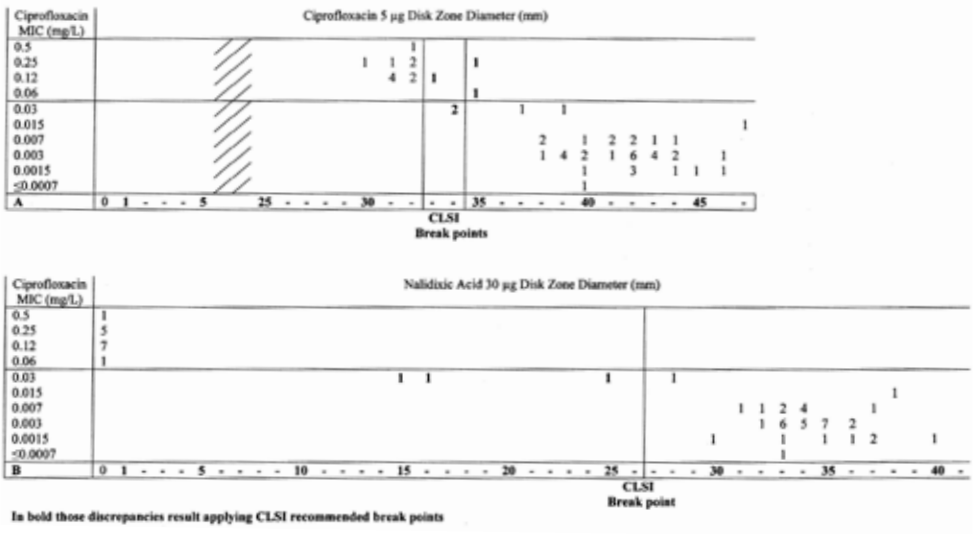
Fifty seven meningococcal strains isolated in Spain over 2000-2007 period were included. MICs for ciprofloxacin ranged from ≤ 0.0007 to 0.5 mg/L, including some representative of all dilutions of the antibiotic. Those gyrA mutations associated with reduced susceptibility to the drug were present in both CipI and CipR isolates and absent in susceptible ones. In all strains the single-zone-diameter (SZD) was determined using both, NA and Ciprofloxacin discs of 30 μg and 5 μg and the MIC to NA by agar dilution method was determined.

Results and discussion

All susceptible strains (MICs ‹ 0.06 mg/L) showed SZD using NA disc ranging from 28 to 40 mm, except three strains with MIC of 0.03 μg/ml displaying SZD lower than 26 mm (defined as cipR strains using the CLSI recommendations). For all meningococci with MICs › 0.03 mg/L no single-zone was observed at all (table 1). We suggest defining cipR strains associated with gyrA mutations as those without single-zone using the NA disc. In addition, the data show that the use of ciprofloxacin discs (that has been also suggested) it is not useful to properly define ciprofloxacin resistance because several discrepancies with the recommended zone-diameter were found (table 1). The use of MIC for NA to predict the level of susceptibility to ciprofloxacin presented good general agreement. All the isolates with an MIC for ciprofloxacin ‹ 0.03 mg/L showed MICs for nalidixic acid ranging between 0.25 to 1 mg/L. Four strains with MIC to ciprofloxacin of 0.03 mg/L were included in the study: 2 of them presented MICs for nalidixic acid of 1 and 2 mg/L but the other two showed an MIC for nalidixic acid of 16 mg/L. All the isolates with MICs › 0.06 mg/L presented MICs for nalidixic acid › 256 mg/L with only one exception with an MIC of 16 mg/L. In general terms, the MIC for nalidixic acid might predict the level of susceptibility to ciprofloxacin, but the 3 exceptions (2 with 0.03 mg/L and one with 0.25 mg/L) do not allow its use for that purpose. In conclusion, the most useful method to predict isolates with gyrA mutations that decrease the activity of fluoroquinolones is the use of nalidixic acid disks of 30 μg. The breakpoint recommendations should be revised according with these results for a proper definition.

Table 1:

Figure 1: A) Combined ciprofloxacin MICs and ciprofloxacin disk 5 μg zone diameters for the 57 strains tested. B) Combined ciprofloxacin MICs and nalidixic acid disk 30 μg zone diameters for the 57 strains tested.



In bold those discrepancies result applying CLSI recommended break points

P066

Prevalence, phenotypic and genetic characterisation of *Neisseria meningitidis* comprising reduced susceptibility or resistance to rifampicin and/or ciprofloxacin in Sweden

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In most countries, chemoprophylaxis is recommended to prevent secondary cases of invasive meningococcal disease. Commonly used prophylactic agents are rifampicin and ciprofloxacin. However, reports of reduced susceptibility or resistance to both rifampicin and ciprofloxacin in several countries have been published. Resistance to rifampicin is mainly due to alterations in the rpoB gene, and alterations in especially the gyrA gene, but also the parC and parE genes, may cause resistance to ciprofloxacin.

Objectives

The aims of the present study were to develop and apply genetic methods for identification of reduced susceptibility and resistance to rifampicin and ciprofloxacin in *Neisseria meningitidis*, to investigate the prevalence and characteristics of reduced susceptibility to both antibiotics in Sweden, and to discuss establishment of breakpoints on a genetic basis.

Methods

All Swedish *N. meningitidis* isolates (invasive, n=5; and carrier, n=18), displaying MIC › 0.19 mg/l of rifampicin (n=22, all with MICs ≤ 0.5 mg/l) or MIC › 0.03 mg/l of ciprofloxacin (n=1, MIC = 0.125 mg/l), received by the Swedish Reference Laboratory from 1995 to 2008 were included. All isolates were genetically characterised, using genosubtyping, fetA sequencing, penA allele identification and multi locus sequence typing (MLST). Furthermore, the rpoB gene was real-time PCR amplified and sequenced in all isolates with reduced susceptibility to rifampicin and the gyrA, parC and parE genes were real-time PCR amplified and sequenced in isolates with reduced susceptibility to ciprofloxacin. Six reference strains were included, one fully susceptible (MC58) to both antibiotics, three rifampicin resistant and two ciprofloxacin resistant strains.

Results

The previously identified rifampicin resistance associated amino acid alteration in RpoB, His552 → Tyr552, was found in all three rifampicin resistant reference strains, but in none of the clinical isolates. No other amino acid alterations in RpoB were found to be associated with elevated MIC values to rifampicin. In gyrA, one previously identified nucleotide substitution, causing the Thr91 → Ile91 amino acid alteration, was found in all ciprofloxacin resistant isolates (the two reference strains and one Swedish pharyngeal isolate). No other alterations in gyrA, parC or parE were associated with elevated MICs to ciprofloxacin.

The genetic diversity was high among the clinical isolates (n=23). In total, 19 different genosubtypes and three isolates non-subtypeable, 12 fetA variants and 12 penA alleles were identified.

Conclusion

Real-time PCR and sequencing are effective methods for detection of alterations in the rpoB, gyrA, parC and parE genes. The vast majority of the Swedish *N. meningitidis* isolates is still fully susceptible to rifampicin and ciprofloxacin. Among the Swedish isolates with reduced susceptibility to rifampicin, none comprised any of the previously described resistance associated amino acid alterations. One Swedish isolate was resistant to ciprofloxacin, and displayed a previously identified resistance mutation in gyrA, but no alterations in parC or parE. In order to establish breakpoints for rifampicin and ciprofloxacin on a genetic basis, substantially more isolates with diverse, elevated MIC values for both antibiotics are crucial, and a European initiative is in progress.

P067

Impact of penA polymorphisms on penicillin-susceptibility in *Neisseria lactamica* and *Neisseria meningitidis*

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Objectives
Commensal *Neisseria lactamica* and pathogenic *Neisseria meningitidis* colonize the human upper nasopharynx. Interspecies horizontal gene transfer has been described (Spratt et al., 1989). *N. lactamica* isolates are intermediate susceptible to penicillin in contrast to predominantly susceptible meningococci (Arreaza et al., 2002). In meningococci, reduced penicillin susceptibility is conferred by five specific mutations within the penicillin-binding protein 2 (PBP2) which is encoded by the penA gene. In this study, penA alleles of *N. lactamica* and *N. meningitidis* strains were compared and related to the corresponding minimal inhibitory concentrations (MIC) towards penicillin.
Methods
123 *N. lactamica* strains (Germany, 1999/2000) and 129 invasive meningococcal strains (Germany, 2006) were analyzed for their MIC towards penicillin G by Etest® (Inverness Medical, Germany). The 3' region of the penA gene encoding the transpeptidase domain of PBP2 was sequenced and penA alleles were determined by comparison to the penA database (http://neisseria.org/). Transformation experiments were done with the meningococcal penicillin-susceptible recipient strain alpha-14 harboring penA-2.
Results
The penA sequence analysis of 123 *N. lactamica* isolates identified 63 different alleles comprising 55 new variants. In contrast to meningococci, *N. lactamica* either harbored 3 or 5 of the non-synonymous mutations of PBP2 characteristic for intermediate-susceptible meningococci. Strains with 3 mutations exhibited a MIC between 0.064 and 0.38, whereas strains with 5 alterations exhibited a MIC ›= 0.25. In contrast, meningococci with 5 alterations displayed a MIC range between 0.064 and 0.25. The phylogenetic analysis of all meningococcal penA alleles submitted to the penA database together with the 55 newly identified alleles of *N. lactamica* suggested one clade comprising the penA alleles without alteration representing the susceptible meningococci. The penA alleles with 5 alterations clustered together irrespective of the host species. The MIC of meningococcal strain alpha-14 could be increased by transformation with *N. lactamica* DNA in a single step by acquisition of the respective penA allele. However, the MIC never reached levels observed in *N. lactamica*. PenA alleles with only 3 alterations could not be transformed.
Conclusion
Reduced penicillin-susceptibility in *N. lactamica* as in meningococci is associated to penA mutations. However, the MICs in *N. lactamica* are generally higher than in meningococci, suggesting that other factors contribute to a reduced penicillin-susceptibility in *N. lactamica*. Co-colonization with *N. lactamica* might elevate MICs of *N. meningitidis*, but levels observed in *N. lactamica* are unlikely.

P070

Inhibition of apoptosis by PorB from *N. meningitidis* is independent of NF-κB p65 in HeLa cells

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Objectives
Meningococcal porin has been shown to directly bind to TLR2/TLR1 and to induce a TLR2-dependent NF-

κB activation. Activation of the transcription factor NF-κB regulates the expression of many genes, including genes coding for proteins involved in modulation of both intrinsic and extrinsic apoptosis. The ability of Neisserial porins to prevent apoptosis induced via the intrinsic apoptosis pathway has been demonstrated in various cell models.
N. gonorrohoeae porin PIB-mediated prevention from apoptosis has been correlated with NF-κB-dependent expression of anti-apoptotic genes such as bfl-1, cox-2 and c-IAP-2.
N. meningitidis porin PorB efficiently inhibits intrinsic apoptosis by blocking mitochondrial cytochrome c release, caspase activation and DNA degradation. We have previously proposed that PorB direct interaction with mitochondria and consequent prevention from mitochondrial membrane depolarization is the underlying anti-apoptotic mechanism of PorB. However, it is not clear whether activation of the intracellular anti-apoptotic machinery is induced by Nme PorB in terms of NF-κB activation and anti-apoptotic genes expression.
Methods, results and conclusions
HeLa cells are very efficiently protected by Nme PorB against staurosporine (STS)-induced apoptosis, but they lack TLR2 expression and thus fail to respond to TLR2 ligands, including Nme PorB. Low levels of IL-8 secretion are induced in HeLa cells by Nme PorB or Pam3CSK4, while they respond to TNF-α secreting high IL-8 levels. Furthermore, NF-κB p65 nuclear translocation was not induced in HeLa cells by Nme PorB, as well as IκBα degradation. However, the analysis of a panel of intracellular proteins involved in modulation of apoptosis demonstrated a time-dependent expression of selected anti-apoptotic proteins, including members of the Bcl-2 family and of the inhibitors of apoptosis family, IAPs, in response to Nme PorB. Bcl-xL and c-IAP-2 levels are transiently increased after 1h incubation with Nme PorB, followed by return to baseline levels as early as after 4h. Bfl-1 expression is also increased and remains sustained after 24h, which is the optimal incubation time for Nme PorB to induce full protection from apoptosis. Our data suggest that the anti-apoptotic effect of Nme PorB is not dependent on a TLR2-mediated cell activation via the NF-κB canonical pathway and that the expression of selected anti-apoptotic genes might contribute to the anti-apoptotic effect of Nme PorB. Whether other NF-κB subunits are activated by Nme PorB and whether Nme PorB can interact with different cellular receptors other than TLRs in HeLa cells is under investigation.

P071

Neisseria meningitidis depletes endogenous S-nitrosothiol in activated macrophages

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Objectives
S-nitrosylation is the coupling of Nitric Oxide (NO) with a reactive cysteine thiol to form an S-nitrosothiol (SNO) and is regulatory of numerous mammalian cellular functions. Macrophages produce NO and thus endogenous SNO via the inducible NO synthase (iNOS), which is expressed in response to infection. *Neisseria meningitidis* (Nm), the causative agent of meningococcal disease, is exposed to NO during natural colonisation and disease. The Nm genome includes the gene norB (NMB1622), encoding a nitric oxide reductase (NorB) that converts NO to nitrous oxide (N_2O). NorB detoxifies NO and enhances survival of Nm within monocyte-derived macrophages (MDM). NorB has also been demonstrated to modulate the cytokine output of MDM and influence entry of these cells into apoptosis. This work tested whether infection of activated macrophages with Nm is accompanied by depletion of endogenous host-cell SNO; as a consequence of NO metabolism by the organism.
Methods
J774.2 murine macrophages were stimulated to produce SNO with exposure to lipopolysaccharide (LPS) and mouse interferon-γ (IFNγ) for 18 h. The cytoplasmic and nuclear SNO concentrations of Nm-infected, LPS/IFNγ-stimulated J774.2 murine macrophages were measured using tri-iodide dependent, ozone-based chemiluminescence.

Results
NorB increased the rate of SNO decomposition *in vitro*. Cytoplasmic SNO concentration in activated J774.2 cells (139.661 + 52.24 pmol/mg) was significantly reduced during 4 h infection with log phase, wild type Nm (57.34 + 23.71 pmol/mg). This effect was ablated in cells infected with either heat-killed wild type bacteria (125.474 + 34.61 pmol/mg) or NorB-deficient mutant bacteria (116.062 + 37.28 pmol/mg). A mutant strain overexpressing the partial denitrification pathway (nsrR-) also significantly reduced SNO concentration (77.24 + 19.16 pmol/mg). A similar pattern was observed for the concentration of NO-adducted moieties in MDM nuclear extracts.
Conclusion
NorB of Nm causes a significant reduction in the concentration of endogenously produced, host cell SNO. This work implies a novel role for nitric oxide detoxification in meningococcal pathogenesis, representing a hitherto unrecognized mechanism of meningococcal disease.

P072

Immunoproteomic analysis of the development of immunity in response to colonisation by *Neisseria meningitidis*

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Objectives
The immune response to existing serogroup B vaccines based on outer membrane vesicles (OMVs) is largely subtype specific. In contrast, meningococcal colonisation is known to stimulate the production of cross-reactive antibodies. In this study, modern proteomic methods were used to investigate the dynamics of immunity in response to meningococcal carriage with the aim of identifying potential vaccine targets. A previous longitudinal carriage study of university student volunteers provided us with a resource of matched sera and carriage strains obtained at four time-points over a 31 week period.
Methods
GeLC-MS/MS was used to study the protein composition of OM and OMV preparations. Screening for immunogenic proteins was then achieved by 2-D gel electrophoresis combined with immunoblotting. Panels of sera obtained prior to and coincident with colonisation were selected to investigate the development of an immune response to homologous and heterologous serogroup B strains.
Results
A total of 236 proteins were identified by GeLC-MS/MS, revealing the complex nature of the meningococcal cell surface not previously elucidated by 1-D SDS-PAGE and immunoblotting with antibodies specific for known components of the outer membrane. Several proteins that have previously been identified as potential vaccine candidates were not detected.
In the second phase of this study, homologous and heterologous OM preparations of serogroup B meningococcal carriage strains were separated on 2-D gels. Circa 80 protein spots were detected after silver staining, fifteen dominant protein spots were apparent, some present as spot trains extending across the isoelectric spectrum.
2-D gel electrophoresis was then combined with immunoblotting and the blots probed with students' antisera obtained prior to and post colonisation detection. The acquisition of meningococcal carriage accompanied raised bactericidal antibody activity and was associated with increased immunoreactivity visible on the immunoblots. This development of an immune response was reflected both in a greater number of spots and increased intensity of existing spots. The number, range and relative intensities of immunoreactions to meningococcal OM preparations varied between students and between strains. Twenty five discrete immunoreactive spots were identified and submitted to mass spectrometry analysis. The common spots detected on all blots regardless of strain or serum sample included PorA and PorB. Additional immunoreactive proteins included proteins predicted 'in silico' as of cytoplasmic or of unknown cellular location.

Conclusions
Both GeLC-MS/MS and 2-D gel electrophoresis are complementary techniques well suited to resolve the complex protein composition of OM and OMV preparations. In addition, 2-D SDS-PAGE can be combined with Western blotting to provide a powerful tool for the study of immunity. To our knowledge, this study is the first to use 2-D western blotting to analyse the development of natural immunity to meningococci. This powerful technique permitted the identification of antigens capable of inducing cross-reacting antibodies. Identification of such antigens with the ability to induce cross-reactive SBA to a range of meningococcal strains may be a major step in the production of an effective vaccine against infections caused by serogroup B meningococci.

P073

Mucosally delivered *Neisseria meningitidis* native outer membrane protein elicits potent immune responses against a foreign antigen in neonatal mice born to naive and immune mothers

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Objectives
Meningococcal disease affects individuals of all age groups, but the highest incidence is in children under 5, and especially among infants aged 3 to 12 months. One of the most critical stages in immune system development occurs in neonatal life. During the first days and months following birth, newborns are exposed to countless new antigens to which they need to mount appropriate immune responses. The development of effective vaccines for neonates and very young infants has been impaired by their weak, short-lived, and Th-2 biased responses and by maternal antibodies that interfere with vaccine take. We investigated the ability of native outer membrane vesicles (NOMV) of *N. meningitidis* B, nasally as a model in neonatal mice.
Methods
In this study we used NOMV of *N.meningitidis* B:4:P1.15 strain. BALB/c mice (8 to 10 weeks old) purchased from Adolfo Lutz Institute were bred to produce pups. Breeding cages were checked daily, and new births were recorded. Experimental groups contained two litters (average of six pups per litter). NOMV doses ranging from 10μg were administered intranasally (i.n.) in a 5-μl volume that was gradually introduced into the pup's nare with a micropipette. The first dose was given on day 7 after birth, and a second dose was given in an identical manner on day 22 after birth. For immunological comparison, 7-day-old mice are believed to approximate the stage of immune maturation of a newborn human. In experiments with neonates born to naive mothers, preimmunization sera were obtained from age-matched pups. Neonates from immune mothers were bled prior to antigen immunization and further bleedings were performed from the retro-orbital sinus every 2 weeks up to day 64 after birth (6 weeks after boost). Pups were nursed by a seronegative foster mother to avoid additional transfer of maternal antibodies through milk, which occurs in rodents but not in humans. Sera were stored at -20ßC until tested. An enzyme-linked immunosorbent assay was performed with polystyrene maxisorb microtiter plate. Electrophoresis and Immunoblotting were performed on a 13% polyacrylamide gel.
Results
We demonstrate for the first time that intranasal immunization of newborn mice with NOMV on days 7 and 22 after birth elicits high titers of bactericidal antibodies, previously found to protect against *N. meningitidis* and similar to those observed in adult mice. Mice immunized as neonates induced NOMV-specific mucosal and systemic immunoglobulin A (IgA), IgG, and gamma interferon secretion. A mixed Th1- and Th2-type response to NOMV was established after the boost and was maintained thereafter. NOMV induces specific antibodies and cell-mediated immunity in the presence of high levels of maternal antibodies.
Conclusion
Only a few studies in animal models have addressed the efficacy of neonatal immunization to protect

against bacterial pathogens. The higher incidence of disease among infants, from 3 months of age, is related to a reduction in maternal antibody titers that had been passively acquired during pregnancy. From 12 months on, children develop naturally acquired immunity, with increased protective antibody titers and, consequently, reduced incidence rates.

P074

The design of new adjuvants for mucosal immunity to *Neisseria meningitidis* B in nasally primed neonate mice for a strong adult immune response

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Objectives
The development of a safe and effective mucosal adjuvant is a crucial step in a mucosal vaccine. As *Neisseria meningitidis*, *Escherichia coli* (STEC) strains invade and infect the host via mucosal surfaces, and it would be important to induce an effective immune response at the mucosal sites. The mucosal immunization has been shown to be effective for the induction of antigen-specific immune responses in both the systemic and mucosal compartments.
The aim of this study was to determine the value of detoxified shiga toxins Stx1 and Stx2 of *Escherichia coli* as a mucosal adjuvant in neonate mice.
Methods
Outer membrane protein (OMP) of *N. meningitidis* B:4:P1.15 was administered nasally with Stx1 or Stx2 as adjuvant in a prime-booster immunization. There are various assays for evaluating the cytotoxic effects on cultured cells of toxins. We have used Neutral Red (NR) a quantitative colorimetric method based on the uptake of NR dye which accumulates in the lysosomes of uninjured cells. After detoxified Stx1 or Stx2 was not cytotoxic to Vero or HeLa cells. Neonate mice (5-day-old) were primed nasally with one dose of 5 μg of OMP with 1μg of detoxified Stx1 or Stx2 and boostered 4 weeks later with one dose of 10 μg OMP and 2 μg of Stx1 or Stx2 by intramuscular immunization. An enzyme-linked immunosorbent assay was performed with polystyrene maxisorb microtiter plate. Electrophoresis and Immunoblotting were performed on a 13% polyacrylamide gel.
Results
Stx1 or Stx2 given nasally in neonates and an intramuscular immunization in adult life triggered a stronger immune response, including significant Th1 polarization. Th1-associated IgG2a and IgG2b levels, to a lesser extent with Th2-associated IgG1 levels, and weakly with thiocyanate elution as a measure of avidity. IgG,IgM and IgA antibodies were produced and were analyzed by immunoblot and ELISA.
Conclusion
The priming effect of OMP and Stx1 or Stx2 used as adjuvants did not seem to be similar in intensity and quality of antibodies produced for OMP antigens. Compared with prime Balb/c mice, spleen cells from immunized adults mice produced more nitric oxide (3-6 fold), IFN-gamma (10-12 fold) and interleukin IL-6 (5-8 fold) respectively with Stx1 or Stx2 as adjuvants. These results suggest that STxs can be safely used as a priming stimulus in neonate animals in a prime-boost strategies to control meningococcal infection. However, priming with OMP Stx1 or Stx2 triggered a homologous booster in adult life, with a mixed Th1/Th2 response pattern. Here, for the first time, Stx1 or Stx2 was shown to be an effective and safe mucosal adjuvant for the development of a nasal meningococcal immune response. The altered toxicity of Stx1 or Stx2 toxin might be closely related to the potent adjuvant action on antibody responses to OMP antigens of *N.meningitidis* B.These data suggest the possibility of intranasal immunization with meningococcal antigens and detoxified STx1 and STX2 adjuvants as a new strategy in mucosal immunization.

P075

Molecular characterization of the interaction between porins of *Neisseria gonorrhoeae* and factor H

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Objective
Binding of factor H (fH), a key inhibitory protein of the alternative pathway of complement, to *Neisseria gonorrhoeae* constitutes an important mechanism of complement evasion. fH comprises 20 short consensus repeat (SCR) domains. We have shown previously that only human fH, but not fH from other primates (such as chimpanzee and rhesus macaque), binds to *N. gonorrhoeae*. Binding sites for *N. gonorrhoeae* porin (Por) B.1A in human fH reside in SCR 6 and SCR 20. Sialylated Por B.1B strains bind fH mainly through SCR 20. In this study, we used the differences in amino acid sequence between human and chimpanzee fH SCR 20 to define the amino acids in this domain that are important for binding to *N. gonorrhoeae*.
Methods
A chimeric protein containing human SCRs 18-20 (N-terminal) fused to the Fc fragment of mouse IgG2a (C-terminal) (SCR 18-20/Fc) binds to *N. gonorrhoeae*. Human and chimpanzee fH SCR 20 differ by 11 amino acids. We created human-to-chimpanzee point mutations (some mutant proteins contained mutations at two positions) in the background of SCR 18-20/Fc and examined binding of these mutant proteins to a *N. gonorrhoeae* Por B.1A and a sialylated Por B.1B strains by flow cytometry; the murine Fc region served as a tag to facilitate detection of bound proteins.
Results
Thus far, we have created mutant proteins that target seven of the 11 amino acids that define differences between human and chimpanzee SCR 20. Human-to-chimpanzee mutations at amino acid positions 1200 and 1203 (V1200I and R1203N) resulted in abrogation of SCR 18-20/Fc binding to Por B.1A as well as sialylated Por B.1B strains. None of the other mutant proteins that we have analyzed thus far (N1176K, T1184K and Q1187E) had a significant impact on the binding. A previously published crystal structure of SCR 19-20 has shown that the arginine (R) residue at position 1203 is part of the heparin binding region in this domain. Accordingly, we showed that binding of human SCR 18-20/Fc to gonococci could be blocked by heparin and another highly charged anion, sucrose octasulfate.
Conclusions
We have defined two amino acids in SCR 20 that are critical for binding to gonococci. Additional mutations to cover the remaining human-chimpanzee differences are being created. Gonorrhea is a disease that is restricted to humans. Understanding the basis for the human-specific binding of fH to gonococci may advance our understanding for the host-specificity of this infection. Such studies may aid in the development of molecules that block fH-gonococcal interactions and provide therapeutic option against this pathogen that has rapidly become resistant to multiple antibiotics.

P076

Prime-boost protocols of immunization with native outer membrane antigens of *Neisseria lactamica* in neonate mice

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Objectives
Immunological and epidemiological evidences suggest that the development of natural immunity

to meningococcal disease results from colonization of the nasopharynx by commensal Neisseria sp, particularly *N. lactamica*. The aim of the present study was to investigate the immunogenicity of antigens of Native outer Membrane Vesicles (NOMV) of *N. lactamica* with or withouth *Bordetella pertussis* (BP) as an adjuvant in neonates mice.

Methods

Strains of *N. lactamica* were isolated from the oropharynx of health carriers in our community. Groups of neonate BALB/c mice were immunized intranasally with a final volume of 5μl of NOMV containing 20μg antigens of *N. lactamica* or *N. lactamica* with $1x10^2$ of BP(whole cells). Four doses of antigen of *N. lactamica* with or without BP adjuvant were administered intranasally to these animals in the interval of 12 days after birth. On the 35th day, the animals were immunized intramuscularly with NOMV of *N. lactamica* with or without BP. Th1 and Th2 immune response to NOMV were analysed in adult mice. NOMV of *N. meningitidis*, *N. lactamica* and other species of the genus Neisseria were analysed by SDS-PAGE, Dot-ELISA and Immunoblot in order to analysed the cross reactive antigens. INF gamma (Th1 cytokine), and (Th2 cytokine), IL-6 were examined by ELISA using specific reagent for each cytokine according to manufacture's protocol.

Results

The results showed a predominance of IgG and IgM,with avidity, after i.n/i.m immunization. Immunoblot analysis of serum showed cross-reactive antigens between the species of the genus Neisseria to 50 to180kDa antigens. More importantly, the IgG subclasses of the antibodies generated by the NOMV prime-peptide boost regimen with or without B. pertussis as adjuvant were primarily of the IgG2a isotype, suggesting that the immunization strategy would be advantageous in eliciting a more beneficial Th1-type immune response.

Conclusion

Prime-boost protocols consisting of *N.lactamica* or *N.lactamica* and B.pertussis as adjuvant had a Th1 profile and were characterized by the production of large quantities of IFN -gamma and IL-6 cytokine of which is significant for the host's ability to fight off a bacterial infection. Therefore, prime-boost protocols that use *N. lactamica* or *N. lactamica* and *B. pertussis* are also very efficient at inducing humoral immune responses. Also, we observed an increase in antibody avidity using this schedule, indicating that affinity maturation has occurred. In conclusion, we found that specific priming, rather than specific boosting with NOMV of *N. lactamica*, gave a significant rise in immune response after intramuscular immunization against NOMV antigens of *N. lactamica*, with high avidity antibodies in an extended immunisation schedule.

P077

Monoclonal antibody on LPS selection antigens of *Neisseria meningitidis* B: PrimeBoost protocols using *Bordetella pertussis* cellular and acellular preparation as adjuvants in neonate mice

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Objectives

The natural habitat of *Neisseria meningitidis* is the human nasopharynx and the bacterium is transmitted by direct mouth-to-mouth contact or by the excretion and inhalation of mucous particles during close contact. *N. meningitidis* in current studies searching vaccinal antigens that are derived from (OMP). However, vaccines based on OMP are considered weak, being necessary the use of adjuvant by nasal immunization. The present study had the aim of evaluating the specificity and protective activity of the antibodies induced in neonate mice by the native outer membrane vesicles (NOMV) obtained from strain of *N. meningitidis* B:4:P1.15.

Methods

The strain of *N. meningitidis* used for immunization of mice were selected through colony-blot, using anti L3,7,9 monoclonal antibody, for the highest expression of the immunotype L3,7,9. First we selected the strains using colony blot, then we performed the extraction of the NOMV of the bacteria belonging to the selected strains B:4:P1:15 L3, 7, 9 followed by, the intranasal (IN)in neonate and IN/(IM) immunization in adult BALB/c

mice with the purified NOMV. As mucosal adjuvants we used heat-killed *B. pertussis* (strain 18323, ATCC (whole cells) or acellular supernatant of *B. pertussis* after 48h culture. Sera of immunized mice were evaluated by ELISA in order to compared the different immune response. We also verified the avidity index of them. A final volume of 5μl of 10μg of NOMV was gradually introduced with a micropipette in groups of animals that received 1, 2, 3 and 4 doses of antigens in 3, 7, 9 and 12 days after birth with *Bordetella pertussis* (whole cells)$1x10^2$ or 10μg of culture supernatant of this bacteria after 48h culture as adjuvants.
At the 35th days the animals were immunized with one dose intramuscullary (IM) with *Bordetella pertussis* (whole cells)$1x10^2$ or 10μg of culture supernatant of this bacteria after 48h culture as adjuvants.

Results

The present study analysed the reactivity of the antibodies (IgM, IgG and IgA) found in the serum of mice who were immunized IN or IN/ IM, as well as the presence in the serum of antibodies with bactericidal activity. Expressive titers of IgG and IgM were detected in the serum of mice after the immunization, with avidity indexes that from intermediary to high. All adjuvants were capable to increase the immune response against NOMV of *N. meningitidis* in a homologous prime/boost schedule used.

Conclusion

Data suggest that the NOMV with L3,7,9 selected is important in the induction of mucosal immunity to *N. meningitidis* B and the quality and magnitude of the immune responses generated by mucosal vaccines are influenced by the antigen as well as the adjuvants. In conclusion, the nasal delivery of NOMV with BP as adjuvants has considerable potential in the development of a mucosal vaccine against serogroup B meningococci. Thus, this antigen may be useful in prime/boost immunization protocols designed.

P079

Implications of human species-specific binding of complement factor H to *Neisseria meningitidis* for assaying functional activity of vaccine-induced serum antibodies using non-human complement

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Objectives

Serum bactericidal activity is the serologic hallmark of protection against meningococcal disease. However, the choice of complement source (rabbit or human) remains controversial. Complement factor H (fH), a negative regulator of the alternative pathway, binds to *Neisseria meningitidis* and increases resistance to serum bactericidal activity. Binding was reported to be specific for human fH. The objective of the present study was to determine the effect of human fH on bactericidal titers measured with rabbit complement. We also determined the effect of human fH on meningococcal bacteremia in infant rats since this animal model also has been used to measure the ability of antibodies to confer passive protection against meningococci *in vivo*.

Methods

Sera from 7 children, ages 4 to 5 years, who had been immunized with quadrivalent meningococcal polysaccharide vaccine and who were identified as high group C bactericidal responders using rabbit complement were re-assayed with rabbit complement using 0, 5 or 25 μg/ml of purified human fH in the reaction. We also determined the effect of treatment of infant rats with human fH given at the time of IP challenge with group B strain H44/76.

Results

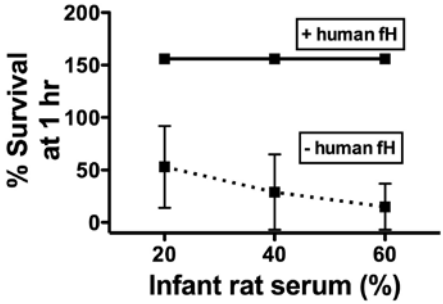
The addition of human serum (as a source of factor H), but not rat or rabbit serum, inhibited binding of biotinylated human fH to live encapsulated *N. meningitidis* bacteria as measured by flow cytometry. These results were consistent with human-specific fH binding to meningococci. When measured with rabbit complement, the geometric mean group C bactericidal titers of the human sera obtained immediately

before and one month after immunization were ‹1:16 and 1:475, respectively. The addition of as little as 5 μg/ml of human fH decreased the post-immunization GMT from 1:475 to 1:14 (P‹0.001). For 6 of the 7 sera, the decrease in titer was ›10-fold (titers of ‹1:16). Only one of the 7 sera had a positive bactericidal titer (≥1:4) when measured with human complement. In experiments with the infant rat model, the group B strain was partially killed by infant rat serum but not by non-immune human serum. The strain was resistant to killing by the infant rat serum when purified human fH was added to the reaction (figure 1). When rats were challenged IP with 10^4 CFU, co-administration of human fH at doses of 0, 2, 10 or 50 μg/rat resulted in a dose-dependent increase in CFU/ml of bacteria in blood 8 hrs later (P‹0.02). The CFU/ml of blood in rats given the 50 μg dose of fH was higher than that of control animals given 50 μg of C1 esterase inhibitor (1050 vs. 43, P‹0.005).

Conclusions

Collectively the data underscore the importance of binding of human fH for survival of *N. meningitidis in vitro* or *in vivo*. The data indicate that the results of serum bactericidal assays using non-human complement, or clearance of bacteremia in animal models by passively administered antibody, do not reflect the actual functional activity of antibodies against *N. meningitidis* during human exposure when complement activation is highly down-regulated by bound fH.

Figure 1: Survival of *N. meningitidis* in infant rat serum



P080

Homologous primeboost Immunization in neonate mice with purified class 3 protein of *Neisseria meningitidis* B using *Bordetella pertussis* as adjuvant

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Objectives

All meningococci express PorB, an outer membrane porin protein (OMP) porB genes have been assigned to either class 2 or class 3 homology allele groups. Antibodies against both proteins are bactericidal, making serotyping results useful, not only for epidemiologic surveillance of meningococcal disease, but also for identifying potential vaccine components. The aim of this study was verify the immunogenicity of the purified class 3 of 44/76 strain and "whole cells"(wc) of *Bordetella pertussis* (Bp) as adjuvant, in a neonate mouse model using a (IN/IM)prime-boost immunization.

Methods

BALB/c or outbred neonates mice between 3 to 12 days were immunized with 1 to 4 doses of the purified class 3 proteins with or without adjuvant, by the IN and in the 21ºday the animals received a IM dose with the class 3 protein with or without Bp. Immunized mice were evaluated by ELISA to detect the presence of specific IgG, IgM and IgA antibodies in serum and saliva in adult life. We also determined the

avidity index of IgG and IgM produced.The specificity of the antibodies were analysed by Immunoblot.

Results

The results demonstrated that after 2 doses intranasally and 1 dose intramuscullary we get a rapid stimulation of the cells in adults mice as much as in neonates meanwhile with the production of IgG and IgM antibodies of low avidity. The immunization schedule using 4 doses delivered during neonatal stage with the production of antibodies of high to intermediate affinity and bactericidal activity in adult life. A primary series of two doses i.n. was found to be important for increasing IgG level.The number of doses of the PorB antigen could be compared in Balb/c and outbred neonates in adult life.

Conclusion

These results suggest that purified class 3 protein of *N.meningitidis* B can use as a priming stimulus in neonate animals to control meningococcal infection in adult life. However, priming with class 3 protein and Bp as adjuvant,directed the ensuing immune response triggered by homologous booster, to a mixed Th1/Th2 pattern of response. Like many other vaccines designed for human use, it must be considered that it is important to study the class 3 purified protein in a prime-boost that in the future cam be administered to newborn children. With this in mind we evaluated class 3 protein immunogenicity using neonate mice. Since the nasopharynx is the only natural habitat of meningococci, intranasal immunization with meningococcal antigens has been suggested to be an effective way of inducing both mucosal and systemic immunity against purified class 3 protein. Natural infection with *Bordetella pertussis* has long been considered to induce strong and long-lasting immunity that wanes later than vaccine-induced immunity. Furthermore, infection with *B. pertussis* induces measurable antigen-specific Th1-type immune responses even in very young children (as young as 1 mo of age) These observations suggest that antigens of *B. pertussis* as adjuvant applicable by the nasal route, in order to mimic as closely as possible natural infection, may be attractive alternatives over the currently available vaccines to meningococal infection.

P081

Design and use of B cell tetramers to study ultra-low frequency PorA specific B cells

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Objectives

Consistently lower and less functional PorA-specific IgG titers are found after immunization with P1.7-2,4 Outer Membrane Vesicles (OMV) than after immunization with P1.5-1,2-2 OMV [1,2]. We previously reported that poor PorA specific serological outcome is not associated with a limited size of specific B cell subpopulations involved [3], urging the study of other qualitative cellular characteristics. Currently no methods are available to isolate and characterize specific B cell populations after immunization, other than through spot-based B cell enumeration (B cell elispot). Here we set out to develop fluorescent tools that can be used for flow-based-characterization, in situ localization and, potentially, the isolation of P1.7-2,4 and P1.5-1,2-2 specific B cells.

Methods

Biotin-conjugated cyclo- and linear synthetic peptides were designed to mimic hypervariable loops P1.4 and P1.2-2, respectively. The peptides were used to manufacture PorA-specific fluorescent B cell reagents, through tetramerization using streptavidin-PE or -APC. In combination with other fluorescent markers, tetramers were used for flowcytometric analysis of the following surrogate or genuine specific B cell populations: i) THP-1 monocytic cells, coated with either P1.4 or P1.2-2 specific MoAbs via FcR binding, ii) P1.4 and P1.2-2 loop-specific hybridoma's, and iii) ex-vivo murine splenocytes at various time points after P1.7-2,4 and P1.5-1,2-2 OMV vaccination. PE-tetramers were also used, after signal-amplification, for confocal microscopy of splenic sections or cytospins made from bone marrow suspensions, derived from P1.7-2,4 and P1.5-1,2-2 OMV vaccinated mice.

Results

Rational epitope-design led to synthetic P1.4- and P1.2-2-mimicking loops, that could be tetramerized to form specific fluorescent B cell detecting reagents. In a series of FACs experiments using various monoclonal and polyclonal B cell populations, optimized P1.4 and P1.2-2 constructs were selected showing highest specificity and sensitivity for their respective target populations. As few as 0.1% specific B cells could easily be detected among non-specific B cells. PorA specific B cell tetramers stained vaccine induced specific murine B cells, in cell suspensions and in situ. Studies to visualize and isolate human circulating PorA specific B cells are in progress.

Conclusion

In sharp contrast to the successful use of MHC-peptide tetramers to track particular T cell specificities within the repertoires of mice and humans, the analysis of low-frequency antigen specific B cells in immune responses such as those induced by vaccines have been hampered by the lack of specific reagents. In this study we succeeded to develop PorA specific B cell tetramers. These may prove to be important in probing human antigen specific B cells after meningococcal infection and vaccination, and can be used for rescuing epitope specific B cells via EBV transformation for human monoclonal antibody production.

References

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P082

Identification of blocking antibodies against serogroup B *N. meningitidis* in normal human serum

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Objective

The classical complement pathway is essential for antibody (Ab) mediated bactericidal and opsonophagocytic immunity against *N. meningitidis.* Colonization is an immunizing process and natural Ab may play a key role in protection against invasive disease. Sera from non-immunized individuals exhibits a large variation in complement-dependent bactericidal activity; in some instances, despite high levels of Ab binding, killing does not occur. Ab directed against certain bacterial targets may not activate complement, and in some instances, may even block killing by otherwise bactericidal Ab. The ability of the bacteria to elicit such blocking Ab may provide an important means to evade the host. As an example, reduction modifiable protein (Rmp) of *N. gonorrhoeae* has been shown to be a target for blocking Abs. Here, we provide evidence for the presence of blocking Abs against *N. meningitidis* in the serum of certain healthy individuals.

Methods

Sera obtained from 19 healthy individuals were screened for bactericidal activity against serogroup B strain H44/76. Sera that decreased CFU by ›70% were termed bactericidal while sera that killed ‹20% were termed non-bactericidal. Heat-inactivated non-bactericidal serum was added to bactericidal sera to screen for blocking activity. IgG purified from blocking sera were used to confirm that this class of Ab possessed blocking activity. In addition, we determined if blocking Ab was directed against the *N. meningitidis* Class IV outer membrane protein, a homologue of gonococcal Rmp, by comparing the level of blocking directed against wild type strain H44/76 to its isogenic Class IV protein deletion mutant.

Results

Out of the 19 individual sera 4 were non-bactericidal, 4 were bactericidal and the remaining 11 showed intermediate killing. Three of the 4 non-bactericidal sera blocked killing of serogroup B H44/76 by two of the bactericidal sera. The remaining two bactericidal sera were not blocked by any of the non-bactericidal sera. One of the non-bactericidal sera failed to block killing of any sera tested. Experiments using purified

IgG confirmed that blocking dose-dependent and mediated by IgG. Furthermore, blocking serum depleted of IgG did not have any residual blocking activity, affirming IgG as the blocking factor. Blocking was also seen with a serogroup A strain, suggesting that blocking Ab was not directed against capsule.
Deleting the class IV protein from *N. meningitidis* did not abrogate blocking, strongly suggesting that, distinct from *N. gonorrhoeae*, the blocking Ab was not directed against the Class IV protein. Western blot experiments showed novel IgG targets recognized by blocking sera that were not otherwise identified by non-blocking sera.
Conclusions
Presence of blocking Ab in serum may enhance susceptibility of individuals to invasive meningococcal infection. Furthermore, such blocking Abs could undermine the efficacy of meningococcal vaccines.

P083

Immunization with *Francisella tularensis* LPS with neisserial PorB induces a high level of protection against respiratory challenge and induces BALT in lungs of mice

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Francisella tularensis causes severe pneumonic disease that can lead to fatality if left untreated. Due to the potential use of *F. tularensis* as a biological weapon, research is being conducted to understand correlates of protection and to develop an effective vaccine against the pneumonic form of the disease. Efforts are also being made to select and study adjuvant molecules able to generate a better and long-lasting protective effect. PorB from *Neisseria meningitidis* is a well established TLR2 ligand and has been shown to be a promising vaccine adjuvant candidate due to its co-stimulatory capacity both *in vitro* and *in vivo*.
BALB/c mice were immunized three times with lipopolysaccharide isolated from *F. tularensis* live vaccine strain (LVS) (Ft-LPS) mixed to neisserial PorB and challenged intranasally with 10^6 CFU of LVS. Survival was observed and lungs were dissected from mice at different time points post-infection, and processed for histopathology and immunohistochemistry to determine tissue changes.
Seventy percent of the animals immunized with Ft-LPS+PorB were fully protected from respiratory challenge. Lungs from mice recovering from infection presented prominent lymphoid aggregates, which revealed to be areas of induced bronchial associated lymphoid tissue (iBALT) as shown by the presence of proliferating B cells, germinal centers and T cell infiltration in perivascular areas. Organization of iBALT, lymphocyte proliferation and lymphoid follicle size were found to be increased over time. Interestingly, vaccinated survivors sacrificed at later time points post-challenge also presented classic BALT, which is mostly present around blood vessels in the upper bronchi.
These data indicate that neisserial PorB might be an optimal adjuvant candidate for improving the protective effect of Ft-LPS, but there is a still need to test its efficacy against virulent type A and type B *F. tularensis* strains. The presence of BALT and iBALT in the lungs of mice induced by vaccination and subsequent challenge might play a role in recovery from respiratory tularemia and possibly long-term protection, although more research is needed to clarify their function.

P084

Mannose-binding lectin is present in human semen and modifies cellular pathogenesis of *Neisseria gonorrhoeae in vitro*

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Objectives
Mannose Binding Lectin (MBL) is a collectin present in blood and some mucosal tissues, which can influence microbial attachment and inflammatory responses of host cells during infection. The objective of this study is to determine the presence and concentration of MBL in human semen and its effects on gonococcal pathogenesis.
Methods
Semen concentration of MBL amongst 14 men attending a fertility clinic was measured by ELISA. Additionally, the effect of MBL upon gonococcal attachment and uptake by human epithelial cells and primary human monocyte derived macrophages (MDMs) *in vitro* and release of cytokines/chemokines by those cells was measured.
Results
MBL was present in all semen samples in the range 1.2-24.9 ng/ml. MBL bound to *N. gonorrhoeae* but there was evidence of strain to strain variation in the nature of the bacterial receptor; binding to strain FA1090 was serum-inhibitable whereas binding to strain MS11 was carbohydrate-inhibitable. Pretreatment of bacteria with MBL modulated adhesion of *N. gonorrhoeae* strain FA1090 but not strain MS11 to Chang epithelial cells and MDMs. This effect was dose dependent with an increase in binding at lower concentrations of MBL (0.4-1.6 μg/ml) and a decrease in binding at 5 μg/ml MBL. In addition MBL (5 μg/ml) reduced internalization of strain FA1090 and intracellular killing of both FA1090 and MS11 by MDMs. MBL also reduced pro-inflammatory cytokine production by MDMs in response to strain FA1090.
Conclusions
MBL is present in human semen and modifies cellular responses to *N. gonorrhoeae* in a concentration dependent manner.

P085

Investigation on the effect of immune selection on resistance to bactericidal antibodies to Group B Meningococci *in vitro*

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Objectives
A study was performed to assess the possible induction of resistance by immune selective pressure in meningococcal group B strains (MenB) to bactericidal antibodies from humans immunized by a 5-component recombinant MenB vaccine (5CVMB) with or without an outer membrane vesicle component. Surviving bacterial colonies that were treated with serum dilutions resulting in 90% killing of bacteria were pooled and used to prepare a new culture for re-assay with the same serum sample from which the bacteria were initially tested. Immune sera treatment of bacteria was repeated in this manner through four rounds of selection, which would represent a selection event frequency of one in 104 assuming one log of selection each round. The criterion used to assess induction of resistance to

vaccine-induced bactericidal antibodies was a four-fold or more reduction in titer as compared to control bacteria passaged in the same concentration of non-immunized human serum and complement lacking bactericidal antibodies. Possible changes in antigen expression were examined by Western blots.

Methods

Sera were obtained from adult human subjects one month following immunization with three, four, or five doses. Control sera were obtained before the first immunization and were tested beforehand to ensure that they lacked naturally acquired bactericidal antibodies against the strains tested, H44/76 and 2996. Frozen stock cultures of MenB bacteria were grown for 15 to 18 hours on chocolate agar at 37ºC in 5% CO_2. 10-20 isolated colonies were re-suspended in Mueller-Hinton broth (Becton Dickinson) with 0.25% glucose and cultured for 1.5 to 2 hours to mid-log. Sera were serially diluted from a starting dilution of 1:2 and mixed with 25% exogenous human serum complement and bacteria. The reaction mixture was incubated for 60 minutes at 37ºC in 5% CO_2 in 96 well plates, spread on to chocolate agar using the spot and tilt method, then counted the next day. Surviving bacterial colonies were pooled and used to prepare a new culture for re-assayed.

Results

In no case was a four-fold or greater reduction in titer observed as a result of immune selection compared to titers obtained with the strains passaged in nonimmune serum. Some variation in titers between days was seen but was within the fourfold range. Comparison of titers obtained against bacteria passaged in antibody-negative serum and human complement, with titers against bacteria passaged 4 times in bactericidal serum and human complement, showed an average difference in titer of approximately 2.5-fold. None of the pairs of results reached the pre-set criterion of a fourfold titer reduction. Immunostaining indicated that antigen levels for all of the antigens examined in the antibody-selected cultures were comparable to wild type bacteria.

Conclusion

The criterion chosen as an indicator of immune-induced resistance to bactericidal activity of a four-fold reduction of titer relative to the control strain was not met for either of the two MenB strains tested. Additionally, selection with antibody and complement resulted neither in loss of expression of the antigens nor in loss of recognition by bactericidal antibodies.

P086

Incorporation of LPS adjuvants into liposomes with Neisseria opacity protein does not improve their adjuvant effect

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Objectives

LpxL1 and PagL lipopolysaccharides (LPS) are penta-acylated derivatives derived from wildtype meningococcal LPS by genetic engineering of the lipid A biosynthetic pathway. Inactivation of lpxL1 leads to the loss of the secondary acyl chain at the 2' position, while expression of the PagL deacylase from *Bordetella bronchiseptica* in *Neisseria meningitidis* leads to removal of the primary acyl chain at the 3-position. These modifications reduce the endotoxic activity over wildtype LPS while retaining adjuvant effect in mice. Since liposomes are a useful delivery system for maintaining conformational epitopes of outer membrane proteins (OMPs) used in meningococcal vaccine development, the purpose of this study was to investigate if co-incorporation of mutant LPS and OMPs in liposomes is required for an optimal adjuvant effect, or alternatively whether free LPS is equally effective.

Methods

The meningococcal Opacity protein (Opa) was used as model antigen. Recombinant OpaJ protein was purified from inclusion bodies in *E.coli* and refolded *in vitro*. LPS extractions were performed from *Neisseria meningitidis* mutant strains using the phenol methodology. Adjuvant and antigen were co-incorporated in liposome

particles using the dilution method. Liposomes were characterized using FACS and SDS-PAGE to confirm that protein and LPS were incorporated in the same particles. IL-6 induction in the murine macrophage cell line J774A.1 was used to assay the effect of LPS liposome co-incorporation on the endotoxic activity. Balb/c-Rivm mice were immunized with two doses of 5 μg of OpaJ incorporated in liposomes containing 0.1 μg or 1 μg of either free LPS (FLPS) or LPS incorporated in liposomes (LLPS), including as control groups OpaJ liposomes without adjuvant or with AlPO4. The antibody response was tested by determining total IgG and IgG subclasses against whole cells in ELISA, and bactericidal assays using Opa-expressing *Neisseria meningitidis*.

Results

A reduced *in vitro* LPS endotoxic activity was found upon LPS incorporation into liposomes as compared to the same amount of free LPS. The immunization experiments showed a clear adjuvant effect for both concentrations of LpxL1 and PagL LPS, as evidenced by higher total IgG titers against Opa+ meningococci, altered IgG2a,b/IgG1 ratios and higher bactericidal titers. However, co-incorporation of LPS did not lead to an improved adjuvant effect; in most cases the reverse was seen i.e. greater effect of free LPS. By contrast, the antibody response against LPS itself was higher upon liposome incorporation; this response was much higher with PagL LPS than with LpxL1 LPS.

Conclusions

This study demonstrates that co-incorporation of both mutant LPS and Opa protein into the same liposome vaccine formulation does not improve the adjuvant effect as compared to free LPS addition.

P087

The macrophage scavenger receptor A plays an important role in the clearance of *Neisseria meningitidis in vivo*

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Macrophages are a first line of defence against invading pathogens and express the class A scavenger receptor (SR-A), one of several pattern-recognition molecules capable of recognising microbial ligands. Previously, the role of SR-A in the phagocytosis of *Neisseria meningitidis* was investigated and a comparison of bone marrow macrophages from wild-type and SR-A-/- mice showed that nonopsonic phagocytosis of *N. meningitidis* was mediated almost exclusively via SR-A. Initial investigations into the Neisseria ligand for SR-A showed that the presence or absence of bacterial pili and capsular polysaccharide did not alter microbial binding to the receptor. Using inner and outer core lipopolysaccharide (LPS) mutants and an LPS-deficient mutant (H44/76lpxA) we found no evidence to implicate LPS as a ligand for SR-A. In this study we investigated the possibility that the ligand was a protein using a novel ELISA-based assay. We identified three potential SR-A ligands on the *N. meningitidis* surface. The candidate proteins were evaluated further using *in vitro* endocytic uptake assays and blocking assays where they were evaluated for their ability to inhibit the uptake of a known ligand by SR-A. Our results show that all three Neisseria proteins are ligands for SR-A and we therefore present the identification of the first naturally occurring unmodified protein ligands for SR-A. Furthermore, *in vivo* studies in wild-type and SR-A knock-out animals indicate reduced clearance of *N. meningitidis* MC58 and an increased cytokine production in animals lacking SR-A. We also constructed a mutant *N. meningitidis* strain lacking two of the ligands, and for this strain the SR-A-mediated effects were abrogated *in vivo*. This work represents the first step in delineating the role of macrophages in the immune response to *N. meningitidis*.

P088

Experimental colonisation of adult volunteers with *Neisseria lactamica* induces mucosal IgA and IgG responses

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Objectives
Previous studies have suggested that nasopharyngeal colonisation with the commensal *Neisseria lactamica* may prevent colonization with the pathogen *N. meningitidis*. We have performed a human challenge model to characterize the immune responses following *N. lactamica* carriage. Subjects were given an intranasal challenge with live *N. lactamica* and then swabbed periodically to detect carriage. Serum IgG and mucosal IgA immune responses to *N. lactamica* have been determined.
Methods
41 volunteers were given an intranasal challenge with *N. lactamica*. Saliva and serum samples were taken prior to inoculation and at 2, 4, 7 and 12 weeks post inoculation. Serum samples were tested with an *N. lactamica* outer membrane vesicle (OMV)-specific IgG ELISA. The ELISA plates were coated with *N. lactamcia* OMVs overnight. The plates were washed and blocked for 1 hour. The block was removed and sample dilutions prepared in duplicate with a 3-fold dilution series and then incubated for 90mins. The plates were washed and then incubated with anti-human IgG-AP conjugate for 90 mins, washed, and then incubated with AP-Yellow substrate for 55 mins. The plates were read using Softmax Pro Software and titres calculated against the reference serum.
Saliva samples were tested in an *N. lactamica* OMV-specific IgA ELISA and a Total IgA ELISA. The *N. lactamica*-specifc ELISA plates were coated with *N. lactamica* OMVs and the total IgA ELISA plates were coated with anti-human IgA, overnight. ELISAs were then performed as above except for the use of anti-human IgA-AP conjugate. Mucosal IgA results were expressed as *N. lactamica* OMV-specific IgA titre units/μg total IgA.
Results
Colonisation with *N. lactamica* was detected in 26/41 volunteers. Colonised subjects showed increases in geometric mean serum IgG titres from the pre-inoculation sample, to 4 weeks post inoculation, then a slight drop to 12 weeks post inoculation. The non-colonised and control subjects had no significant change in their IgG titres. There was a statistically significant difference between the titres of the colonised and both the non-colonised and control subjects (p=0.0027 and p=0.0028, respectively). Preliminary mucosal IgA results show that the colonised subjects exhibit significant increases in *N. lactamica*-specific: total IgA over the 12 weeks post inoculation when compared to the non-colonised subjects (p=0.0061) or the controls (p=0.0039). The control subjects showed no change in titre and the non-colonised subjects showed a decrease in *N. lactamica*-specific: total IgA, although this was not statistically significant.
Conclusions
Intranasal inoculation of adult volunteers with live *N. lactamica* followed by colonisation causes both local and systemic immune responses shown by increases in both serum IgG and mucosal IgA titres. Further work will show if this is sufficient to prevent re-colonisation of volunteers with *N. lactamica*.

P089

The effect of the meningococcal nitrite reductase, AniA, on the interactions of *Neisseria meningitidis* with human macrophages

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Objectives

Neisseria meningitidis (Nm), the causative agent of human meningococcal disease, is exposed to nitrosative stress in its biological niche. Invasion of the nasopharyngeal mucosa, antecedent to fulminant meningococcal disease, brings the organism into contact with cells of the innate immune system, most notably tissue macrophages. Monocyte-derived macrophages (MDM) produce nitric oxide (NO) in response to infection via the inducible NO synthase (iNOS). The genome of Nm includes the genes aniA and norB, which encode a nitrite reductase and nitric oxide reductase, respectively. Together these constitute a partial denitrification pathway, which the meningococcus uses to supplement its growth under microaerobic conditions. Previous work has shown that expression of NorB enhances survival of *N. meningitidis* within nasopharyngeal tissue explants and MDM, influences the entry of MDM into apoptosis and modulates MDM secretion of cytokines. These data suggest that the concentration of NO during a meningococcal infection influences host-cell physiological processes. As a source of NO, generated through the reduction of nitrite (NO2-), we investigated the effect of AniA expression on a number of MDM:Nm interactions. Methods: Fluorescence microscopy was used to compare the binding and internalization interactions of wild type and aniA- meningococci with MDM. Gentamycin protection assays were used to investigate killing of wild type and mutant bacteria by MDM following phagocytosis. We also investigated the effect of Nm infection on the expression of cytokines by MDM, using cytokine protein arrays. Selected cytokines, whose expression was strongly modified by infection with Nm, were then studied further by ELISA to determine differences between wild type and aniA- infection. Results: In capsulate Nm, expression of AniA had no significant effect on the binding, internalization or killing of the bacterium by human MDM. Expression of AniA also had no effect on the secretion of the pro-inflammatory cytokines, TNF-alpha, IL-1beta and IL-6; the immunomodulatory cytokine, IL-10; or the chemokine, MCP-1. Conclusion: The expression of the meningococcal nitrite reductase, AniA, has no effect on the interaction of the meningococcus with human MDM.

P090

Neisseria meningitidis and *Neisseria lactamica* infection induce different host response pathways in human bronchial epithelial cells

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Objectives

Neisseria meningitidis is a bacterium commonly found at the human nasopharyngeal mucosal surface. However in some people it can invade to cause potentially life-threatening meningitis and septicaemia. In contrast, closely related bacteria such as the commensal *Neisseria lactamica*, colonise the nasopharynx but do not cause invasive disease. Characterising the host response to these two different bacteria via genome wide microarray expression studies may uncover how pathogenic and commensal bacteria modulate the host in different ways. In this study we have determined the gene expression signatures of a human bronchial epithelial cell line in response to *N. meningitidis* and *N. lactamica.*

Methods
The extent of adherence and invasion of *N. meningitidis* MC58 and *N. lactamica* Y92-1009 to a human bronchial epithelial cell line (16HBE14) was established. The transcriptome of 16HBE14 cells in response to both bacterial species was determined by whole genome wide microarray technology (Illumina HumanRef-8 V2 BeadChip). Quantitative RT-PCR was used to verify expression of genes of interest. Pathway analysis was used to identify host gene networks specifically associated with either *N. meningitidis* or *N. lactamica*.
Results
N. lactamica adhered to 16HBE14 cells to a greater extent than *N. meningitidis* although there was no significant difference between the two bacteria in the numbers that invaded. Host processes commonly associated with both *N. meningitidis* and *N. lactamica* infection included phosphate metabolism and glycolysis. An early suppression of the host immune response was specifically associated with live *N. meningitidis*. In contrast, live *N. lactamica* was specifically associated with changes in host gene expression of cytokine and chemokine mediated signalling pathways and cell proliferation and differentiation.
Conclusion
Infection of human bronchial epithelial cells with *N. meningitidis* and *N. lactamica* was associated with a down-regulation of host defence genes and an increase in the expression of host proinflammatory genes respectively. Active bacterial processes may be involved in the differential response of human bronchial epithelial cells to either commensal or pathogenic bacteria.

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Response of human mucosal dendritic cells to bacterial colonisers of the upper respiratory tract

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Objectives
The nasopharynx is constantly exposed to a wide variety of harmless food antigens and allergens. This mucosal surface is also the gateway to bacteria such as *Neisseria meningitidis* (Nm) that colonise the upper respiratory tract without causing harm, but occasionally can invade to cause meningitis and septicaemia. We have previously shown that mucosal T cell immunity to Nm is acquired naturally with age. Dendritic cells (DC) orchestrate the balance between tolerance and active immunity to such pathogens at this important inductive site. We have therefore investigated how human tonsil DC regulate immunity to Nm, comparing them to blood monocyte-derived DC (MoDC).
Methods
The ability of purified tonsil DC to take up meningococcal antigen was determined by monitoring internalisation of FITC-labelled Nm. Tonsil DC and MoDC were incubated with Nm antigens and expression of DC surface activation markers was analysed by flow cytometry. Levels of proinflammatory and immunomodulatory cytokines in the cell cultures were measured by Luminex assay. The ability of DC to stimulate antigenic-specific T cell proliferation in co-cultures of DC and purified autologous T cells was investigated by 3H-thymidine incorporation.
Results
Tonsil DC are a mixed population containing both plasmacytoid and myeloid-type DC. Both populations internalised FITC-labelled Nm. However, although there was a small increase in CD83 expression, there was no detectable upregulation of mucosal DC HLA-DR, CD40, CD80 or CD86 after overnight incubation with Nm. In contrast, stimulation of MoDC with Nm showed upregulation of HLA-DR, CD40, CD80, CD83 and CD86. Whereas this MoDC activation was associated with production of a variety of cytokines (IL-12p40, IL-12p70, IL-6, TNF-α, IL-10) and the chemokine MCP-1, Nm induced only low levels of tonsil DC cytokines (IL-6, TNF-α and

IL-10). MoDC showed a greater ability than tonsil DC to induce antigen-specific T cell proliferative responses.
Conclusion
Tonsil DC are able to internalise Nm antigen but in comparison to MoDC are only moderately activated by this antigenic encounter and are less effective inducers of antigen-specific T cell proliferation. This relative indifference may promote colonisation by Nm but until an effective T cell response is generated, may also leave the gate open to invasive disease.

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Neisseria gonorrhoeae elicits IL-17 and Th17-dependent responses in a murine model of genital tract infection

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Objectives
Host immune responses to gonorrhea are poorly understood. Consistent with a lack of protective immunity against repeated infection, adaptive immune responses are modest at best and innate rather than specific immune defense mechanisms may be responsible for the elimination of infection. We propose that the newly described "Th17 axis of immunity", which links the adaptive and innate arms of the immune system, has a critical role in the response to and defense against *Neisseria gonorrhoeae*. We have tested the ability of N. gonorrhoeae to induce IL-17 and other aspects of Th17-dependent immunity using murine and human cells *in vitro*, and a murine model of vaginal gonococcal infection.
Methods
Mouse spleen mononuclear cells, mouse bone marrow-derived dendritic cells, or differentiated human monocytic THP-1 cells were cultured with either whole gonococci or gonococcal outer membrane vesicles. Supernatants were assayed for production of Th17-associated cytokines by ELISA. Mouse genital tract tissue from either wild type or IL-17-receptor knockout (IL-17R-KO) mice (Amgen) was cultured *ex vivo* with *N. gonorrhoeae* and supernatants were assayed for CXC chemokines and IL-6. The role of IL-17 in a mouse model of vaginal infection was tested by treating mice with a blocking antibody to IL-17 (Amgen) or control rat IgG, and infected vaginally with *N. gonorrhoeae*. Duration and extent of infection were monitored by swabbing and plating, and neutrophil influx was counted by microscopic examination of vaginal smears.
Results
Mouse spleen cells cultured with gonococcal antigens produced IL-17, and other inflammatory cytokines associated with a Th17 response, including IL-6, IL-22, TNF-alpha, and IL-1beta. Mouse bone marrow-derived dendritic cells cultured with gonococcal antigens produced IL-6 and IL-23, and human THP-1 cells produced IL-6, TNF-alpha, IL-1beta, IL-23 and IL-8. Vaginal tissues cultured *ex vivo* produced IL-6, IL-17, and neutrophil-attracting chemokines KC, LIX, and MIP-2 in response to gonococci. Production of CXC chemokines and IL-6 was lowered in IL-17R-KO mice stimulated with N. gonorrhoeae compared to wild type mice. In mice challenged with gonococci and treated with IL-17-blocking antibody, neutrophil influx was significantly diminished compared to the control mice, and clearance of the infection was delayed by 2-3 days.
Conclusions
Gonococci stimulated in both mouse and human cells a cytokine profile consistent with the induction of a Th17 response. Normal genital tract tissue cultured with gonococci released CXC chemokines which are secreted by IL-17R bearing cells in response to IL-17, whereas tissue from IL-17R deficient mice produced lower levels of CXC chemokines and IL-6 in response to gonococci, suggesting that IL-17 is critical for neutrophil recruitment in response to *N. gonorrhoeae*. Blockade of IL-17 *in vivo* diminished neutrophil influx and hindered clearance of vaginal gonococcal colonization, indicating the relevance of IL-17-dependent responses to *N. gonorrhoeae* infection. These results provide evidence for the development of a Th17 response to *N. gonorrhoeae* infection that may be crucial for host defense.

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Evaluation of antibody responses in pre- and post-infection sera from cases of childhood meningococcal disease

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Objectives

To evaluate specificity and levels of antibody response in sera derived from childhood cases of meningococcal disease by comparing these responses to those of children from similar ages groups involved in the porA strain-specific meningococcal vaccine (MeNZBTM) clinical trials (vaccinees).

Methods

Serum samples from childhood cases presenting with epidemic strain-specific meningococcal disease were tested by serum bactericidal assay (SBA), ELISA and immunoblotting. Samples comprised paired acute and convalescent sera from 2 unvaccinated individuals and paired sera from 16 children who had received between one and three doses of vaccine prior to infection. Serum bactericidal antibody (SBAb) responses were measured by the standard assay used in the New Zealand vaccine trials [1]. Sera were evaluated for the presence of inhibitory antibiotics pre-SBA testing, and where the inhibitory effect was unable to be neutralised, an SBA was not performed. Anti-outer membrane vesicle (OMV) IgG was determined by ELISA assay as described [2] and immunoblotting performed according to the methods as described [3].

Results

For this investigation, a total of 36 sera from cases of disease were compared with the corresponding age group's pre- and post-vaccination responses, as analysed during the MeNZBTM clinical trials. None of the vaccine trial-derived serum samples demonstrated the presence of inhibitory antibiotics whereas 12 of 18 acute samples were unable to be SBA tested.

Comparison of pre-vaccination sera from vaccinees and acute phase sera from disease cases indicated few had elevated levels of SBAb which was consistent with IgG ELISA levels of ‹80 Units. Varying levels of SBAb were found in convalescent serum samples, but the range was consistent with that obtained from vaccinees receiving three doses of vaccine as part of the trials. It is likely that some of the titre differences are attributable to the varied number of vaccine doses the children had received before becoming infected. Immunoblotting demonstrated that in those cases where acute phase SBAb could not be determined because of antibiotics, the blots showed response patterns against the PorA protein consistent with their ELISA results and vaccine history.

Conclusion

Early treatment with antibiotics for patients with meningococcal disease is important for case management, however, their inhibitory effect in acute phase sera has implications for assessing pre-existing antibody levels. By using a multi-test approach including SBA, ELISA and immunoblotting, a clearer picture is gained of pre- and post-infection functional antibody responses.

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P094

Secreted complement inhibitors by *Neisseria meningitidis*

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Neisseria meningitidis can evade complement-mediated lysis. This resistance is attributed to its polyschaccharide capsule, LPS, and the ability to bind human complement regulators (factor H, C4BP) to the bacterial surface. In contrast to a growing armamentarium of secreted complement inhibitory proteins in *Staphylococcus aureus* and Group A Streptococcus, little is known about such inhibitors in Gram-negative bacteria. To get more insight into meningococcal complement evasion, we investigated small secreted proteins in Neisseria and their interaction with the human complement system.
Using the sequenced genomes Z2491 (serotype A), MC58 (serotype B) and FAM18 (serotype C) we searched for open reading frames (ORFs) with a putative role in complement modulation. Selection criteria were based upon hallmarks of many Gram-positive complement modulators: the open reading frame is (1) on a mobile element, (2) secreted and (3) smaller than 60kD. The proteins were cloned and expressed in *Escherichia coli* as His-tagged proteins and purified by nickel affinity chromatography. Recombinant proteins were screened for immunomodulatory functions in several *in vitro* assays.
Genome analysis resulted in a list of 18 open reading frames (ORFs). Immunoprecipitation of NB3 (12.4 kD) with human serum revealed a specific interaction with a 30kD serum protein that remains to be identified. Functional complement assays showed that NMB0976 (13kD) is a very potent complement inhibitor. NMB0976 blocked all complement pathways specifically at the level of C5b-9, while C3b deposition was not affected. The fact that NMB0976 also blocked C5b-9 formation on zymosan, strongly suggests that this protein blocks formation of the bacteriolytic membrane attack complex. Here we present NMB0976 as the first secreted complement modulating molecule in *Neisseria meningiditis.* NMB0976 specifically prevents C5b-9 deposition, implicating a crucial role in the Neisserial evasion of complement-mediated lysis.

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Early life immunity elicited by meningococcal protein antigens examined in a murine animal model

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Objectives
At present, young children are the intended recipient of the vast majority of the anti-meningococcal vaccines under development. However, the immunogenicity of meningococcal antigens is usually studied in adult mice. In the present work, we employed a different approach, and wanted to explore the early life immunity of individual protein antigens by using an animal model that resembles the immature immune state of human infants.
Methods
The antigens NMB2134, NMB0848, NMB0109, NMB0938, NMB0928, NMA0939 and NMB0606 were obtained as fusion proteins. The recombinant proteins were extracted from the insoluble fraction with chaotropic agents and purified by Ion Metal Affinity Chromatography. Neonatal OF1 mice (7 days-old) were immunized by intraperitoneal route with a first dose of antigen, by using aluminum salts as adjuvants. In two-dose experiments, the second dose was administered 7 days later. In three-dose schedules, the second dose was given on day 10, and the third one was administered on day 14 after birth (10 or 15 μg/

mice). Immune sera, collected on day 21 after birth, were tested by ELISA. The immune serum reactivity with whole bacteria from several meningococcal strains was determined by Whole Cell ELISA (WCE).

Results

All the mentioned antigens were immunogenic in mice sensitized as early as 7 days after birth, when two doses of protein (10 μg each) absorbed onto aluminium hydroxide were administered. The IgG titers against the immunizing antigen did not improve significantly when mice received three doses of it. Sera elicited against NMB0928 reacted with whole meningococci, as ascertained by WCE. According to this assay, the antibodies generated against NMB0928 were cross-reactive. Among the rest of the evaluated antigens, NMA0939 and NMB0606 were selected for further evaluation, due to the encouraging results obtained by WCE. The immunogenicity of NMB0606 was confirmed by using aluminium hydroxide and aluminium phosphate as adjuvants, all neonatal mice seroconverted, and no statistically significant difference was found between both groups. Sera obtained after murine early life immunization with this protein reacted with antigens present in isolates belonging to different serogroups. When the immunogenicity of NMA0939 was re-examined employing doses of 10 μg and 15μg/mice, both amounts of antigen elicited similar antibody titers in neonatal mice. However, when the reactivity of the antisera with whole meningococci was assessed, only those sera produced with the highest dose of NMA0939 were able to recognize antigens present in four phenotypically different meningococcal strains.

Conclusions

The early life immunity of meningococcal protein antigens can be conveniently explored in a murine animal model. Among the antigens studied in this work, NMB0928, NMB0606 and NMA0939 elicited the broadest cross-reactive response at this age.

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Binding of Opc to vitronectin contributes to increased serum resistance of *Neisseria meningitidis* isolates

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Vitronectin is a multifunctional serum glycoprotein. One of its regulatory functions involves its participation in the immune system by binding to terminal complement components and modulating the function of membrane attack complex (MAC). This property of vitronectin provides protection to bystander host cells from complement mediated lysis. *Neisseria meningitidis* (Nm) Opc protein interacts with a conformational form of vitronectin that binds to MAC. Binding of Opc-expressing Nm to vitronectin should therefore render bacteria more serum resistant than those bacteria not expressing the Opc protein.

Objectives

To assess relative serum sensitivity of Nm either expressing or lacking the expression of Opc and to analyse the role of vitronectin in serum resistance of Nm.

Key methods

In vitro serum bactericidal assays (SBA) used normal human serum at a range of concentrations in the presence or absence of additional purified vitronectin. Well-defined isolates of distinct serogroups with or without capsule and/or Opc expression and strains of distinct lineages were assessed in SBA. In addition, antibodies that blocked the interactions of Opc with vitronectin were used to confirm the roles of vitronectin and Opc in serum resistance.

Results

The results indicate that Opc-expressing Nm are more serum resistant than Opc- bacteria. In addition, vitronectin concentration-dependent increase in serum resistance of Opc-expressing Nm can be demonstrated on addition of vitronectin to normal human serum. Bactericidal effect of serum on all Opc-expressing phenotypes was dramatically reduced by vitronectin independently of Nm capsule expression. Phenotypes not expressing Opc remained as serum sensitive in the presence of added vitronectin as in its absence.

Conclusions
Nm isolates of distinct serogroups can interact with serum vitronectin via the Opc protein, a property that enhances their serum resistance. *In vitro*, added activated form of vitronectin enhances this serum resistance but only if Opc is expressed. Since activated form of vitronectin increases during sepsis, binding to such a form of vitronectin may add to the ability of Nm to overcome complement mediated killing during disseminated infection.

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Local innate and early immune response of 17 beta-estradiol-treated BALB/c mice to *Neisseria gonorrhoeae* and differential host susceptibility and response to gonococcal infection

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Objectives
Gonorrhea is an acute inflammatory disease of the urogenital tract, which is characterized by an exudate containing polymorphonuclear leukocytes (PMNs) with intracellular gonococci. Infections can also be asymptomatic, particularly in females. Experimental infection of BALB/c mice given β-estradiol to promote long-term colonization mimics many aspects of gonococcal infection, including an influx of PMNs in 30-70% of mice. Here we characterized the murine proinflammatory response to *N. gonorrhoeae*. We also tested C57BL/6 and C3H/HeN mice to determine if genetic backgrounds differ in susceptibility or host response.
Methods
Groups of estradiol-treated female BALB/c mice were inoculated intravaginally with ~ 1 x 10^6 colony forming units (cfu) of *N. gonorrhoeae* strain FA1090 or saline (control). Vaginal mucus was quantitatively cultured for *N. gonorrhoeae* for 7-10 days and the percentage of PMNs among 100 vaginal cells was determined by examination of stained vaginal smears. Levels of IL1-α, IL-6, TNFα, Mip2, and KC (the murine IL-8 homolog)] were measured in vaginal swab suspensions on days 1, 3, 5, and 7 by relative real time RT-PCR. Vaginal washes collected on day 5 of infection were analyzed by multiplex ELSA to confirm RT-PCR results. We also inoculated groups of estradiol-treated BALB/c, C57BL/6, and C3H/HeN mice with strain FA1090 or saline as above. Recovery of gonococci over time was compared between test groups by ANOVA and percentage of vaginal PMNs was compared between test and control groups for each mouse strain.
Results
We detected a proinflammatory response in infected mice compared to control mice, the kinetics of which showed a slight elevation in IL1-α expression (˜ 2 fold) compared to the uninfected controls on days 1, 3 and 5 and declined on day 7. Expression of TNF-α and IL-6 were also slightly elevated (˜2 fold) on days 1 and 3 and peaked on day 5 (˜8 fold), and declined on day 7. Similar expression patterns were observed for chemokines KC and Mip2. Mip2, but not KC production was significantly associated with an influx of vaginal PMNs. RT-PCR results were confirmed by ELISA. We also found that C3H/HeN mice were resistant to colonization compared to BALB/c and C57BL/6 mice. The duration of colonization in BALB/c and C57 BL/6 was longer (› 7 days) compared to C3H/HeN (2 days, $p < 0.01$). Interestingly, PMN influx was strongest in BALB/c mice and was observed from days 5-8, while C57BL/6 and C3H/HeN had a basal level of PMN influx during that time period (‹ 5%).
Conclusions
Experimental gonococcal infection of 17 β-estradiol-treated BALB/c mice induces a robust inflammatory response with elevated TNFα, IL-6, KC and Mip2 levels detected on day 5 of infection. The trafficking of murine PMNs to the lower genital tract is associated with Mip2 production. Experimental infection of BALB/c mice can therefore serve as a valuable tool to elucidate how *N. gonorrhoeae* triggers receptors of the innate response. The identification of mouse strain differences in host susceptibility and response can also be exploited to understand interactions between *N. gonorrhoeae* and the host immune response.

P098

Differential activity of the α-2,3-sialyltransferase (*lst*) promoters contribute to the rate of LOS sialylation in the pathogenic neisserial subspecies *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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Gonococci scavenge 5′-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) from the host to sialylate their lipooligosaccharide (LOS). This sialylation is catalyzed by the enzyme α-2,3-sialyltransferase (Lst). LOS sialylation of gonococci renders serum-sensitive strains resistant to killing by non-immune normal human serum by enabling gonococci to bind the complement regulator, factor H. Most virulent strains of *Neisseria meningitidis* endogenously sialylate their LOS; however, this does not result in increased binding of factor H and does not predictably increase resistance to serum. In the current study we begin to examine whether the differences in Lst expression between *Neisseria gonorrhoeae* and *N. meningitidis*, which result from differences in the *lst* promoters (Infect Immun. 2006. 74:5;2637-50), contribute to the role of LOS sialylation in the pathogenicity of each species.

Objective

The sequence of the *lst* promoters in *N. gonorrhoeae* F62 and *N. meningitidis* MC58 is substantially different. A salient difference is the presence of a 105-bp Correira repeat-enclosed element (CREE) in the 5′ region of *N. meningitidis lst*, which is absent in *N. gonorrhoeae*. This CREE results in a ~5-fold decrease in *lst* transcript levels (Packiam et al, Infect Immun, 2006). The objective of our work was to define the relative efficiencies of LOS sialyltransferases across the two pathogenic neisserial species and its effect on binding of the alternative pathway complement inhibitor, factor H, to *N. gonorrhoeae*.

Methods

We selectively replaced the *lst* promoter in *N. gonorrhoeae* F62Δ*lgtD* with the *lst* promoter (containing the CREE) from *N. meningitidis* MC58. Lst activity was assessed using uptake of tritium-labeled CMP-NANA while factor H binding to bacteria was measured by flow cytometry.

Results

Incorporation of tritium-labeled CMP-NANA showed that the F62 Lst expressed from the meningococcal *lst* promoter in the background of F62Δ*lgtD* incorporated 4-fold less radiolabeled CMP-NANA than F62Δ*lgtD*, as did a meningococcal strain that contained its own Lst and promoter. This result was confirmed with a factor H binding assay, whereby the F62Δ*lgtD* strain with the meningococcal *lst* promoter bound less human factor H (4-fold lower fluorescence intensity) than F62Δ*lgtD*, due to decreased levels of sialylation of the bacteria.

Conclusions

From our data it appears that the decreased expression of F62 Lst expressed from the meningococcal *lst* promoter results in incorporation of less CMP-NANA and decreased factor H binding. Factor H binding to bacteria is proportional to Lst expression and LOS sialylation. Gonococci lack a capsule and serum sensitive strains must scavenge CMP-NANA very efficiently from the host to bind factor H and evade complement and this is likely facilitated by high Lst expression. In contrast, meningococci rely on capsule expression for high-level serum resistance and a fine balance between capsule expression and LOS sialylation may be required for optimal virulence. Decreased Lst expression because of the CREE 5′ to meningococcal *lst* could increase availability of sialic acid for adequate capsule biosynthesis. Our data indicate that a ~5-fold decrease in *lst* transcription is biologically significant and impacts binding of factor H to gonococci.

P099

CD46 in meningococcal disease; gene sequence results and effect of the rs2724374 SNP on cytokine production

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Human Membrane Cofactor Protein (MCP or CD46) is a protein, present in 4 different alternatively spliced isoforms, that is pivotal in the pathogenesis of invasive meningococcal disease. Binding of meningococcal pili to CD46 on nasopharyngeal mucosa cells facilitates adherence and invasion of meningococci. CD46 present on all nucleated cells regulates complement activation. CD 46 on T-cells influences T_{reg} cell development and cytokine production.
Thus far, mutations in the *mcp* gene have been linked to the chance to acquire atypical Hemolytic Uremic Syndrome. Mutations in the human *CEACAM*-genes, involved in meningococcal adhesion and invasion by the binding to Opa proteins, were been found to be related to the susceptibility to meningococcal disease. We hypothesized that mutations in the *mcp*-gene, or the presence of the SNP rs2724374 that presumably influences alternative splicing, affects susceptibility to or severity of invasive meningococcal disease.
To this end, 1) We sequenced all 14 exons of the *mcp*-gene from patients with clinically well defined meningococcal disease. 2) We developed a Taqman SNP assay, for the intronic SNP rs2724374 and analyzed 150 patients, 240 parents and 9 sibs. 3) We tested with a Transmission Disequilibrium Test, using data from 113 complete trios (= patients plus both parents), whether this SNP influences susceptibility to disease. 4) We measured cytokine production in whole blood stimulated with heat-killed meningococci in patients and parents, and related the production to the presence of the intronic SNP rs2724374.
Results
Among the 145 completely sequenced individuals only one (0.7%) mutation (not previously known) was found that might have an important effect on the structure or expression of CD46 (p.arg367ser); 137 patients (94.5%) were wildtype, 7 patients (4.8%) had a known mutation with none or only minimal effect on protein structure. Thus, in patients with meningococcal disease, exonic mutations in the *mcp*-gene are rare.
The rs2724374 SNP was present in 59/150 patients heterozygously and 6/150 patients homozygously, equally distributed over different disease manifestations i.e. severity. The allele frequency (23.7%) of this SNP was somewhat higher than in published healthy controls. TDT test results (in 113 complete trios) were not significant. Thus we could not show a significant effect of the presence of this SNP on susceptibility to or severity of meningococcal disease. The presence of the rs2724374 SNP did not influence TNF, IL-1 and IL10 production in whole blood cultures stimulated with heat-killed meningococci, whereas IL-12 production was significantly ($P = 0.039$; n= 309) lower in the presence of this SNP. In view of the lack of effect on susceptibility and severity of disease the significance of this latter finding deserves further research.
We conclude that the vast majority (99.3%) of patients with meningococcal disease have an intact or nearly intact *mcp(CD46)*-gene. The presence of the rs2724374 SNP does not influence susceptibility to or severity of disease, but decreases meningococci induced IL-12 production.

P099a

NarE is a novel immunomodulatory protein from *Neisseria meningitidis* Serogroup B

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Neisseria meningitidis is a major cause of both meningitis and septicaemia worldwide. The meningococcus resides in the upper respiratory tract of a high percentage of the population without clinical consequences but in some individuals the bacterium invades the tissue leading to meningitis and septicaemia. The ability of *N. meningitidis* to reside in the respiratory tract for long periods suggests that it can evade immune defences. Thus, it is important to establish the mechanisms that underlie this immune evasion. Unlike many other pathogenic bacteria, *N. meningitidis* has no known toxin. The availability of the bacterial genome sequence allowed a process of genomic screening to be carried out for features of a group of known bacterial toxins, the ADP-ribosyltransferases (ADPRTs). Members of this group including cholera toxin are pathogenicity factors and also potent immunomodulators. A novel ADPRT was identified and cloned (Neisserial ADP-ribosylating enzyme (NarE)). NarE has both ADP ribosyltransferase and NAD glycohydrolase activity. Given the fact that NarE shows structural homologies with CT, we investigated if it too has immunomodulatory properties. We have investigated the response of murine bone marrow derived dendritic cells (DCs) stimulated with NarE alone and/or in the presence of meningococcal lipopolysaccharide (LPS) or heat killed *N. meningitidis* (HkNm). NarE induced the maturation of DC as measured by increased surface expression of the costimulatory molecules CD80, CD86 and CD40 and MHC class II. In addition to this, NarE modulated DC cytokine production. It was shown to induce IL-6 alone, but pre-treatment of DCs with NarE inhibited LPS- and HkNM-induced IL-1β, IL-10 and IL-12 production. We also investigated the ability of NarE to modulate T type responses *in vivo*. We found modulation of cytokine production in response to NarE. Our findings suggest that the meningococcal toxin NarE unlike other ADPRTs, is not very immunogenic and does not display adjuvant properties although it does exert immunomodulatory effects on cells of the innate and adaptive immune system. In conclusion, our results suggest that NarE is a novel immunomodulator and may play a role meningococcal evasion of the immune system.

P100

Functional cross-reactive antibodies are elicited by a Group B *Neisseria meningitidis* bivalent recombinant lipidated LP2086 vaccine in Cynomolgus macaques

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Objectives
LP2086 (recently named as Factor H binding protein, fHBP) is a novel vaccine candidate for *Neisseria meningitidis* serogroup B. In this study we evaluate the imunogenicity of an experimental vaccine containing two recombinant lipidated fHBP (rLfHBP) representing the two sequence subfamilies (A and B) in cynomolgus macaques. Experiments were conducted for the rLP2086(A+B) vaccine to evaluate dosage and the effect of an aluminum phosphate adjuvant.
Methods
Cynomolgus macaques (n = 5/group) were immunized intramuscularly with rLP2086 (A + B) proteins

adsorbed to AlPO4 at dosages of 20, 60 and 200 μg of total rLP2086 (A + B). In a separate study, four groups of 6 macaques were immunized intramuscularly with either 2 or 20 μg of total rLP2086A + B formulated with or without AlPO4. In both studies, animals were vaccinated at weeks 0, 4 and 24, and rLP2086-specific IgG and functional antibody titers were determined prior to vaccination and two weeks after both the second and third vaccination. Serum rLP2086-specific IgG titers were determined against rLP2086A and B. Functional antibody titers were examined by serum bactericidal assay (SBA) against MnB strains expressing either LP2086 with sequences homologous or heterologous to those contained in the vaccine.

Results

All rLP2086 vaccine dosages elicited IgG titers to both rLP2086 proteins. Results suggested the doseages resulted in statistically significant lower titers were obtained using the 2 μg and 20 μg dosage in comparison to either the 60 or 200 μg dosages. Bactericidal antibodies were elicited that killed both strains within each MnB LP2086 subfamily. Significantly higher SBA and IgG titers were observed with the inclusion of AlPO4 adjuvant.

Conclusion

Overall, the immunogenicity data in nonhuman primates were consistent with the results previously noted for both mice and rabbits; (1) Bivalent vaccine elicits subfamily-specific antibodies with bactericidal activity, and (2) the immunogenicity of a bivalent vaccine consisting of both rLP2086 subfamily A and subfamily B antigens is enhanced with the inclusion of the aluminum phosphate adjuvant.

P101

Physicochemical and immunological characterization of the drug product (final lot) of a meningococcal group A conjugate vaccine

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Objectives

The meningococcal group A organism is responsible for 90% of the cases of endemic and epidemic meningitis caused by *Neisseria meningitidis* bacteria. The Meningitis Vaccine Programme (MVP) is developing an affordable monovalent meningococcal A conjugate vaccine (PsA-TT) consisting of periodate-activated group A polysaccharide (PsA) conjugated to hydrazide-derivatized tetanus toxoid (TT). The labile nature of the PsA phosphodiester linkage means that special attention needs to be paid to the integrity/stability of the conjugate vaccine. Typically stability-indicating assays involving size analysis and free saccharide determination are performed on the bulk conjugate, however, in keeping with the increasing emphasis being placed on the quality of the vaccine administered, the WHO Recommendations for the group A conjugate vaccines includes these assays on the final lot. For a lyophilized final product, the presence of large amounts of excipients means that appropriate methods need to be developed for the full physicochemical characterization and testing of the Drug Product. These physicochemical data may be complemented by the use of immunogenicity assays in an animal model.

Methods

Meningococcal A conjugate vaccine (PsA-TT) conjugate and final fills (formulated with mannitol) were obtained from Serum Institute of India Limited. Proton and carbon NMR spectra were recorded on the final fill dissolved in D_2O using a Varian Inova 600 MHz NMR spectrometer. High performance size-exclusion chromatography (SEC-HPLC) was performed with ultra-violet (UV) or refractive index (RI) detection on a Waters or an Agilent 1200 instrument. PsA content was determined using the phosphate assay or following hydrolysis and quantification of mannosamine-6-phosphate by use of high performance anion-exchange chromatography with pulsed amperomeric detection (HPAEC-PAD). Free/unbound saccharide was separated from conjugate by use of deoxycholate/acid (DOC/HCl) precipitation or by ultrafiltration. Stability studies of

the final fill were conducted on the final fill stored at 2-8ºC and samples subjected to elevated temperatures. Immunogenicity studies were performed in mice immunized with 3 doses of vaccine (1 ug PsA/dose); total PsA-specific IgG and functional bactericidal activity (SBA) of sera were determined.

Results

The structure and identity of the excipient and meningococcal A polysaccharide antigen in the final fill were confirmed by NMR spectroscopy. SEC-HPLC showed that the molecular size distribution indicates the integrity of the conjugate; samples stored at 40ºC showed peak broadening and the presence of a new lower molecular weight peak. The free saccharide assay was shown to be a sensitive indicator of conjugate integrity; the method using DOC/HCl and phosphate detection was validated. The final fill was shown to meet specifications after storage for two years at 2-8ºC. The immunogenicity study showed that antibody (Ab) titres increased significantly ($P<0.05$) following a second dose of vaccine, but not significantly after a third dose. There was a partial correlation ($\rho 0.6$) between total IgG and functional Ab at post-dose 2 & 3.

Conclusion

A variety of physicochemical methods and immunogenicity confirmed the quality of the meningococcal A conjugate final fill vaccine; this accords with the successful clinical Phase II results reported for this vaccine.

P102

A DNA fusion vaccine induces bactericidal antibodies to a peptide epitope from the PorA porin of *Neisseria meningitidis*

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Objectives

DNA vaccines have been the focus of intense investigation over the past two decades and are in development for therapies against auto-immune diseases, allergies and cancers. DNA vaccines offer the advantages of facile production, safety, stability and as the antigens are produced *in vivo*, they can be modified by the host and have the potential to assume native conformation. In addition, DNA vaccines have been reported to induce antibody responses against bacterial pathogens where humoral immunity to protein antigens is believed to be essential. In the current study, a strategy was developed to focus the humoral antibody response towards a defined meningococcal outer membrane protein epitope, the P1.16b serosubtype peptide from PorA, known to be essential for inducing functional, bactericidal antibodies.

Material and methods

DNA vaccine constructs were prepared encoding the entire surface-exposed loop4 (36 amino acids) containing the protective MC58 P1.16b epitope (pPorALoop4), with and without the presence of the Fragment C (pPorALoop4-FrC) immunostimulatory sequence from tetanus toxin. Additional cysteine codons were included at both N- and C-terminus in order to provide the potential conformational constraint of a disulphide bridge. Control DNA plasmid (pFrC) contained the FrC fragment without the pPorALoop4 DNA fragment. Mice were immunised intra-muscularly with 50 ug of plasmid in saline without adjuvant at day 0, 21 and 42. Individual murine antisera were then i) reacted in ELISA against MC58 OM; ii) reacted with whole meningococci with antibody binding detected by immuno-fluorescence (IF); iii) tested for bactericidal activity against the homologous strain MC58 and heterologous strains (H44/76, MC50, MC106 and MC168).

Results

Antisera from mice immunised with the pPorALoop4-FrC DNA plasmid construct showed significant reactivity against MC58 OM in ELISA, reacted with homologous meningococci as determined by IF and were bactericidal (a 50% end-point titre of 1/16-1/64 serum dilution). The reactivity was serosubtype-specific as no IF cross-reactivity or bactericidal activity was demonstrated by the antisera towards the heterologous meningococcal strains. By contrast, antisera from mice immunised with pFrC control plasmid, pPorALoop 4 DNA plasmid or saline showed little or no reactivity against MC58 OM in ELISA, did not react with meningococci as determined by IF and were not bactericidal.

Discussion
Our current study provides proof of the feasibility of a peptide epitope-based DNA vaccine strategy for inducing humoral antibodies to a defined, conformation-dependent epitope derived from a bacterial porin. The presence of an intrinsic immuno-stimulatory sequence, the FrC fragment, was essential for inducing a protective immune response in the absence of an exogenous adjuvant. However, current studies are aimed at increasing immunogenicity through the addition of exogenous adjuvant and/or the use of novel plasmid delivery systems. Finally, a significant advantage of the DNA plasmid vaccine approach is that it readily enables the production of many constructs in response to any changes in the immuno-dominant protein epitope sequences occurring through immune selection within a given population.

P103

Formulations of five conserved meningococcal B virulence proteins, 5CVMB, with safe adjuvants elicit broadly protective antibody responses in neonatal mice

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Objectives
No universal vaccine is available against meningococci of serogroup B (MenB), but several vaccine candidates have been identified by reverse vaccinology. The objectives of this study were to evaluate whether the conserved MenB virulence proteins GNA2131, Factor H binding protein (fHbp) or GNA1870, GNA2091, GNA1030 and NadA were immunogenic in neonatal mice when given as a pentavalent vaccine, 5CVMB. Furthermore, to assess whether their immunogenicity in neonatal mice could be enhanced by the adjuvants, LT-K63, MF-59, CpG1826 with or without Alum.
Methods
NRMI mice were vaccinated subcutaneously at one week of age, boosted 16 days later and again 14 days after the first booster. Blood samples were collected from the tail vein 2 days before the 2nd immunization and weekly thereafter. Protein specific IgG antibodies were measured by ELISA and the results expressed in ELISA units (EU/mL). Protective efficacy of the antibodies induced by the different vaccine formulations was evaluated by serum bactericidal activity (SBA) expressed as serum titer causing 50% killing of the bacteria.
Results
The 5CVMB vaccine alone elicited significant IgG serum antibody titers against each protein in the vaccine compared to unvaccinated mice (1.77- 4.18 log EU/mL, p ‹0.001 - 0,005, two weeks after 3rd immunization) and detectable SBA (›16) against two of the six MenB strains tested. Mixing and coadministration of an adjuvant with 5CVMB significantly enhanced specific IgG antibody responses to all the proteins (p ‹ 0.001- 0.005) compared to the 5CVMB vaccine alone, except for response to GNA1030 in the groups receiving 5CVMB with CpG1826 and/or Alum (p = 0.083-0.38). In agreement with the adjuvant enhancement of antibody titers SBA was increased 5 to 7 fold. All the adjuvants (except Alum) increased the SBA coverage of MenB strains compared to the 5CVMB alone. The strongest adjuvant effect was observed for CpG1826+Alum providing a significant increase in antibody responses to four of the five proteins (increase in log EU/mL: 1.2 for GNA2131, 1.43 for fHbp (GNA1870), 2.43 for GNA2091, and 2.15 for NadA, p‹ 0.001 for all four proteins, but 0.65 for GNA1030, p = 0.083). SBA titers to the various Men B strains were increased 6- 7 fold compared to 5CVMB alone, with SBA strain coverage to five of the six strains tested. Of all the vaccine formulations tested 5CVMB with CpG1826+Alum elicited the best SBA coverage of Men B strains.
Conclusion
The results of this study demonstrated that the meningococcal B virulence proteins GNA2132, fHbp (GNA1870), GNA2091, GNA1030 and NadA were immunogenic in neonatal mice when administered as

a 5CVMB. Antibody responses were significantly enhanced by safe and effective adjuvants resulting in higher SBA titers and broader MenB strain coverage indicating that these formulations can provide a broad and effective protection against MenB infections. The results encourage us to further study the neonatal immune responses to 5CVMB and adjuvant formulations to help to design optimal vaccination strategies against meningococcal disease in the young.

P104

The effects of co-administration and pre-vaccination with BCG on neonatal response to MenC-CRM197

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Objectives
To assess the ability of BCG to act as an adjuvant in newborn, by analysing whether BCG could enhance neonatal murine responses to a monovalent meningococcal C conjugate vaccine, MenC-CRM197, and direct the immune response from a Th2-type towards a Th1-type.
Methods
Neonatal mice were primed subcutaneously (s.c.) or intranasally (i.n.) with MenC-CRM197. BCG was either administered concomitantly, a day or a week before MenC-CRM197 was administered. The mice were reimmunised either 16 days later with MenC-CRM197 by the s.c. or i.n. route. Antibody (Ab) levels were measured by ELISA and results expressed in ELISA units per mL (EU/mL). Generation of immunological memory was assessed by the kinetics of the Ab response. Serum bactericidal activity (SBA) was measured to evaluate protective efficacy.
Results
After the MenC-CRM197 booster mice primed with MenC-CRM197 alone s.c. showed low but significant increase in MenC-specific IgG Ab levels compared to unimmunised mice (1.4 fold increase, P=0.005,). Mice primed with MenC-CRM197 and BCG concomitantly s.c. showed significantly higher MenC-specific IgG antibody titers than the mice primed with MenC-CRM197 alone (2 fold increase, P=0.002). However, there was no difference in MenC-specific Ab levels between the mice receiving BCG one day or one week prior to the MenC-CRM197 priming and mice primed with MenC-CRM197 only. SBA was only detectable (SBA titer ›16) after the MenC-CRM197 booster in the group primed with MenC-CRM197 and BCG concomitantly s.c., and not in the group primed with MenC-CRM197 plus BCG i.n. Mice primed with MenC-CRM197 plus BCG s.c. and reimmunised with MenC-CRM197 i.n. showed significantly higher MenC-specific IgG responses than mice reimmunised with MenC-CRM197 s.c. (P=0.038). Accordingly, the mice primed with MenC-CRM197 plus BCG s.c. showed a higher SBA titer when boosted i.n. than when boosted s.c (512 vs 128).
Discussion and conclusions
Mice primed with MenC-CRM197 and BCG concomitantly s.c. elicited a strong MenC-CRM197 specific IgG antibody response already 16 days after priming, indicating that BCG activated the immune cells in neonatal mice and thus acted as an adjuvant. They also showed a strong and rapid response to the MenC-CRM197 booster given by either the s.c. or i.n. route, indicating efficient generation of memory cells by the priming. Only the mice primed with MenC-CRM197 and BCG concomitantly s.c. showed detectable SBA 2 weeks after reimmunisation with MenC-CRM197. Mice pre-treated with BCG a week before MenC-CRM197 immunisation showed comparable response to those receiving MenC-CRM197 alone, indicating that BCG did not induce maturation of neonatal immune cells enough to enhance their responses to a subsequent immunisation. These results contradict results from human neonates showing that BCG given at birth was able to induce Th1-type responses to unrelated vaccines administered several weeks later. In this neonatal murine model BCG only functions as an adjuvant if given concomitantly with the MenC-CRM197 conjugate.

P105

Use of gold nanoparticle glycoconjugates to generate IgG specific for the carbohydrate

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Objective
Neisseria meningitidis is the leading cause of bacterial meningitis and is a worldwide health problem. *N. gonorrhoeae* F62)deltalgtD has been genetically modified to produce a single lipooligosaccharide (LOS) (the lacto-N-neotetraose LOS), an LOS expressed by almost all meningococcal strains. Our goal was to determine if we could isolate OS from the gonococcus, and make a glycoconjugate vaccine that would elicit an IgG response specific for gonococcal OS.
Method
We developed a glycoconjugate vaccine (TRIAD) that contains an oligosaccharide derived from N. gonorrhoeae F62)deltalgtD and a peptide that possesses the ability to bind to a large number of HLA class II molecules, chemically conjugated to a gold nanoparticle. TRIAD was chemically characterized and found to possess both OS and peptide on each gold nanoparticle. We were able to control the relative amount of OS and peptide on the particle by adjusting the ratio of the two conjugates used in the synthesis of TRIAD. Immunization of mice with our vaccine construct produced no observable adverse effects in C57 BL6 mice. We performed as series of biweekly or triweekly immunizations. Blood was periodically collected and assayed by ELISA for the presence of antibody.
Results
Our ELISA data demonstrated that significant levels of antibody were elicited after a single immunization, with the predominant isotype being expressed being IgG; IgM levels were minimal and comparable to those elicited by LOS when used to vaccinate control groups of mice. Furthermore, subsequent immunizations did not result in an increase in anticarbohydrate titer.
Conclusions
From our results, we concluded that TRIAD is capable of eliciting a potent IgG response that is directed against polysaccharide, and that a single dose of vaccine is sufficient to generate a maximal antibody response.

P106

Evaluation of meningococcal candidate vaccines by DNA immunization

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Gene immunization was recently used to quickly discover new antigens for meningococcal vaccine and handle the immunogenicity of a protein at the sequence level without requiring any protein production and purification. Expression Library Immunization was shown to be an effective means to screen the meningococcal genome for valuable protective antigens (Yero et al., 2007, 2005).
In this study, about 20 individual meningococcal antigens encoding putative surface-exposed proteins were tested as DNA vaccine vectors and analyzed for their capacity to induce functional antibodies. A vaccine candidate, the human transferrin-binding protein B, TbpB, was used as control together with other well studied outer membrane proteins such as NspA and the hemoglobin receptor HmbR.
Each individual meningococcal sequence of interest was cloned into the pCA/myc-His DNA vector and used for immunization of ten outbred CD1 mice via the intramuscular route. Sera were analyzed for their

bactericidal activity against the homologous strain grown in iron-rich and iron-poor conditions.
Except TbpB, all antigens administered by DNA immunization were not able to induce bactericidal antibodies in the experimental conditions tested in this study.
On the other hand, gene immunization of TbpBs from both tbpB-isotypes I and II, was capable of eliciting the production of bactericidal antibodies. The bactericidal activity was heterogeneous between mice but it was already effective after one dose of injected DNA.
The expression of the protein TbpB in a native-like conformation has been earlier reported to be of crucial importance for the induction of an efficient immune response against *Neisseria meningitidis*. Our data suggest that the bacterial surface-exposed TbpB was also correctly folded in the eukaryotic environment and this was likely not the case with the other antigens evaluated in this study. In the past, successful DNA vaccination has been reported for bacterial antigens that were shown to be highly immunogenic when injected as proteins, such as OspC from *Borrelia burgdoferi*. Here we report the successful DNA immunization approach using the TbpB vaccine candidate, previously shown to be highly immunogenic when administered as a protein. Monoclonal antibodies against TbpB were also successfully produced after DNA immunization and their characterization is on going.

P107

Immunogenicity of a native outer membrane vesicle vaccine for *Neisseria meningitidis* serogroup B in rabbits

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Objectives
We are developing a group B vaccine consisting of native outer membrane vesicles (NOMV). NOMV contain outer membrane proteins (OMPs), lipooligosaccharides, and phospholipids in their native conformation, and thus enable the generation of antibodies that can recognize antigens on viable bacteria. Our vaccine contains NOMV derived from three laboratory strains of group B meningococci. We have evaluated the immunogenicity of the vaccine and of its three separate NOMV components in rabbits.
Methods
Three antigenically diverse serogroup B strains were genetically modified to decrease endotoxicity and increase the breadth of the immune response induced by the vaccine. NOMV were produced from the genetically modified vaccine strains 44/76-HOPS-D (B1), 8570 HOPS-G1 (B2) and B16B6 HPS-G2 (B3). Rabbits received three intramuscular immunizations four weeks apart of either an individual NOMV component or the complete vaccine (containing B1, B2 and B3 NOMV). Concentration of specific antibodies and bactericidal activity against serogroup B strains were evaluated in pre- and post-immunization sera.
Results
Immunogenicity of the individual components was evaluated in sera from rabbits that received three immunizations of 10 μg to 60 μg of a single NOMV component. Immunization with NOMV induced increases in bactericidal antibodies in the range of 8 to 128-fold against homologous strains. A moderate dose response was observed. As determined by ELISA, each NOMV component induced a 100-fold or greater increase in IgG antibodies. Antibody responses measured against homologous and heterologous NOMV were similar. Immunogenicity of the complete vaccine was compared to that of the individual components. Immunization with single components or the complete vaccine induced similar total IgG antibody concentrations and similar bactericidal titers against the homologous test strains. This indicates immunization with the complete vaccine does not result in a lower antibody response to the individual components. Minimal differences were observed when the combined vaccine was delivered with or without aluminum hydroxide adjuvant.
Sera from rabbits immunized with the B2 NOMV component were evaluated for bactericidal activity

against isogenic mutants of the homologous strain expressing different PorA serosubtypes. Bactericidal activity was similar against strains expressing homologous or heterologous PorAs, indicating that the bactericidal antibodies induced were not principally porA specific. The post vaccination sera were also bactericidal against a heterologous strain expressing different PorA, PorB and LOS antigens.

Conclusion

Immunization of rabbits with B1, B2 or B3 NOMV vaccine components or the complete vaccine resulted in increased specific antibody levels by ELISA and increased bactericidal activity against both homologous and heterologous bactericidal test strains. Results were similar when rabbits were immunized with or without adsorption to aluminum hydroxide indicating that NOMV vaccines can provide protective immune responses without the use of adjuvant. The vaccine induced bactericidal activity against several heterologous strains indicating that immunization can provide protection against divergent serogroup B strains. Further analysis of the cross reactive bactericidal antibody response is in progress.

P108

Immunological evaluation and sequence constancy of two new meningococcal vaccine candidate proteins

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Objectives

A growing number of high-throughput approaches have been employed to screen the meningococcal genome for potentially protective antigens. Recent developments in genomic technology, together with the availability of genome sequences provide the opportunity to examine such proteins, which might form the basis of a truly universal vaccine, if they were present on all strains irrespective of serogroup. In our laboratory, the novel meningococcal antigens NMA0939 and NMB0938 were identified by mining meningococcal genomic sequence databases. In this work, we evaluated these proteins as potential vaccine candidates to control meningococcal disease.

Methods

We investigated the conservation of the deduced amino acid sequence of NMA0939 and NMB0938 in different meningococcal strains, by combining PCR and nucleotide sequencing. Sequences codifying for the two antigens were amplified by PCR, from purified chromosomal DNA, using specific primers. A total of 42 strains, with representation of serogroups A, B and C were tested, including strains isolated from healthy carriers. Additionally, the recombinant variants of these proteins, which were cloned and expressed in *Escherichia coli*, were easily purified in one step by immobilized metal ion chromatography after solubilization. After immunization with the recombinant antigens, the immune response was evaluated by ELISA, Fluorescence-activated cell sorter (FACS)-related assays and Western blotting of total cell extracts from different Neisseria strains. Functional activity of antibodies was evaluated by serum bactericidal activity and infant rat protection assays.

Results

The genes were present in 100% of the strains evaluated. It was found, after the sequence analysis, the overall identity of the deduced polypeptides ranged from 95 to 100%. When recombinant variants of the antigens were used as immunogens, animals developed cross-reactive IgG antibodies in their sera, as determined by ELISA and Western blotting using whole cells of homologous and heterologous strains. FACS analysis showed binding of mouse polyclonal sera to live *N. meningitidis* from the CU385 strain, suggesting that these proteins are exposed on the surface of the cells. Besides, the immunization induced a functional response characterized by bactericidal antibodies and protective activity against meningococcal bacteremia in the infant rat model.

Conclusion
Taking into account these findings, NMA0939 and NMB0938 have several attributes that make them promising candidates to be included in a future vaccine against meningococcal disease.

P109

Analysis of the immune response to an investigational three component *N. meningitidis* NOMV vaccine in mice

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Background and objectives
Meningococcemia and meningitis caused by *N. meningitidis* serogroup B is still a major problem in both the developed and developing world. Although there are a few candidate vaccines that are currently undergoing clinical trials, to date we do not have an approved broadly cross protective vaccine that would prevent the disease. The objective of this study was to assess the effectiveness of a three component *N. meningitidis* native outer membrane vesicle (NOMV) investigational vaccine in mice and analyze the immune response to the antigens expressed in the NOMV vaccine.
Methods
The *N. meningitidis* parent strains 44/76, 8570, and B16B6 were genetically modified for the production of the investigational NOMV vaccine. The modifications were undertaken to induce broad cross protection against group B *N. meningitidis*. Each of the strains was modified to express an additional porA allele and one of the potentially protective outer membrane conserved proteins such as NadA, FHBP1 and FHBP2. NOMVs obtained from each of the genetically modified strains constituted one component of the three component NOMV vaccine used in this study. CD1 mice were immunized intraperitoneally on day 0, and boosted on day 28, and day 56 with either individual components or various combinations of NOMV components. The sera collected from the immunized mice two weeks after their final boosts were analyzed for the presence of antibodies to antigens present in the NOMV vaccine and their bactericidal effectiveness.
Results
The sera collected from the immunized mice showed an increase in the presence of antibodies to over-expressed antigens present in the NOMV vaccine. As compared to the control parental strains, we saw a 6 fold increase in antibodies to NadA and a 5 fold increase in Abs to FHBP1. Bactericidal depletion assays show that the LOS, porA, and conserved proteins like FHBP1 in the NOMVs induced bactericidal antibodies in mice. Furthermore, sera from mice that received the complete, three component NOMV vaccine showed significant bactericidal activity against a high percentage of heterologous *N. meningitidis* clinical isolates.
Conclusions
The truncated LOS, as well as porA and conserved surface proteins present in the NOMV vaccine do indeed induce bactericidal antibodies in mice. The three component *N. meningitidis* native outer membrane vesicle (NOMV) investigational vaccine appears to induce broad crossreactivity and bactericidal activity against group B *N. meningitidis* in mice and therefore is a good candidate vaccine for further investigation.
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This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

P110

Purification, characterization, and immunogenicity of native NadA from *Neisseria meningitidis* strain B11

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Objectives
Neisseria adhesion A (NadA; NMB1994) is an adhesin/invasin that binds human epithelial cells. NadA forms a stable, SDS- and heat-resistant trimer with its C-terminal domains anchoring the protein to the outer membrane of the bacteria. Literature studies have shown that recombinant, truncated forms of NadA in which the membrane interacting domain is deleted can generate anti-sera with serum bactericidal activity (SBA). The objective of this study is to purify and characterize native, full length NadA from *Neisseria meningitidis* strain B11 and to evaluate its immunogenicity in mice.
Methods
We have purified a full-length NadA homolog from the outer membrane protein complex (OMPC) from *Neisseria meningitidis* strain B11 (a serogroup B strain). OMPC is a manufactured (MMD; West Point, PA) vesicular detergent bacteria extract and is used as a carrier for conjugate vaccines. In brief, OMPC was pelleted by ultracentrifugation and NadA was extracted from the pellet using a buffer containing the detergent zwittergent® 3,12. Solubilized NadA was purified by a combination of ion-exchange and size-exclusion chromatography. We immunized CD1 mice with purified NadA in detergent fos-choline® 12 after adsorption to aluminum hydroxyphosphate sulfate adjuvant. For comparison, we also immunized CD1 mice with OMPC from strain B11 adsorbed to aluminum hydroxyphosphate sulfate. We applied three serological assays to evaluate the mouse immunogenicity of fos-choline® 12-solubilized NadA: sera were analyzed by anti-NadA ELISA, whole bacteria strain B11 binding, and SBA titer against strain B11.
Results and conclusions
Analysis with electrospray tandem mass spectrometry confirmed that we obtained a NadA homolog (allele 2) as the purified product. Approximately 5 mg of NadA could be purified starting with 700 mg of OMPC protein from *Neisseria meningitidis* strain B11. Purified NadA precipitated with 80% ethanol could be readily solubilized with a buffer containing the detergent fos-choline® 12 for immunogenicity studies. ELISA results using purified NadA as the coating antigen showed that high anti-NadA antibody titers are present in the mice sera immunized with purified NadA. FACS experiments showed that the anti-NadA sera bound strongly to whole strain B11 bacteria. Anti-NadA sera displayed high SBA titers against strain B11. These results suggest that native source purified, full-length NadA has immunogenic properties consistent with a serogroup B vaccine component. Furthermore, ELISA results using purified NadA as the coating antigen showed that high anti-NadA antibody titers are also present in the OMPC (strain B11) immunized mice sera. NadA immunogenicity as a component of OMPC versus a purified protein will be discussed.

P111

Protective antigens in the immune response to *Neisseria meningitidis* Native Outer Membrane Vesicles (NOMV)

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Objective
Deoxycholate-extracted OMV (outer membrane vesicles) vaccines have been used effectively to combat

epidemic outbreaks of clonal group B *N. meningitidis* (eg., in Norway, Cuba, and New Zealand). The protective, bactericidal response was directed to outer membrane proteins present in these OMV vaccines, especially PorA and Opc. However, non-detergent extracted, native outer membrane vesicles (NOMV) are composed of a greater variety of outer membrane proteins and contain a greater proportion of LOS. Would the differences in composition between deoxycholate-extracted OMV and NOMV affect the major targets of bactericidal antibodies by the immune response? Is the bactericidal immune response of animals immunized with NOMV similarly directed against PorA or are other antigens such as LOS important?

Methods

Our group has examined the bactericidal response of animals and humans to NOMV and other investigational vaccines derived from group B *N. meningitidis* by determining bactericidal titers against a variety of strains and by bactericidal depletion assays. In addition, human convalescent and normal sera with protective titers (› 1/4) to group B *N. meningitidis* strains have been tested by bactericidal assay and bactericidal depletion assays to specifically identify protective antigens arising from natural infection or carriage. Protective antigens (targets of bactericidal antibodies) are inferred by comparing bactericidal titers against strains differing in PorA subtype or expression of other variable surface components. Bactericidal depletion assays more directly reveal bactericidal antibodies to a particular component because specific antibodies are adsorbed out of the serum prior to incubation with meningococci and complement; the removal of specific bactericidal antibodies results in more survival. Aliquots of sera (diluted to ~50% survival for the test strain) are preincubated with a dilution series of antigen in solid phase, and then the sera are incubated as in a bactericidal assay with fresh complement.

Results

In many cases, preincubation of the sera with purified LOS could deplete the majority of bactericidal antibodies from animal and human sera. In other cases, purified LOS could only deplete a portion of the bactericidal antibodies as compared to depletion with an NOMV preparation containing homologous LOS. In the latter case, one or more other components, presumably proteins, were also the targets of bactericidal antibodies. In at least one case, it appears that after immunization of mice with NOMV, PorA was not the primary target of bactericidal antibodies; the titer of pooled sera was similar against isogenic strains expressing antigenically distinct PorA, one of which was not present in the NOMV.

Conclusion

In animals, the protective response to NOMV was directed against LOS and conserved proteins, in addition to PorA. Human convalescent and normal sera showed a similar pattern: that LOS was commonly a major, but not the exclusive target of bactericidal antibodies. The pattern for the bactericidal response to NOMV is somewhat different from what has been reported for deoxycholate-extracted OMV vaccines and may be a consequence of the difference in composition of NOMV and OMV.

P112

Characterisation of the Norwegian 44/76 outer membrane vesicle vaccine cultivated in Frantz' and modified Catlin-6 growth media

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Background

Outer membrane vesicles (OMVs) from the Norwegian vaccine strain 44/76 have so far been prepared, after fermentation of meningococci in the rich organic Frantz' medium (FM) and in the synthetic modified Catlin-6 medium (MCM). An initial study, using SDS-PAGE and serum bactericidal assays, demonstrated differences in antigen expression and in bactericidal activity of the two vaccine preparations. (IPNC 2002; p. 269).

Objectives
The aim of this study was to perform a more comprehensive characterization of the vaccines' protein composition and to undertake a detailed comparative analysis of the two vaccine preparations.
Methods
Three batches of 44/76 (B:15:P1.7,16; ET-5) were grown in a 2.5 litre fermentor in FM or MCM. OMVs were extracted with deoxycholate and analysed by two-dimensional electrophoresis and Differential Gel Electrophoresis (2D-DIGE). Samples with labelled with cyanine NHS ester dyes. IPG strips (24cm, pH 3-11 non-linear) were used for first dimension isoelectric focusing under an optimal 45KV·h total focusing time. Second-dimension SDS-PAGE was carried out in 12% acrylamide gels (26 x 20 x 0.1 cm) and scanned images were analysed by DeCyder image analysis software (GE Healthcare). Protein identification was carried out using tryptic digestion, tandem mass spectrometry sequening and database searching.
Results
The application of 2-D electrophoresis technology using large-format gels offered the resolving power to reveal the true complexity of the vaccines. Gel spot patterns demonstrated similarities in the overall protein composition between samples with PorA and PorB being the dominant components. However, DIGE analysis also revealed significant differences in the expression of certain proteins in different growth media. The proteins were of varying molecular weight, pI, and abundance. Differences also varied in fold-change in quantity and appeared not to follow a trend towards one of the growth media. Protein characterisation is in progress to identify the proteins composition of the OMV vaccines including those that change in expression level due to different growth media.
Conclusion
Improved protein separation was obtained with 2-D electrophoresis allowing the construction of a comprehensive map of the Norwegian meningococcal OMV vaccine from strain 44/76. DIGE technology also revealed significant changes in protein expression after fermentation in the different growth media.

P113

Rational engineering of Neisseria factor H binding protein to induce cross-protective immunity

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Objectives
The factor H binding protein (fHbp) of *Neisseria meningitidis* (or GNA 1870) is a component of a multivalent vaccine against *Neisseria meningitidis* serogroup B which is currently in clinical trials This protein has been named fHbp due to its ability to bind human factor H, a negative regulator of the alternative complement pathway. Previous studies demonstrated the presence of allelic polymorphism of fHbp in the meningococcal population. Sequence variability correlated with antigenic variation, as polyclonal sera obtained immunizing with recombinant proteins of each variant were demonstrated protective versus strains carrying the homologous variant, but in some extent ineffective against those displaying heterologous variants. The NMR structure of the carboxyterminal domain, containing most of the bactericidal epitopes, has been solved. This structure has been taken as model to graft putative epitopes from one fHbp variant to another and test the ability of these new molecules to elicit protective immune response proving the potential of such novel molecular engineering. This approach should also allow to identify new epitopes in fHbp variants and understand their respective contribution to the immunological effect.
Methods
The fHbp gene devoid of the putative leader peptide coding sequence was used as "receiver". Codons were modified in silico in order to encode desired amino acids changes. Synthetic genes were purchased and resulting mutants expressed in *Escherichia coli* as C-terminal histidine fusions. Recombinant proteins

were purified and used for mouse immunization as already described. The recombinant proteins were administered intraperitoneally, together with different adjuvants. The corresponding antisera were used for testing bactericidal activity against *N. meningitidis* strains, with pooled baby rabbit serum used as complement source.

Results

The mutant design started on the base of several data. Potentially exposed residues available for antibody binding were preliminary identified when the NMR structure of the C-terminal immunodominant domain of fHbp of MC58 strain was obtained. Moreover, it was assumed that, for the successful transplant of heterologous epitopes into fHBP of MC58, it was necessary to modify a part of the surface of the "receiver"protein in a way to adequately reproduce the corresponding portion of the "donor". Therefore groups of amino acids to concurrently mutate were selected to create such mimetic heterologous areas that we called patches. By this way, a series of mutants displaying individual patches scattered on the surface of the protein were obtained.

The immunological potential of the mutants resulting from epitope grafting was assessed by evaluating the ability of corresponding antisera to induce bacterial killing on a collection of clinical isolates carrying different alleles of fHBP and able to represent the existing biodiversity of meningococcus serogroup B. Some mutants showed different levels of cross-reactivity and some promising engineered antigens were identified.

Conclusion

We implemented a new approach in order to create novel cross-protective candidates of fHbp. The first results obtained with corresponding antisera indicate that the graft of patches containing heterologous epitopes from one fHbp variant to another can lead to a wider cross-reactivity, proving the power of this approach.

P114

Immunogenicity of meningococcal Omp85 protein

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Objectives

The highly conserved Omp85 protein family is essential for the assembly of βbarrel proteins into the bacterial outer membranes and thus for bacterial viability (Gentle et al. 2005). In meningococci, a role for the transfer of lipids in the outer membrane was also postulated (Genevrois et al. 2003). Increased levels of Omp85 specific antibodies are found in patients with meningococcal disease and in vaccinees receiving OMV vaccines (Wedege et al. 1998; 2007). With postvaccination sera, Omp85 binds more IgG antibodies per weight than other OMV proteins. The aim of our study was to investigate whether Omp85 specific antibodies induced in humans and mice possessed functional activity.

Methods

Strain 44/76 was transformed with a plasmid carrying Omp85 controlled by an IPTG-inducible promotor (kindly donated by J. R. Dillon and M. Bos). The resulting OMV (Omp85+ OMV), obtained after fermentation in modified Catlin medium, expressed 5-fold more Omp85 than the control OMV. It was adsorbed to aluminum hydroxide and injected into groups of 12 Balb/c and 12 C57BL mice in two 2.0 ug doses as well as in groups of 10 OFI mice with three 5.0 ug doses. Mice sera were analysed for Ig levels by immunoblotting and for functional activity in serum bactericidal assay (SBA) and in opsonic assays (OPA) with live meningococci measuring respiratory burst. Target strains were strain 44/76 and a 44/76 PorA-mutant. His-tagged recombinant Omp85, bound to magnetic Dynabeads® Talon™, was used to adsorb specific antibodies from pooled sera from mice, patients with meningococcal disease and human vaccines receiving MenBvac.

Results
On blots, the various mouse strains responded with different levels of Omp85 specific Ig antibodies after immunisation with Omp85+ OMV. C57BL mice showed 3 times higher Ig specific levels than Balb/c mice following a 2.0 ug dose, while the more heavily immunized OFI mice had 1.2 higher levels than C57BL mice. Levels in OFI mice were similar to NMRI mice receiving 2 ug of a control OMV vaccine. Differences in Ig binding to other OMV proteins between the mouse strains were also observed. OFI mice given Omp85+ OMV formulated with aluminium hydroxide alone or in combination with MPL showed similar levels of OMP85 Ig. Although Omp85 specific Ig was higher in C57BL mice than in Balb/c mice, SBA titres against 44/76 were significantly lower. Preliminary adsorption experiments with pooled mice sera did not indicate opsonic activity of Omp85 antibodies. The SBA titres were found to correlate significantly with Ig binding to PorA, which may possibly mask the contribution of antibodies with lower activities. The functional assays were therefore also performed with the PorA- strain for both mice and human sera and will be reported.
Conclusion
The levels of Omp85 antibodies induced in mice depends on the mouse strain. Although our studies so far do not indicate a distinct functional activity of Omp85 antibodies, the masking of such antibody activity by the dominant PorA antibodies cannot be excluded.

P115

Epitope mapping of a bactericidal monoclonal antibody against the factor H binding protein of *Neisseria meningitidis*

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Objectives
The factor H binding protein (fHbp) is a 27 kDa membrane-anchored lipoprotein of *Neisseria meningitidis* that allows the survival of the bacterium in human plasma and is also a major component of a universal vaccine against meningococcus B.
In the present study we used nuclear magnetic resonance spectroscopy, mutagenesis and in silico modelling to map the epitope recognized by MAb502, a bactericidal monoclonal antibody elicited by fHbp. The identification of the MAb502 epitope is valuable information for the rational engineering of fHbp, aimed to create a chimeric protein presenting a wider collection of neutralizing epitopes to the host immune system.
Methods
To test the binding of MAb502 to fHbp produced by natural isolates representative of the diverse meningococcal population, we selected 10 strains carrying non-redundant fHbp sequences. MAb502 was able to recognize fHbp in 6 out of 10 strains in western blot and FACS experiments, and had a strong bactericidal activity only against MC58.
Hints on residues involved in the formation of the MAb502 epitope were searched by analyzing the sequence differences between the fHbp protein produced by MC58 strain and those of the other clinical isolates. Sequence alignment of such fHbp alleles allowed to attempt a first prediction on which residues were could be essential or dispensable for the epitope. The predictions on fHbp residues interacting with MAb502 were tested through nuclear magnrtic resonance spectroscopy, by analysing the perturbations caused in the 1H-15N HSQC and CRINEPT spectra of fHbp upon addition of the FAb fragment (FAb502). To model the structure of the complex between fHbpC and the hypervariable antigen-binding fragment of MAb502 (Fv502), docking calculations were performed using as input the NMR structure of fHbpC and a computer homology model of Fv502.
Results
The data show that the antibody recognizes a conformational epitope within a well defined area of the

immuno-dominant carboxyl-terminal domain of the protein, formed by two loops connecting different beta strands of a beta barrel and a short alpha helix brought in spatial proximity by the protein folding. The epitope Arg204, a residue previously already identified as essential for binding to the antibody.

Conclusion

The detailed knowledge of epitopes recognized by protective antibodies is a necessary requisite for the understanding of molecular mechanisms for establishing the effective immune response. In addition, epitope mapping of bactericidal monoclonal antibodies provides a powerful tool to identify a priori bacterial strains carrying susceptible antigens. The approach here used, which combined immunological, FACS, Western blot and NMR characterization resulted to be particularly powerful in defining the epitope region. These results represent the first step of an experimental strategy in which vaccine candidates can be designed to contain broad repertoires of natural protective epitopes identified by epitope mapping.

P116

Factor H-binding protein is important for meningococcal survival in human whole blood and serum, and in the presence of the antimicrobial peptide LL-37

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Objectives

Factor-H binding protein (fHBP; GNA1870) is one of the antigens of the five-component vaccine against Meningococcal serogroup B, which has been developed using 'Reverse Vaccinology' (5CVMB; Giuliani et al., 2006). Binding of factor h (fH), an inhibitor of the alternative complement pathway, to fHBP enables *N. meningitidis* to evade killing by the innate immune system. The significance of the level of fHBP expression by different strains with respect to killing by factors of the human immune response has been assessed.

Methods

N. meningitidis MC58, BZ83, NZ98/254, and 67/00 wild type and derivative strains lacking the expression of fHBP (ΔfHBP) were analysed with respect to survival in *ex vivo* human serum and whole blood models of meningococcal bacteraemia, as well as *in vitro* survival assays in the presence of antimicrobial peptide.

Results

The MC58 (high fHBP expressor), BZ83 (high fHBP expressor), NZ98/254 (medium fHBP expressor), and 67/00 (low fHBP expressor) ΔfHBP mutant strains were all sensitive to killing by human whole blood and serum, with respect to the isogenic wild type strains. The MC58, BZ83 and NZ98/25 ΔfHBP mutant strains were also sensitive to killing by the antimicrobial peptide LL-37.

Conclusions

The expression on fHBP by *N. meningitidis* strains is important for survival in human blood and human serum regardless of the level of its expression. High, medium and low expressing strains of fHBP all had increased survival in human blood and serum when compared to isogenic mutant strains lacking fHBP expression. fHBP also plays an as yet uncharacterised role in protection against the antimicrobial peptide LL37. We conclude that the vaccine antigen fHBP is an important component of the 5CVMB due to its functional significance in survival of *N. meningitidis* in *ex vivo* models.

P117

Influence of serogroup B meningococcal vaccine antigens on strain growth and survival *in vitro* and *ex vivo*

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Objectives
'Reverse vaccinology' has led to the development of a 5-component vaccine against Meningococcal serogroup B strains (5CVMB; Giuliani et al., 2006) which is currently in clinical trials, and initial results indicate that this vaccine is well tolerated and induces bactericidal antibodies. The combination of five antigens in one multicomponent vaccine was pursued not only to increase the breadth of vaccine coverage but also to avoid the selection of vaccine escape mutants. To determine if the protein antigens in the MenB vaccine are critical for survival of *N. meningitidis*, and to evaluate the outcome of possible escape mutants lacking these vaccine antigens, a series of growth and survival experiments were conducted.
Methods
N. meningitidis MC58 derivative strains were generated lacking the expression of individual antigens (ΔfHBP (ΔGNA1870), ΔNadA (ΔGNA1994), ΔGNA2132, ΔGNA1030, ΔGNA2091) contained in the 5CMBV vaccine. In addition, MC58 derivative strains lacking three (3KO; ΔfHBP, ΔNadA and ΔGNA2132) or all five antigens (5KO) were generated using a strategy of stepwise deletion by allelic replacement. These strains were analysed with respect to growth and survival in various media and *in vitro* stress assays (including antimicrobial peptide and serum bactericidal activity (SBA) assays), as well as *ex vivo* human serum and whole blood models of meningococcal bacteraemia.
Results
All single and multiple knockout strains were viable when grown in culture media (GC and Mueller-Hinton (MH) broth and agar). However, the 5KO and ΔGNA2091 strains had decreased growth in MH broth but not MH supplemented with Isovitalex or glucose, suggesting that the GNA2091 protein may be involved in nutrient uptake. The 3KO and 5KO mutant strains were significantly more sensitive than the wild type strain to killing by human serum and whole blood. fHBP, which has recently been shown to bind factor H, was a major contributor to the bacterial survival in human blood. The ΔfHBP, 3KO, 5KO strains are also sensitive to the antimicrobial peptide LL-37. As expected the 3KO and 5KO strains are less sensitive to the bactericidal activity of polyclonal antibodies generated against 5CVMB.
Conclusions
N. meningitidis mutant strains lacking the genes encoding for fHBP, three or five vaccine antigens are more sensitive to killing by innate factors of the human immune response, as displayed in the *in vitro* antimicrobial peptide killing assays, and the *ex vivo* human serum and whole blood models of meningococcal bacteraemia. Mutant strains lacking GNA20291 or all five vaccine antigens also have decreased growth capabilities *in vitro* with respect to the wild type strain. We conclude that vaccine escape mutants are unlikely to be successful since they have reduced survival and virulence.

P118

Vaccination with a gonococcal oligosaccharide epitope configured as a peptide mimic fused with complement component C3d

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Objective
The 2C7 epitope is a conserved oligosaccharide (OS) structure, a part of lipooligosaccharide (LOS) on *N. gonorrhoeae*, present in 95% of clinical gonococcal isolates. We identified a peptide mimic (PEP1) as an antigenic surrogate of the 2C7 epitope by screening a random display library with mAb 2C7, which is specific for the 2C7 epitope. Conversion of the OS epitope to PEP1 provides a strategy to overcome a TI (T-cell independent) immune response to OS when the peptide is used as an immunogen. Previously, we reported that 50 μg of PEP1 formulated as a multiple antigenic peptide (MAP1) emulsified in Complete Freund's adjuvant (CFA), elicited a ≥ 10 fold increase in cross reactive bactericidal anti-LOS antibodies. The specific killing activity of anti-LOS antibody was calculated to be 1.53 μg/ml of IgG anti-LOS necessary to kill 50% of *N gonorrhoeae* strain 15253. (Vaccine 24: 157-170, 2006). In the present study we describe the immunostimulatory effect of a natural adjuvant, C3d, fused to PEP1, when this construct was used to immunize mice.
Methods
Complement C3 plays an important role in cell-mediated immune responses. Binding of the C3 derived fragment, C3d, to a foreign antigen facilitates its binding to CR2 (CD19/CD21) and membrane IgG antibody on B cells thereby facilitating uptake, antigen processing and presentation to T cells. We constructed PEP1-3,C3d that contains a PEP1 gene linked in tandem with 3, C3d genes and then expressed the fusion peptide in 293F mammalian cells. Four BALB/c mice were immunized (ip) with 10 μg of purified PEP1-3,C3d (containing 0.14 μg of PEP1 protein) and boosted 3 times at 4-weekly intervals. Two additional mice received 10 μg of LOS purified from gonococcal strain 15253 as a control. Anti-LOS antibody in mouse sera was quantitated by ELISA using purified gonococcal LOS from strain 15253 as the target antigen. Bactericidal assays were performed to assess C-mediated bactericidal activity of pre and post immune mouse sera against gonococcal strain 15253.
Results
Mice immunized with PEP1-3,C3d elicited ≥ 3 fold increase in cross-reactive anti-LOS antibodies. The specific killing activity of anti-LOS antibody was calculated to be 0.075 μg/ml of IgG anti-LOS antibody necessary to kill 25% of *N. gonorrhoeae* strain 15253 (0.165 μg/ml when data were extrapolated to 50% killing). Mice receiving 10μg LOS purified responded with a ≥ 10 fold increase in anti-LOS antibodies. Interestingly, anti-LOS antibodies induced by immunization with LOS demonstrated no bactericidal activity against gonococcal strain 15253.
Conclusion
PEP1-3,C3d elicited cross reactive anti-LOS antibodies that were specific for the OS epitope and were also bactericidal against a strain of *N. gonorrhoeae* that ordinarily resists killing by NHS. Furthermore, the adjuvant effect of C3d when used together with peptide resulted in an immune LOS antibody response that possessed 11.7-fold higher specific bactericidal activity than that elicited by CFA and peptide when both responses were normalized to 25% bactericidal activity (9-fold higher when 50% bactericidal activity was used). The use of a natural adjuvant such as C3d directly linked to peptide compares favorably in experimental immunization with the traditional adjuvant, CFA.

P119

Exploitation of fungal mannosylation to boost immunogenicity in mice of a peptide vaccine candidate that mimics an oligosaccharide epitope of *Neisseria gonorrhoeae*

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Objective
Peptide mimics of oligosaccharide (OS) antigens may offer an alternative strategy to convert T-cell independent (TI) immunogens to T-cell dependent immunogens. We identified a peptide mimic (PEP1) as a surrogate of the 2C7 OS epitope on *N.gonorrhoeae* by screening a random display library with mAb 2C7. Conversion of the OS epitope to PEP1 provides a strategy to overcome the TI response of OS when used as an immunogen. Mannosylation of antigen represents a promising strategy to augment vaccine immunogenicity by targeting antigen to mannose receptors on dendritic cells (DCs). In this study we mannosylated PEP1 and evaluated its effect on DC maturation and activation using an *in vitro* assay. We also evaluated immune responses to mannosylated PEP1 in mice.
Methods
Molecular characterization of *Cryptococcus neoformans* mannoproteins (MPs) indicate that serine/threonine (S/T) C-terminal regions serve as sites for extensive O-linked glycosylation. We fused the PEP1 gene with the S/T rich gene of cryptococcal MP and expressed mannosylated PEP1 antigen in *Pichia pastoris*. Activation of mouse DCs was measured by the capacity of MPs to promote IL-12 and TNF-α production by these cells. Four BALB/c mice were immunized intraperitoneally with 100 μg of purified mannosylated PEP1 (containing 10μg of PEP1). As a control, two additional mice received 10μg of LOS purified from the 2C7 epitope bearing *N. gonorrhoeae* strain 15253. Mice were boosted with the same immunogen at the same dose used initially 3 times at 4 weekly intervals. Anti-LOS IgG antibodies in mice sera were quantitated by ELISA using gonococcal LOS from strain 15253 as the target antigen. The function of anti-LOS antibodies was tested in bactericidal assays against strain 15253, which otherwise resists killing by normal human serum (NHS).
Results
Bone marrow DCs incubated with 1 μg of mannosylated PEP1 induced 173.8- and 57.3-fold more TNF-α and IL-12 respectively compared to PEP1 formulated as a multiantigenic peptide, thereby confirming the role of mannosylation of antigen and its engagement of the mannose receptor on DCs. Mice immunized with 100 μg mannosylated PEP1 (containing 10μg of PEP1) elicited $\geq$ 8-fold increase in cross-reactive IgG anti-LOS antibodies. The specific killing activity of anti-LOS antibody was calculated to be 0.15 μg/ml of IgG anti-LOS necessary to kill 25% of *N. gonorrhoeae* strain 15253 (0.6 μg/ml when data were extrapolated to 50% killing). Mice receiving 10μg purified LOS elicited $\geq$ 10-fold increase in anti-LOS antibodies. Interestingly, anti-LOS antibodies induced by immunization with LOS demonstrated no bactericidal activity against gonococcal strain 15253.
Conclusion
We found that mannosylated PEP1 promoted DC activation and maturation. PEP1 mimics the OS epitope antigenically and immunogenically. Mannosylated PEP1 elicited cross reactive anti-LOS antibodies that were specific for the nominal OS epitope and were also bactericidal against a 2C7 epitope bearing strain of *N. gonorrhoeae*. Furthermore, mannosylated PEP1 resulted in an immune LOS antibody response that possessed 5.9-fold higher specific bactericidal activity than that elicited by CFA and peptide when both responses were normalized to 25% bactericidal activity (2.6-fold higher when 50% bactericidal activity was used)-- even when a "formal" adjuvant was not used.

P120

Mapping immunologically relevant regions of *Neisseria meningitidis* TbpA by phylogenetic analysis and peptide-based arrays

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Meningococcal meningitis is a life-threatening disease. In developed nations the majority of cases are caused by virulent meningococcal strains belonging to serogroup B. Presently there is no effective vaccine against serogroup B strains, as traditional vaccine antigens such as polysaccharide capsules are unusable against this serogoup. The transferrin binding protein A is a potentially ideal vaccine target due to its demonstrated critical role for survival of pathogenic Neisseria in humans.
The current study was implemented to explore the reactivity of predicted surface regions of the TbpA protein with various sera and analyze its correlation with the detection of positive selection at codon level, in order to aid in vaccine design and development strategies. Regions involved in binding transferrin and hypervariable regions proposed to be under strong immune selection were two types of predicted surface regions selected for this analysis. The former were identified by screening overlapping synthetic peptide libraries representing TbpA with labeled transferring, and the latter were identified through sequence comparisons of a collection of tbpA genes. Both of these regions were primarily localized to predicted surface loops in topology and structural models based on comparisons with other Ton-B dependent outer membrane receptors. The peptide libraries were screened for reactivity with human convalescent sera or sera produced in mice and rabbits against purified TbpA to evaluate reactivity of these predicted surface regions. The results demonstrated that sera reacted with peptides from the hypervariable regions but there was relatively little reactivity with peptides representing the transferrin binding regions. Through phylogenetic analysis using a Maximum Likelihood based framework we have identified 20 codons where clear evidence of positive selection is present. These codons were located in hypervariable domains and close to transferrin binding regions. In general, while there was good correlation between the presence of positive selection and the human immune response, the lack of such correlation with peptides identified to be responsible of transferrin binding highlights the difficulty of targeting the most promising epitopes. This points to the need of a completely new approach for the development of a truly broad cross-protective neisserial vaccine.

P121

Evidences of positive selection in new vaccine candidates NMB1125, NMB0928 and NMB0088

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Objectives
To explore the incidence of positive Darwinian selection on potential Neisserial vaccine candidates identified by genomic approaches with the use of a Maximum Likelihood based framework.
Methods
With the objective of extract and use the evolutionary information contained in the target gene sequences, the determination of the sequence corresponding to 15 different isolates per gene was performed. Multiple sequence alignment was performed by means of the program CLUSTAL X version 1.8. and

corrected manually to avoid gross errors. In order to estimate the most plausible phylogeny the following steps a selection of the model of nucleotide that provided the best fit to the data, was determined using the program MODELGENERATOR. Secondly, we employed the maximum likelihood method for the estimate of the phylogeny implemented in the program PHYML, using the heuristic hill-climbing method, to search the best tree, adjusting the topology and the distance of the branches simultaneously. Estimated trees and parameter values found during tree reconstruction phase are available on request.
Analysis of Selection Pressures: A maximum likelihood (ML) approach was used to examine selection pressures acting on the meningococcal nmb1125, nmb0928, and nmb0088 genes. Here, dN and dS were examined codon-by-codon, using different models of codon substitution that differed in how dN/dS ratios (parameter ω, omega]) varied along sequences, as well as incorporating information about the phylogenetic relationships of the sequences in question so that comparisons are independent (Yang et al. 2000). The M1a (Nearly Neutral), the M2a (Positive Selection), the M7 (Beta), and the M8 (Beta + μ) [3], all them included in the program codeml found inside the PAML 3.15 package were explored. Nested models (M1a - M2a and M7 - M8) were compared using a likelihood ratio test (LRT) in which twice the difference in log likelihood between models was compared with the value obtained under a X2 distribution (degrees of freedom equal to the difference in the number of parameters between models, in this case gl=2). Finally, Bayesian methods were used to determine the probability that a particular codon site fell into the positively selected class. All these analyses used the CODEML program from the PAML package (version 3.4, Yang 1997)

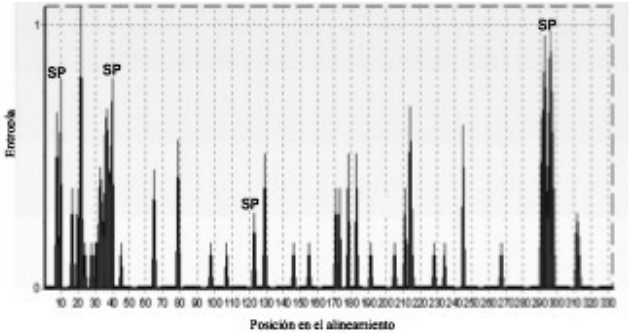
Results

A total of four sites (codons) were detected for nmb0928 and 14 sites were detected for nmb0088 and all are proposed to be under positive selection. No sites with such atributes we detected in the more conserved and smaller nmb1125.

Conclusions

The detection of positive selection in nmb0928 and nmb0088 genes is an additional evidence of the exposition of their respective coded products to the action of the human immune system. Potential explanations in the case of the vaccine candidate NMB1125, for which no significant variability and no positive selection was detected, are that either is very conserved or is not accessible at all to the antibody response elicited by the host during the course of natural infection.

Figure 1: NMB0928 Sequence Variation, SP-positive selection



P122

Mapping immunologically relevant regions of *Neisseria meningitidis* TbpA by phylogenetic analysis and peptide-based arrays

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Meningococcal meningitis is a life-threatening disease. In developed nations the majority of cases are caused by virulent meningococcal strains belonging to serogroup B. Presently there is no effective vaccine against serogroup B strains, as traditional vaccine antigens such as polysaccharide capsules are unusable against this serogoup. The transferrin binding protein A is a potentially ideal vaccine target due to its demonstrated critical role for survival of pathogenic Neisseria in humans.

Objectives

The current study was implemented to explore the reactivity of predicted surface regions of the TbpA protein with various sera and analyze its correlation with the detection of positive selection at codon level, in order to aid in vaccine design and development strategies.

Methods

Regions involved in binding transferrin and hypervariable regions proposed to be under strong immune selection were two types of predicted surface regions selected for this analysis. The former were identified by screening overlapping synthetic peptide libraries representing TbpA with labeled transferring, and the latter were identified through sequence comparisons of a collection of tbpA genes. Both of these regions were primarily localized to predicted surface loops in topology and structural models based on comparisons with other Ton-B dependent outer membrane receptors.

Results

The peptide libraries were screened for reactivity with human convalescent sera or sera produced in mice and rabbits against purified TbpA to evaluate reactivity of these predicted surface regions. The results demonstrated that sera reacted with peptides from the hypervariable regions but there was relatively little reactivity with peptides representing the transferrin binding regions. Through phylogenetic analysis using a Maximum Likelihood based framework we identified 20 codons where clear evidence of positive selection is present. These codons were located in hypervariable domains and close to transferrin binding regions.

Conclusion

In general, while there was good correlation between the presence of positive selection and the human immune response, the lack of such correlation with peptides identified to be responsible of transferrin binding highlights the difficulty of targeting the most promising epitopes. This points to the need of a completely new approach for the development of a truly broad cross-protective neisserial vaccine.

P123

Lacto-N-neotetraose lipooligosaccharide affinity purified human IgG contains four subpopulations; three bind internal oligosaccharides and at least two are bactericidal for serogroup B *N. meningitidis*

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Objectives

We previously reported that affinity-purified human IgG that binds a lacto-N-neotetraose-dependent (LNnT) lipooligosaccharide (LOS) structure is bactericidal for serogroup B *N. meningitidis*. The purpose of this

study was to use gonococcal strain 1291 (L7 equivalent; LNnT) and its pyocin LOS mutants with successive saccharide deletions to further characterize the binding and bactericidal activity of this IgG.

Methods

Human LOS IgG antibodies were affinity purified by passage of Intravenous Immunoglobulin (IVIG) through purified LOS coupled to epoxy-activated Sepharose 6B. LOS from strains 1291 (Galβ1→4 GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1→Kdo) and 1291a (GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1 →Kdo), an lgtB deletion mutant, were used. IVIG also was passed sequentially through 1291a LOS and then 1291 LOS to isolate IgG that required the terminal galactose on LNnT for binding. Whole cell ELISA (WCE) was used to measure the binding of 1291 and 1291a IgG to strains 1291, 1291a, 1291b (L1; Galβ1→4Galβ1→4Glcβ1→4Hep1→Kdo), 1291c (Galb1→4Glcb1→4Hep1→Kdo), 1291d (Glcβ1→4Hep1→Kdo), and 1291e (Glcβ1→4Hep1→Kdo). The bactericidal activity of 1291 IgG, 1291a IgG, and 1291 depleted of 1291a IgG was measured against serogroup B *N. meningitidis* 7946 (L3,7) with human complement.

Results

The subpopulation of IgG that required the terminal galactose of LNnT for binding comprised about 15% of the total 1291 IgG pool. The binding by WCE (Figure 1) of 1291 IgG to the pyocin mutants (expressed as the percentage of the binding of 1291 IgG to strain 1291) was 98 ± 5% for 1291a, 66 ± 8% for 1291b, 72 ± 10% for 1291c, 55 ± 6% for 1291d, and 57 ± 16% for 1291e. The binding by WCE (Figure 2) of 1291a IgG to 1291 and pyocin mutants b-e (expressed as the percentage of the binding of 1291a IgG to strain 1291a) was 88 ± 2% for 1291, 65 ± 3% for 1291b, 66 ± 2% for 1291c, 50 ± 3% for 1291d, and 55 ± 6% for 1291e. The percent survival at 60 minutes of strain 7946 with 25% human complement and 100 μg/ml of LOS specific IgG was 3% with 1291 IgG, 1% with 1291a IgG, and 2% with 1291 depleted of 1291a IgG. Survival with complement alone was 153%.

Conclusions

The IgG that is affinity purified by 1291 LNnT LOS contains at least 4 subpopulations; one that requires the full LNnT structure (1291 wild type), one that binds GlcNAcβ1→3Galβ1→4Glcβ1→4Hep1→Kdo (1291a), one that binds the internal lactosyl structure Galβ1→4Glcβ1→4Hep1→Kdo (1291b,c; note- 1291 wild type does not express the L1 structure), and one that binds the internal glucose Glcβ1→4Hep1→Kdo (1291d,e). While the antibody that requires the full LNnT structure is highly bactericidal for an L3,7 meningococcal strain that expresses LNnT, IgG that binds to internal oligosaccharide moieties is also bactericidal for this strain. We conclude that 1291 IgG contains at least 2 different bactericidal subpopulations and the terminal galactose on LNnT would not be required for the induction of bactericidal antibody by an LOS based vaccine. Ongoing work will determine if antibody that binds the internal lactosyl or glucosyl groups is bactericidal for L3,7 meningococcal strains.

Figure 1:

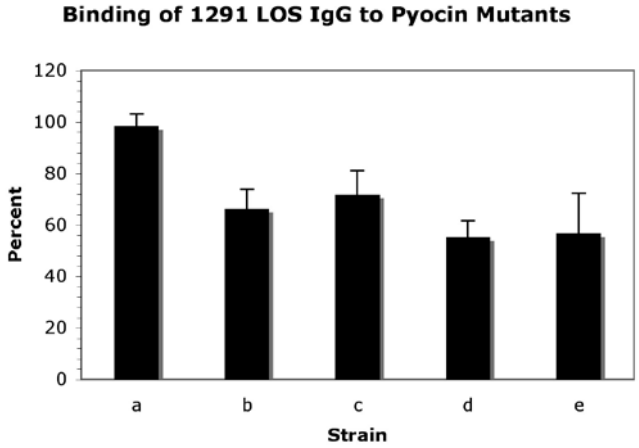
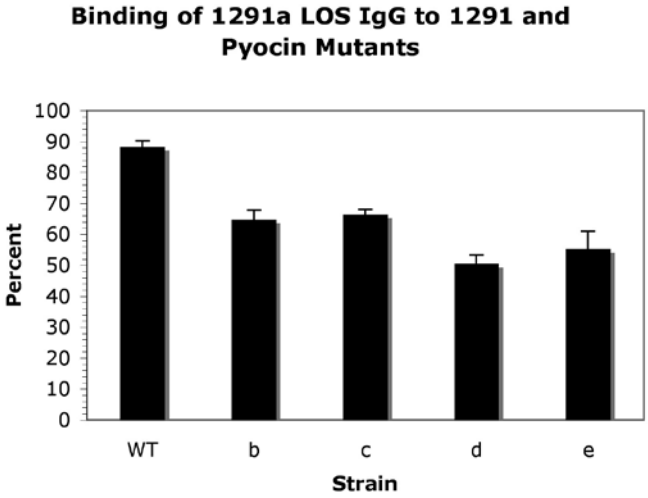


Figure 2:



P124

Structural properties of a multivalent recombinant vaccine against *Neisseria meningitidis*

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Objectives
A multivalent protein vaccine against *Neisseria meningitidis* has shown promising results in early clinical investigations1. The vaccine comprises recombinant NadA antigen (GNA1994) and the fusion proteins GNA2132-GNA1030 (FP-1) and GNA2091-GNA1870 (FP-2). The aim of the present study was to explore structural properties of the vaccine components and investigate their thermal and chemical behaviour in solution.
Methods
Size exclusion chromatography (SEC) coupled with "in line"multi-angle laser light scattering (MALLS), refractive index (RI), and ultraviolet (UV) detection was applied to determine protein molecular mass. The effects of increasing temperature and denaturant concentration (GuHCl), pH, salts and additives on secondary and tertiary structures and folding/unfolding profile were measured by Circular Dichroism (CD) and intrinsic fluorescence spectroscopy. The binding of monoclonal antibodies or mouse polyclonal immune sera to the folded or unfolded protein forms was also studied.
Results
The vaccine components presented a mainly monomeric organisation, with the exception of NadA for which evidence of a trimeric organisation was previously presented2. CD and fluorescence spectroscopy provided valuable tools in the analysis of protein conformation and folding producing consistent results among batches. The secondary structure content calculated for NadA, FP-1 and FP-2 was in broad agreement with the available predicted or solved protein structures. Upon incubation at increasing temperature, a structural transition from a highly α-helical NadA to a more unordered conformation, with mid point at ~35ºC, was observed. In contrast, the incubation at pH ranging from 3 to 10 caused only small changes in the helical profile. FP-1 and FP-2 maintained their conformation up to 50ºC or 6M GuHCl in the

case of FP-1. The proteins' unfolding was not always reversible. Monoclonal antibodies to specific antigens were able to discriminate between unfolded and folded protein forms (anti-NadA, anti-GNA2132), but mouse immune sera (2 doses, 2 μg/ml) recognised the different forms equally.

Conclusion

Solution structural analysis of three meningococcal recombinant vaccine antigens reveals them to contain expected secondary and quaternary structural elements. Folding and unfolding studies have proven useful in better understanding the solution behaviour and antibody binding of immune sera.

References

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2. Martino A., Magagnoli C., De Conciliis G., D'Ascenzi S., Crane D., Chan H., Feavers I.M., Forster M., Bolgiano B. (2007) "Structural characterisation and antibody response of a recombinant protein vaccine against *N. meningitidis*"Meningitis Research Foundation's international conference, Meningitis and Septicaemia in Children and Adults, Royal Society of Medicine, London, UK.

P125

A universal engineered outer membrane vesicle vaccine for prevention of epidemic meningococcal disease in Africa caused by strains from all capsular groups

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Objectives

Explosive epidemics of meningococcal disease in sub-Saharan Africa are generally caused by encapsulated group A strains, but recently strains from capsular groups W-135 or X also have caused epidemics in this region. These latter strains may emerge to become the prominent causes of disease once mass immunization with a group A polysaccharide-protein conjugate vaccine is introduced. The objective of this study was to investigate the molecular epidemiology and vaccine-potential for Africa of prototype engineered outer membrane vesicle (OMV) vaccines that target non-capsular antigens.

Methods

We investigated DNA sequence variability of the factor H binding protein (fHbp) and differences in fHbp expression among capsular group A, W-135 and X strains. We also measured susceptibility of the strains to complement-mediated bactericidal activity of sera from mice immunized with native (non-detergent-treated) OMV vaccines. The vaccines were prepared from mutant group B strains of H44/76 and NZ98/254, which were engineered with LpxL1 mutations to attenuate endotoxin activity and with over-expression of factor H binding proteins in the variant 1 [fHbp-1] or 2 [fHbp-2] groups.

Results

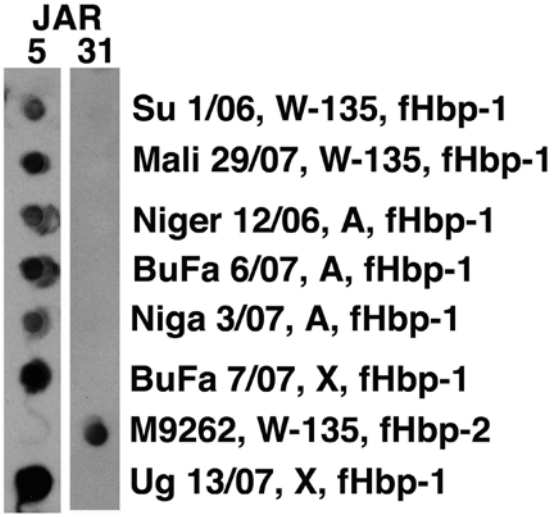
All eight group A isolates, which included strains from each of the clonal complexes responsible for group A epidemics since 1964, had genes encoding fHbp-1. These proteins were highly conserved (≥99.5% amino acid identity). Two group X isolates from Burkina Faso and Uganda, each from different clonal complexes, and three ST-11, W-135 isolates from France, Sudan and Mali, also had genes encoding fHbp-1 with 91 to 95 percent amino acid identity to fHbp-1 of the group A strains. Two other ST-11, W-135 isolates from Burkina Faso and France, had genes encoding fHbp-2 (99.5% amino acid identity to a fHbp-2 from a capsular group C strain). All of the group A, W-135 and X strains with fHbp-1 reacted with anti-fHbp mAb JAR 5 (Figure), which is specific for an epitope on the B domain of fHbp-1, while the ST-11, W-135 isolates with fHbp-2 reacted with JAR 31 (Figure) and 36, which recognize cross-reactive epitopes on fHbp-2 and

fHbp-3. Sera from mice immunized with a monovalent native OMV vaccine prepared from the H44/76 mutant with over-expressed fHbp-1 had bactericidal GMTs ˜1:100 to ›1:1000 (human complement) against all seven group A, X and W-135 strains tested that expressed fHbp-1. A resistant group W-135 isolate that expressed fHbp-2 was susceptible to bactericidal activity of sera from mice immunized with an OMV vaccine prepared from the NZ98/254 mutant with over-expressed fHbp-2 (GMT ›1:200).

Conclusions

All of the group A strains representing each of the major clones spanning 40 years, and recent group X strains, had conserved proteins in the fHbp-1 group, while recent ST-11, W-135 isolates expressed fHbp-1 or fHbp-2. Although additional data are needed, a bivalent native OMV vaccine prepared from mutants with attenuated endotoxin and over-expressed fHbp-1 and fHbp-2 has the potential to elicit broad protective immunity against meningococcal disease in Africa caused by strains from all capsular groups responsible for epidemic disease.

Figure 1: Dot blot activity of anti-fHbp mAbs, JAR 5 and 31



P126

Use of phage display to identify a conserved region of factor H-binding protein (fHbp) that affects the epitope of a protective, cross-reactive mAb

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Objectives

Meningococcal factor H-binding protein (fHbp) is a promising vaccine candidate. The protein binds human factor H (fH), and the presence of fH on the bacterial surface enhances resistance of the organism to complement-mediated serum bactericidal activity. Antibodies to fHbp both activate the classic complement pathway and some also block binding of fH. JAR 4 is an IgG2 mAb from a mouse immunized with fHbp from the variant 1 group (Welsch et al, J Immun 2004). The mAb binds strongly with recombinant fHbps in both the variant 1 or 2 groups but minimally with fHbp in the variant 3 group. JAR 4 does not block

fH binding, and the mAb is not bactericidal with human complement when tested individually. However, JAR 4 elicits cooperative complement-mediated bactericidal activity with other mAbs specific for fHbp in the variant 1 or 2 groups, which individually are not bactericidal (Welsch et al, J Infect Dis 2008). The objective of the present study was to identify residues affecting the JAR 4 epitope, which is important for understanding the basis of cooperative cross-reactive mAb bactericidal activity.

Methods

We screened a library of bacteriophage f1 containing random nonapeptides fused to protein VIII for their ability to bind with JAR 4. The role of the amino acids encoded by the DNA sequences of the positive phage clones in binding with JAR 4 was confirmed by constructing site-specific mutant recombinant fHbps and measuring their reactivity with JAR 4 by ELISA.

Results

The phage display studies identified a tripeptide consensus sequence, DHK, that matched residues 25-27 in the A domain of fHbps in the variant 1, 2 or 3 groups. To date we have performed mutagenesis of two of these residues, which were individually changed to alanine in fHbp from the variant 1 group (gene from MC58). As compared with the wild-type recombinant fHbp, the mutant containing the H26A substitution showed a ˜50 percent decrease in JAR 4 reactivity, while introduction of K27A completely eliminated JAR 4 binding. Both mutants showed identical concentration-dependent binding with two other mAbs, JAR 5 and mAb 502, which are specific for epitopes in the B and C domains, respectively.

Conclusion

A region of the A domain containing the DHK tripeptide is necessary but not sufficient for expression of the JAR 4 epitope since the tripeptide is conserved across fHbp in all three antigenic variant groups while JAR 4 reacts primarily with fHbp in the variant 1 or 2 groups. A truncated fHbp containing only the combined B and C domains was reported to elicit bactericidal activity against strains expressing fHbp from the homologous antigenic variant group. However, since JAR 4 elicits cooperative bactericidal activity with second mAbs reactive with amino acid residues in the B or C domains, antibodies to the A domain also contribute to bactericidal activity. The presence of the A domain, therefore, may be useful for eliciting cross-reactive bactericidal anti-fHbp antibodies against strains from different antigenic variant groups, for example, antibodies elicited by recombinant, chimeric fHbp vaccines.

P127

Heat shock protein complex (HspC) enriched vaccine induces antibody-mediated opsonophagocytosis, indicative of cross strain protection against *Neisseria meningitidis*

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Objectives

Heat shock proteins (Hsp) play a pivotal role in the immune response to infection. Hsp form complexes with pathogen antigens (HspC) that target antigen presenting cells of the immune system resulting in a broad immune response against the target pathogen. The objective of this study was to test the ability of a novel *Neisseria meningitidis* B (MnB) vaccine derived from heat-shocked *N. meningitidis* MC58, to establish cross strain immunological protection. Sera derived from vaccinated mice were tested against a representative panel of seven MnB strains.

Methods

Plate cultures of an acapsulate variant of strain MC58 were heat shocked at 44oC and killed by treatment with the antibiotic gentamicin. Cell pellets were processed to produce two preparations enriched for

heat shock protein complexes (HspC). The presence of Hsp65 (GroEL) and Hsp70 (DnaK) was confirmed by Western blotting. The vaccines were used to immunise mice (10 per group) to generate sera for assessment of cross strain responses. The following groups were established, non-vaccinated control, H44/76 OMV vaccinated (positive control) and two MC58 vaccine immunised groups. Sera (obtained from terminal bleeds) were pooled and then assessed for their ability to elicit antibody-mediated opsonophagocytosis (OP) using the following MnB strains; MC58, H44/76-SL, M01-240101, M01-240013, M01-240149, M01-240185 and M01-240355. For the OP assay, serum samples were incubated with killed bacteria (BCECF-stained) and IgG-depleted baby rabbit complement for 7.5 min at 37oC. HL60 cells differentiated by treatment with 0.8% DMF were added and the samples incubated for an additional 7.5 min before the addition of ice cold DPBS (containing EDTA) to stop the reaction. Samples were analysed by flow cytometry and data expressed as a fluorescence index value.

Results

For all strains, serum from mice vaccinated with the MC58- derived HspC enriched vaccine, induced OP responses that were significantly greater than those obtained with serum from non-vaccinated controls. The control H44/76 OMV vaccine, as expected, generated higher OP values than the HspC vaccine against MC58 and the H44/76 strain from which the OMV vaccine was derived. However, for all other strains (M01-240101, M01-240013, M01-240149, M01-240185 and M01-240355) the MC58-derived HspC vaccine generated OP values that were significantly greater than those obtained using the OMV vaccine, indicating possible cross-strain protection.

Conclusion

We have demonstrated that vaccination with a HspC enriched vaccine derived from *N. meningitidis* MC58, induced significant opsonophagocytic activity against strains representing the meningococcal clonal complexes responsible for the majority of MnB disease in developed countries.

P128

Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: further utilization of amidase from *Dictyostelium discoideum* to prepare glycoconjugates

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Previous studies in our laboratories had identified the potential of LPS-based vaccines to combat meningococcal disease, but subsequent studies intended to improve and afford a more robust and consistent conjugation strategy had failed to produce protective antibodies. The present study describes a novel conjugation strategy that still targets the terminal glucosamine disaccharide as the point of attachment to the carrier protein, and we have further developed the use of amidases produced by the slime mould *Dictyostelium discoideum.* This methodology has facilitated a more robust conjugation strategy which has led to glycoconjugates with much improved carbohydrate loading. These 'high-loading' conjugates were immunised into mice and rabbits and the sera will be evaluated for cross-reactivity and functional activity.

P129

Immunological and physical-chemical characterization of meningococcal group A conjugates prepared by modified reductive amination

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Objectives
This work describes the development of meningococcal group A conjugates (MenAPS) by modified reductive amination, using hidrazide-activated tetanus toxoid as a carrier protein. Current methodology was modified to reduce time of reaction allowing scale-up in near future.
Methods
We use spectroscopy (NMR) and mass spectrometry results to characterize the oxidation and conjugation steps. The identity and presence of aldehyde groups in MenAPS were evaluated by 1H NMR 1D spectroscopy at 400 MHz and 25ºC. The proton spectra of all compounds were fully assigned by combination of homo (native and conjugated MenAPS; 1H NMR 1D) and heteronuclear (oxidized MenAPS) 2D NMR (Hsqc) methods. Phosphate ion mass spectrometry was done to evaluate the integrity of polysaccharide after oxidation and conjugation reactions. These steps were followed by SEC. The mixtures were diafiltrated to remove unconjugated polysaccharides and concentrated by tangential flow ultrafiltration. Total sugar and free polysaccharide and protein contents in the intermediate products and the final conjugates were determined by HPAEC-PAD and Lowry methods, respectively. Immunogenicity of the conjugate lots was evaluated by dose-response studies in Swiss mice (i.m. injection with 0.1, 1 and 10 μg/dose of conjugates or plain polysaccharide) using Al(OH)3 as adjuvant, three times with 15 day interval. Serum samples were assayed by ELISA for IgG antibodies against MenAPS and bactericidal activity.
Results
The periodate oxidation of native MenAPS in aqueous media in pH 5.5 (NaIO4 - 23.4 mM) elicited an oxidative cleavage on C3-C4 bond of α-D-mannopyranoside phosphate to yield the corresponding oxidized MenAPS. We observed two signals at 8.44 and 8.47 ppm in the 1H NMR spectrum. This result is in agreement with the oxidation of the respective hydroxyl group at the same position and showed also that the integrity of PS was kept. The oxidation reaction yield was 75% as calculated by HPAEC-PAD. The results obtained by SEC analysis are in good agreement with NMR data. The integrity of oxidized MenAPS was also shown by mass spectrometry where the phosphate ion was kept. The formation of conjugate was clearly demonstrated by the disappearance of the assignments at 8.44 and 8.47 ppm present in the activated-MenAPS corresponding to aldehyde groups, after the conjugation reaction. The NMR spectra of MenAPS-TT also showed the same assignments described for the native and activated MenAPS. In addition other assignments were observed at 6.00-8.00 and 1.8-0.5 ppm corresponding to the protein aromatic and aliphatic amino acids incorporated, respectively. The free polysaccharide content was around 12% as measured by HPAEC-PAD. The conjugate induced high IgG levels and bactericidal activity showing the efficacy of conjugation.
Conclusion
Altogether, these results show the group A conjugate is potentially useful for human vaccine warranting its eligibility for phase I clinical trial.

P130

Protective anti-N-propionyl *Neisseria meningitidis* group B polysaccharide monoclonal antibodies are reactive with non-capsular antigens expressed by group A, B, C, and W135 strains

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Objectives
To determine whether monoclonal antibodies (mAbs) elicited by immunization with a N-propionyl *Neisseria meningtidis* group B polysaccharide (N-Pr NmB PS)-tetanus toxoid conjugate vaccine recognize capsular or non-capsular antigens and, if non-capsular, whether the antigens are expressed by strains from other meningococcal capsular groups.
Methods
The antigenic specificity of the mAbs was determined by direct binding and inhibition ELISA with capsular polysaccharide derivatives. mAb binding to wild-type group A, B, C, W135, X and Y strains and non-encapsulated, polysialyl transferase knock out (STKO) group B and C strains was measured by flow cytometry. Bacteria were cultured in Muller-Hinton (MH) media or chemically defined media (CDM) without or with supplements that can modulate the expression of sialic acid antigens. The ability of the mAbs to mediate serum bactericidal activity (SBA) and activation of complement protein deposition against wild-type strains was determined using human complement.
Results
The mAbs SEAM 2, 3, 12, and 18 were elicited by immunization with an N-Pr NmB PS-tetanus toxoid conjugate vaccine where the antigen consisted of a mixture of N-propionyl and ¯14% neuraminic acid (Neu) residues. SEAM 3 is specific for a PSA tetramer containing a single Neu residue that is likely at the non-reducing end. Although none of the mAbs bind to PSA, all four mAbs bind to PSA derivatives containing Neu and one or more of several N-acyl modifications including N-acetyl, N-propionyl, N-trichloroacetyl, N-glycoyl, N-acryl, but not to group C or W135 or Neu-containing C or W135 PS as determined by ELISA. By flow cytometry the four mAbs ([mAb]=20 μg/ml) are reactive with group A, B, C, and W135 strains grown in MH but not with X or Y strains. There are differences in binding to bacteria grown in MH compared CDM but the differences are variable. For example SEAM 2 binding generally increases when the bacteria are grown in CDM while SEAM 18 binding is unaffected or decreased in CDM. Supplements to CDM including NeuNAc, NeuNGc, CMP-NeuNAc or PSA have no significant effect on binding. While none of the mAbs bind to a group B STKO strain, both SEAM 2 and 18 bind to a group C STKO strain. Antigens identified by the mAbs do not appear to be associated with LOS as none of the mAbs showed binding on Western blots to LOS resolved by SDS-PAGE. SEAM 2, 3, and 18 showed SBA against group B and C strains but not against W135 despite strong binding and activation of complement protein (C3, C4, C6-C9) deposition equivalent to a bactericidal anti-group W control mAb. SEAM 12 SBA was group B-specific but the mAb did activate complement on a group W135 strain.
Conclusion
Since all four mAbs are highly specific for poly α-(2→8) PSA antigens it appears that *Neisseria meningtidis* either express or acquire externally non-capsular α-(2→8) sialic acid antigens, possibly containing Neu. Vaccines that elicit antibodies reactive with the non-capsular antigens, therefore, have the potential to protect against strains from other capsular groups in addition to group B.

P131

Improved immunogenicity of a group B outer membrane vesicle-based vaccine after the incorporation of a recombinant antigens

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Objectives
Outer membrane vesicles (OMV) vaccines against *Neisseria meningitidis* serogroup B have been efficacious in humans. Although the use of OMV-based vaccines in single-strain epidemic scenarios is generally accepted, it is also understood that this approach is likely to be of limited benefit for endemic serogroup B disease, due to the rapid evolution of antigenic variants among meningococcal populations. In order to increase the cross-immunogenicity of OMV vaccines several strategies have been explored, including the enrichment of OMV with minor conserved meningococcal antigens. We have previously reported the potential of one of these antigens, lipoprotein NMB0928, as a candidate for a cross-protective vaccine. However, NMB0928 is poorly represented in the OMV preparations as shown by immunochemical and proteomic assays. In the present study, we propose a novel strategy to increase the immunogenicity of a group B OMV-based vaccine by incorporating this recombinant antigen.
Methods
In this work we are introducing a method for the incorporation of the recombinant protein NMB0928 into serogroup B OMV, where this antigen forms, by co-folding, a complex with the preparation of outer membrane proteins. The new formulation was used to immunize mice using aluminum hydroxide as adjuvant. Groups of 10 BALB/c mice were immunized subcutaneously with 20 μg of said combination, 20 μg of the recombinant protein or 20 μg of OMV alone. The animals were boosted on days 7, 14 and 28 of the immunization schedule. The animals were bled on days 0 and 38, and the sera were evaluated by immunoassays.
Results
After immunization of mice with the OMV vaccine overloaded with recombinant NMB0928, a broader cross-reactivity with diverse meningococcal strains was found for the antibodies generated against the complex, compared to the response detected for the antisera elicited against the OMV alone. Antiserum from mice immunized with the modified OMV vaccine also elicited greater deposition of human C3 complement on the surface of live *N. meningitidis* bacteria and greater protective activity against meningococcal bacteremia in infant rats.
Conclusion
This work demonstrates the feasibility of this strategy in eliciting improved antibody levels and a protective response against homologous and heterologous neisserial strains. Our method is also a solution to increase the amount of lipoproteins on the surface of OMV, since these antigens appear to be affected after the extraction steps with detergents.

P132

Development of an immunisation strategy to optimize the bactericidal antibody response to meningococcal PorA

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Objectives

Due to concerns about the use of the *Neisseria meningitidis* serogroup B polysaccharide capsule, the search for a vaccine providing comprehensive protection against meningococcal disease has predominantly focussed on outer membrane proteins. These antigens are either delivered as purified proteins or in outer membrane vesicles (OMVs). The aim of this study was to optimise the bactericidal response to protein antigens that can be achieved using these delivery methods and licensed adjuvants. This study focuses on the PorA antigen as this has been shown to be a major immunogen after both disease and vaccination.

The main objectives of this study were:

- To compare the bactericidal responses to the PorA antigen after priming and boosting with various combinations of OMV and purified PorA protein components.
- To compare the effects of aluminium hydroxide adjuvant and MPL adjuvant on these bactericidal responses.

Methods

NIH mice (10 per group) were immunised with primed and boosted with different combinations of OMVs (NZ98/254) and purified PorA (P1.7-2,4), with appropriate controls. Either aluminium hydroxide (Alum) or MPL adjuvant was used. Sera were tested by Serum Bactericidal Assays (SBAs) and by whole cell ELISA to compare total IgG, IgG1, IgG2a and IgG2b titres.

Results

The results showed that the presentation of the antigen used to prime the immune system had a larger effect on the bactericidal response than the presentation of the antigen used as the booster. SBA titres were higher in groups primed with the OMV vaccine, particularly when boosted with purified protein (SBA titre ≥1:2048). When Alum adjuvant was used sera from groups primed with purified protein showed very little bactericidal activity (SBA titre ≤1:8). Use of MPL adjuvant generally resulted in improved bactericidal activity for all groups, although groups primed with OMV vaccine still showed higher SBA titres than groups primed with purified protein. SBA titres positively correlated with the levels of IgG2a and IgG2b, and negatively correlate with levels of IgG1. Use of MPL adjuvant generally induced higher levels of IgG2a and IgG2b. Groups primed with purified protein (with either adjuvant) showed high levels of IgG1, blocking the effects of IgG2a and IgG2b, and so these groups showed a reduced bactericidal activity when compared to groups primed with OMV.

Conclusions

- For both adjuvants priming with an OMV vaccine and boosting with purified PorA was the most effective (of all combinations tested) for producing bactericidal antibodies. This suggests that it is possible to improve the bactericidal response elicited by OMVs by targeting PorA with the booster dose.
- The use of MPL adjuvant resulted in higher bactericidal activity, presumably by ensuring a Th1 type response predominated.

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Prevalence and variations for five genes encoding antigens included in a novel vaccine against meningococcal disease

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Neisseria meningitidis colonises the upper respiratory tract in 8-25% of the human population as a commensal. Occasionally the bacteria disseminate to the bloodstream causing septicemia with or without meningitis, with a mortality rate around 10% despite modern medical treatment. To be able to reduce meningococcal disease ongoing challenging work to generate a broad-range meningococcal vaccine is under progress. By the reverse vaccinology strategy a number of potential candidate antigens for such a vaccine were revealed. Recently a new vaccine was described, namely, the 5 Component Vaccine against Meningococcus B (5CVMB) containing a mixture of the New Zealand OMV vaccine and five genome derived antigens (GNA), i.e. a fusion protein of GNA1870 and GNA2091, a fusion protein of GNA2132 and GNA1030, and GNA1994.

Objectives

Determine the gene prevalence and sequence variations as well as the deduced amino acid sequences of five genes encoding potential vaccine candidates (GNA1030, GNA1870, GNA1994, GNA2091 and GNA2132).

Methods

The analysed isolates represents all identified invasive isolates collected in Sweden during 2001 and 2002 (n=95). The isolates were characterised by serogroup, serotype, genosubtype, multilocus sequence type and antibiogram. The five genes encoding vaccine candidates were amplified using primers external to the coding region and subsequently sequenced. Multiple-sequence alignments and phylogenetic analysis of the nucleotide sequences encoding the mature proteins as well as the deduced amino acid sequences of the mature proteins were performed. The numbers of synonymous and nonsynonymous substitutions and sites, the p distances, the overall mean distance, the intra-group as well as the inter-groups mean distances for the individual genes were calculated.

Results

Four of the examined genes were present in all *N. meningitidis* tested. *gna1994* was only present in 38% of the analysed Swedish isolates.

The extent of sequence variation in the different genes varied substantially. In GNA1030 93% of the deduced amino acids were conserved in all isolates. Corresponding number for GNA1870 was 56%; GNA1994, 82%; GNA2091, 92% and GNA2132, 54%.

The phylogenetic analysis of GNA1870 identified 28 different amino acid sequence types, which clustered into three main variants. GNA1994 was divided into five different amino acid sequence types, clustering into two main variants. GNA2132 showed 20 different amino acid sequence types, no main clustering patterns could be seen. Fourteen and six different amino acid sequence types were found for GNA1030 and GNA2091, respectively. The amino acid conservation was relatively high for these last two antigens, which did not cluster into any obvious main variant.

Conclusion

The prevalence in Sweden of the genes encoding the mature protein of the five antigens included in the 5CVMB vaccine was high. Thirty-six per cent of the isolates encoded all five vaccine antigens, 37% four and 27% three of the included antigens, which makes the theoretical coverage appealing.

The level of amino acid conservation differed between the genes but was relatively high in GNA1030, GNA2091 and within the main variants of GNA1870 and GNA1994. GNA2132 appeared to be a more diversified antigen.

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Safety and Immunogenicity of a new Meningococcal A conjugate vaccine in a healthy African population aged 2-29 years

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Introduction
The new conjugate meningococcal A vaccine (PsA-TT), manufactured by Serum Institute of India Ltd Pune - India has shown to be safe and highly immunogenic in clinical phase I in India. In African children aged 12-23 months one dose of PsA-TT vaccine was able to prime memory, as well as superior immunogenicity as compared to a polysaccharide vaccine. The vaccine is currently being tested in a phase II/III clinical study in an African population 2-29 years of age.
Methods
A total of 900 participants were recruited in The Gambia, Mali and Senegal and were randomized to receive either a single intramuscular injection of PsA-TT vaccine. The primary objective of the study is to evaluate the immunogenicity of a single injection of PsA-TT vaccine during 4 weeks post-vaccination with comparison to the men A component of the tetravalent polysaccharide vaccine. The immunogenicity responses are evaluated in terms of serum bactericidal antibody (rSBA) activity (rabbit complement) and anti-polysaccharide group A (anti-PsA) IgG levels. Safety is assessed through an active and daily follow-up for 4 days after vaccination. All Adverse Events are collected up to 4 weeks after vaccination and Serious Adverse Events are collected for the entire study duration (1 year). The study is conducted according ICH/ GCP guidelines.
Results
At 4-weeks local and some systemic post-immunization reactions were reported more frequently in the PsA-TT group than in the Ps vaccine group (Tenderness 4,8% vs. 1,4% ; Induration 1,2% vs. 0, 3%; Fever 3% vs. 1,7%; Headache 11,2% vs. 10,7%; Diarrhea 1% vs. 0,7%). Whereas other systemic post-immunization reactions were reported more frequently in the Ps vaccine group than in the PsA-TT group (Vomiting 1,7% vs. 1,3%; Lethargy 2% vs. 1,5%; Fatigue 5,1% vs. 1,5%; Irritability 1 % vs. 0). However, none of these differences are statistically significant. The proportion of subjects with at least one Adverse Event reported during the 4-weeks study period are the same in both vaccine groups (9%). No Serious Adverse Events were reported during the 4-weeks study period. A significantly higher proportion of subjects in the PsA-TT group had a 4-fold increase of antibody titers measured with both rSBA and ELISA with respect to subjects in the Ps group (rSBA 78% vs. 46%; IgG ELISA 89% vs. 60%), as well as significantly higher rSBA GMTs titres (4713 vs. 1191) and ELISA GMCs (67 vs. 13). These differences are consistently observed across all age-groups and in the 3 countries.
Conclusions
The results of the present study shows that the new conjugate meningococcal A vaccine PsA-TT vaccine is as safe and well tolerated as a licensed widely used polysaccharide vaccine in subjects 2-29 years old residing in the African meningitis belt countries. PsA-TT vaccine showed higher immunogenicity profile than the Men A component of the licensed tetravalent polysaccharide vaccine at 4-weeks after vaccination. These findings support the approach for a large scale introduction of PsA-TT vaccine in the African meningitis belt for elimination of epidemic meningitis.

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Comparative application of modern molecular methods for *Neisseria gonorrhoeae* typing

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Objectives
The choice of adequate epidemiological method remains an actual problem of *N. gonorrhoeae* (NG) molecular monitoring. A collection of geographically unrelated gonococci isolated in Russian clinics was studied by por-typing, NG-MAST (*Neisseria gonorrhoeae* Multi Antigen Sequence Typing), MLST (Multilocus Sequence Typing) and MALDI-TOF MS (Matrix Assisted Laser Desorbtion/Ionization Time of Flight Mass Spectrometry) direct bacterial profiling to compare the applicability of these different systems.
Methods
Gonococci were grown under standard conditions (ASM, IHO). Single colonies of fresh bacterial cultures were analyzed by MS using saturated solution of alpha-cyano-4-hydroxy cinnamic acid in 50 % acetonitrile/2.5 % TFA as matrix. Mass spectra were collected on a Microflex LT MALDI-TOF mass spectrometer, followed by analysis with the MALDI BioTyper 1.1 software (Bruker Daltonics, Germany). NG genomic DNA was isolated by DNA express kit (Lytech Ltd, Russia). Por typing, NG-MAST and MLST were done by nucleotide sequencing as described earlier (Viscidi, 2004; Martin, 2004; Bennett, 2007). The por type of a particular isolate was determined by identification of the porB1 gene nucleotide sequence in NCBI databases. The sequence type (ST) was established in accordance with NG-MAST and MLST databases. The discrimination power for each typing method was calculated using the Hunter-Gaston discriminatory index (HGDI).
Results
Totally about hundred clinical NG isolates were investigated. Collected MS profiles were rather homogeneous with slight differences between isolates. While the majority of peaks were constant the three ones with m/z 4473, 5051, and 8165 changed their m/z value to 4487, 5081, and 8146, respectively, for several strains. Based on m/z values of these peaks combinations - 4473/5051/8165; 4487/5051/8165; 4487/5051/8146 and 4473/5081/8165 the NG strains were divided into four different groups named as proteotype I, II, III and IV. It should be mentioned that the most clinical strains as well as strain ATCC 49226 belonged to the first group corresponding to wild type sequence. Expectedly the discrimination power was found low for this typing method (HGDI=0.32). Based on serotyping data there were 12 different groups found and the calculated HGDI was 0.82. Por-typing, NG-MAST and MLST allowed divided all strains into 46, 55 and 21 genotypes, and the discriminatory index was shown 0.96, 0.98 and 0.87, respectively. A third of strains carried new porB1 alleles, and more than half samples had new ST by NG-MAST or MLST. Interesting the only MLST data revealed any correlation with geographical distribution of NG strains.
Conclusion
Among modern molecular techniques tested only genotyping systems are suitable for NG molecular monitoring. Moreover the MLST seems to fit for broadcast epidemiological studies while the NG-MAST is more appropriated for local outbreak investigations.

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The changing incidence of meningococcal invasive infections across Canada, IMPACT 2002-2007

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Objectives
Meningococcal infections rank second in Canada among life-threatening bacterial infections in children, adolescents and adults. Universal meningococcal conjugate C infant and adolescent immunization programs were started in 3 provinces in 2002-2003, followed by the remaining provinces in 2005. Our active surveillance network provides the most recent information on meningococcal serogroups in Canada since the implementation of universal meningococcal immunization programs.
Methods
Active population based surveillance was conducted across Canada by the 12 centers of the Immunization Monitoring Program, Active (IMPACT) for hospital admissions in all ages related to *Neisseria meningitidis* from January 2002 - December 2007. Case definition required the isolation of meningococcus or positive PCR test from a sterile site. Annual audits for 2007 were still being completed, so 2007 numbers are not included in trend analyses and incidence rates. Population data were obtained from local public health estimates or from 2006 national census data. All incidence rates are reported per 100,000.
Results
A total of 499 cases were reported; 265 in children (‹20 years) and 234 in adults. Annual totals decreased from 104 in 2002 to 61 in 2007, but the decreasing trend (from 2002-2006) was significant only in adults ($X2=9.9$ $p=0.04$). Overall, incidence ranged from 0.62 (95%CI 0.50 to 0.76) in 2002 to 0.42 (0.32 to 0.53) in 2006. The highest rates were seen in children ‹1 years followed by children 1-4 years. Rates within each age group did not vary significantly over the 5 years.
Serogroups B, C and Y caused the majority of invasive meningococcal disease in Canada, but the distribution of the three serogroups varied significantly among adults and children ($p<0.001$) and by province ($p<0.001$). Serogroup B occurred more frequently in children while serogroups C and Y occurred more frequently among adults. Incidence of group C disease decreased significantly over the 5 years from 0.23 (0.16 to 0.32) in 2002 to 0.08 (0.04 to 0.14) in 2006, whereas incidence remained stable for groups B, Y and W135. The decrease in C is primarily the result of a substantial decrease in provinces that started earlier vaccination programs (British Columbia, Alberta and Quebec), from 0.41 (0.28 to 0.60) in 2002 to 0.07 (0.02 to 0.16) in 2006. In these provinces, incidence decreased across all age groups, and was significant in adults. Provinces that initiated programs in 2005, showed stable rates for serogroup C for 2002-2006.
Conclusion
Incidence rates of group C disease have shown a 6-fold decrease in provinces that were the first to establish universal infant immunization. Rates decreased in children and adults.

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LP2086 and MLST distribution in epidemiologically relevant strains of serogroup B *Neisseria meningitidis*

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Objective

LP2086, also known as Factor H binding protein (fHBP), is a 28 kD lipidated surface protein of *N. meningitidis.* Recombinant forms of this protein are under development as a vaccine against serogroup B meningococcal disease. Our previous work has shown that fHBP occurs in two forms, subfamily A and subfamily B [1]. There is only 60-75% amino acid identity between subfamilies, but greater than 83% identity within subfamilies. To gain a better understanding of the epidemiological distribution of fHBP in a large collection of recent clinical isolates, we undertook a systematic survey of 1,263 serogroup B strains causing invasive disease in Europe and the US.

Methods and results

Sequence of fHBP was determined for strains from the US ABC surveillance sites for 2000-2005, and for a subset of strains from four national surveillance centers in Europe (UK, Norway, France, and the Czech Republic) during the years 2001-2006. MLST analysis was performed for most isolates. The fHBP gene was detected in all 1,263 isolates including one isolate for which the gene contained a premature stop codon. There were 143 unique fHBP variants (62 subfamily A and 81 subfamily B) identified. Overall, 71% of strains contain a subfamily B fHBP and 29% a subfamily A fHBP, a ratio that was similar in all five countries surveyed. Similar to what has been shown previously for PorA and transferrin binding protein distributions [2,3], MLST is not a predictor for fHBP type. Most fHBP variants are found in strains belonging to multiple MLST clonal complexes (eg A19 is found in 6 different complexes), and multiple fHBP variants are found within each of the common complexes. All major MLST complexes include both A and B subfamily strains. This is particularly evident in the ST-41/44 complex, which contains about an equal number of subfamily A and subfamily B isolates, and is genetically quite diverse.

Conclusion

Both MLST and *fhbp* sequencing data showed high but not congruent diversity among a large collection of invasive meningococcal B isolates from different countries and spanning several years. This would reflect the free recombination in this bacterium. These results corroborate the need for large collections of representative isolates to estimate vaccine coverage.

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How to for meningococcal disease in the era after conjugated serogroup C vaccination?

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Background and aims
After introduction in 2002 of routine vaccination for children aged 14 months and a catch up campaign for all 1-18 year olds (coverage ±94%) in June-November 2002 with the conjugated meningococcal C vaccine, an intensified surveillance of meningococcal disease was implemented in the Netherlands. We studied the epidemiology, microbiology, and outcome in relation to early disease evolution and presenting clinical manifestation at admission of all patients with invasive meningococcal disease in the Netherlands, directly after the introduction of Men-C mass vaccination. The aim was to identify opportunities to improve the timeliness of health care delivery to patients with meningococcal disease.
Methods
From January 2003 until May 2005 a voluntary electronic questionnaire was linked to the web-based notification system and combined with microbiological laboratory results. Information was gathered on patient characteristics including vaccination status, the time of first disease symptoms, the time of appearance of haemorrhagic skin lesions, the time of hospital admission, administration of antibiotics before admission, the clinical manifestation at admission, and the outcome.
Results
From January 2003 until May 2005, 752 patients with meningococcal disease were notified. In 58 patients (8%) serogoup C was found, all serogroup C cases occurred in unvaccinated individuals. Serogroup B caused 88% of cases. The case fatality rate (CFR) was 6.7% overall, but reached 16% among adults over 50 years. CFR was similar for serogroup B (6.3%) and C (5.2%).
Admission followed median 17 hours after onset of symptoms, and occurred faster in the 1-3 year olds (12 hrs), than in the ‹1 year olds (22.5 hrs), 4-18 year olds (19 hrs) and in ›18 year olds (20.5hrs). The CFR in patients admitted within 12 hrs, 12-18 hrs, 18-36 hrs or ›36 hrs after first symptoms, was 10.2, 7.8, 3.5 and 2.2%, respectively. Haemorrhagic skin lesions - i.e. petechiae or purpura - were seen on admission in 363/601 (60%) patients and more frequently in children aged 1-3 (74%) and 4-18 (74%) than in young infants (48%) and adults (45%).
Conclusions
We showed that after the highly effective introduction of men-C mass vaccination, no cases were reported in previously vaccinated persons. Nevertheless, serogroup B meningococci, for which no vaccine is available yet, still cause more than 200 cases of severe invasive disease in the Netherlands each year. Meningococcal infections cause disease not only in children but also in adults. Timely admission, early enough to save lifes, requires inclusion of non-petechial clues in the diagnosis. A rapid course correlates to a poor outcome. Simple information about the duration of disease before admission, can be of help in the appraisal and recognition of severe cases.

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The association of respiratory tract infection symptoms and air humidity with meningococcal carriage in Burkina Faso

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Objectives
To evaluate risk factors for meningococcal carriage and carriage acquisition in the African meningitis belt, comparing epidemic serogroup A (NmA) to non-epidemic serogroups.
Methods
During the non-epidemic meningitis season of 2003, pharyngeal swabs were taken via the mouth at five monthly visits in a representative population sample (N=488) of Bobo-Dioulasso, Burkina Faso (age 4-29 years). Swabs were analysed by culture and isolated meningococci grouped by PCR and immune serum testing. Participant characteristics were collected by standardized questionnaires. In 2006, a similar study was performed in 624 individuals (age 1-39 years) during an NmA meningitis outbreak in the rural area close to Bobo-Dioulasso. We evaluated serogroup-specific risk factors for carriage, acquisition and clearance using multivariate multilevel random effects models in multivariate logistic and Poisson regression, and a Cox proportional hazard model.
Results
During the non-epidemic meningitis season in 2003, air humidity (20-39% and ≥40%, compared to ‹20%) during the month before swabbing was positively associated with carriage acquisition of non-groupable meningococci (odds ratio (OR) 2.18 [1.28-3.71] and 1.55 [0.80-3.02]) and inversely with carriage clearance (hazard rate ratio 0.61 [0.27-1.37] and 0.27 [0.10-0.76]. Carriage of virulent serogroups (W135, X, Y) was associated with sex (females, OR 0.32 [0.12-0.91]) and age 10-19 years (compared to 4-9 years, OR 0.29 [0.09-0.95]). During the epidemic in 2006, current carriage of the NmA outbreak strain was significantly associated with recent sore throat (odds ratio, 95% confidence interval [OR, 95% CI], 3.13 [1.65-5.96], current rhinitis (OR 2.72 [1.35 - 5.51]), recent meningitis in the family (OR 2.50 [1.32-4.71]), age (1-3 years compared to 4-9 years (OR 0.22 [0.09-0.54]), female sex (OR 0.41 [0.24-0.69]), and five or more persons sharing the bedroom (OR 0.43 [0.21-0.85]). NmY carriage was positively associated with crowding and cigarette smoke exposure.
Conclusion
Humid climate may favour carriage of non-encapsulated meningococci, thereby decreasing the risk of invasive disease at the end of the meningitis season. Respiratory tract infection epidemics may increase meningococcal transmission and contribute to epidemic risk in addition to hyperendemic disease during the meningitis season in the meningitis belt. These findings may help identifying interventions against hyperendemic and epidemic meningococcal meningitis due to non-vaccine serogroups.

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Serogroup X meningococcal meningitis in Togo during 2007 and 2008

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Objectives
To describe group-specific distribution of meningococcal meningitis in Togo, a country on the southern border of the meningitis belt.
Methods
During the 2007 and 2008 meningitis seasons, we conducted hospital-based sentinel surveillance in 4 towns situated in 3 regions of Togo: Tone (Savanes region) in the North, Kara (Kara region), Sokodé and Sotouboua (Central region). Cerebro-spinal fluid samples from all suspected meningitis cases were analysed by PCR in Burkina Faso (AMP/Centre Muraz laboratory, Bobo-Dioulasso) for confirmation and genogrouping of *Neisseria meningitidis* (Nm). Isolated meningococci were serotyped and genotyped by multilocus sequence typing (2008 isolates ongoing), at the IMTSSA, Marseille, France (CCOMS).
Results
During 2007 and 2008 (data available until February 2008, analyses on following weeks ongoing), 539 (123) suspected cases were included and 513 (121) tested by PCR, of which 58% (26%) were positive for Nm. In 2007, two sentinel sites declared epidemics: in Savanes region, 89% of 219 Nm cases were due to NmA (attack ratio 90/105), and in Kara region, 86% of 92 Nm cases were due to NmX (attack ratio 40/105). In the Central region sites with endemic incidence, cases were exclusively due to group W135, X, Y meningococci and non-groupable strains. NmA strains were sero(sub)type 4:P1.9, sequence type (ST-) 2859, and NmW135, NT:P.1.5,2, ST-2881. No data on NmX are available. In 2008, preliminary results reveal: of the 32 Nm cases, 31% were due to NmA (exclusively in the Savanes region), 25% to NmW135 (equally distributed) and 38% to NmX (mainly in Central region). Overall during 2007 and 2008, 11% and 33% of meningococcal meningitis cases in Savanes region were due to groups other than A, while 100% in the other regions were non-A.
Conclusion
NmA was the main causal agent of meningococcal meningitis in northern Togo, where meningitis shows a distinct seasonal incidence pattern with hyperendemic disease or epidemics during the dry season. In the more southern areas, where seasonality is less pronounced, NmW135 and NmX are responsible for endemic disease and outbreaks. Togo joins Ghana and Niger among African meningitis belt countries experiencing documented serogroup X epidemics.

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Comparison of PorA phenotyping and genotyping of *Neisseria meningitidis* - implications for epidemiological surveillance

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Traditionally, the characterization of *Neisseria meningitidis* has involved the analysis of cell surface structures, such as capsular polysaccharide by latex agglutination, co-agglutination and outer membrane

proteins (OMPs) by enzyme-linked immunosorbant assay (ELISA) methods. Developments in DNA sequencing, combined with limitations of phenotypic methods, have resulted in a natural progression towards genotypic procedures.
Here we describe the concurrent characterization by PorA OMP ELISA and DNA sequencing of VR1, VR2 and VR3 of all *Neisseria meningitidis* isolated in Scotland between1999 and 2002. Seventeen percent of all meningococci were non-subtypeable (NST) by PorA OMP ELISA whereas all meningococci were successfully characterized by nucleotide sequencing. A comparison between phenotypic and genotypic data identified a greater level of discrimination using genotypic characterization, increased reliability and subsequently eliminated NST results.

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Outcome of meningitis: a systematic review

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Objectives
To systematically review the long term sequelae of meningitis.
Methods
We undertook a systematic review of observational studies on the outcomes of meningitis from all causes. We identified studies through searching published databases as well as the grey literature. Data were abstracted by 1 researcher and each publication reviewed by 3 researchers.
Results
Of 2387 abstracts identified initially, 150 studies were selected for reviewing. The majority (37% [56/150]) reported the sequelae of bacterial meningitis from any cause. Sixteen eligible studies (11%) reported the outcome of meningococcal meningitis. Figure 1 outlines studies by organism and year of publication. Hearing loss was the most commonly assessed outcome, reported in 71% (107/150). Hearing impairment ranged from 0-52% (median 10.5%) depending on the type of the organism and the year infections were acquired, with a median of 4.8% (Figure 2). The median prevalence and range for other sequelae were visual impairment 2.8% (0-31%), cognitive dysfunction 15% (0-47.4%), educational difficulties 12.7% (0-57.2%), developmental delay 13.4% (0-60%), motor nerve deficits 4.3% (0-41%), amputation (meningococcal disease only) 3.1% (0.1-12%), seizure disorder 3% (0-13%) and behavioural problems 9.8% (1.2-48.8%). Other sequelae were reported rarely, including cerebellar impairment, hydrocephalus, endocrine disorders, sleep disturbance and impaired quality of life.
Conclusion
Meningitis is associated with a variety of significant sequelae which impair function, health and quality of life.

Figure 1: Summary of the reviewed papers according to organisms and year of publication (vertical lines represent the time when vaccination was introduced in the UK)

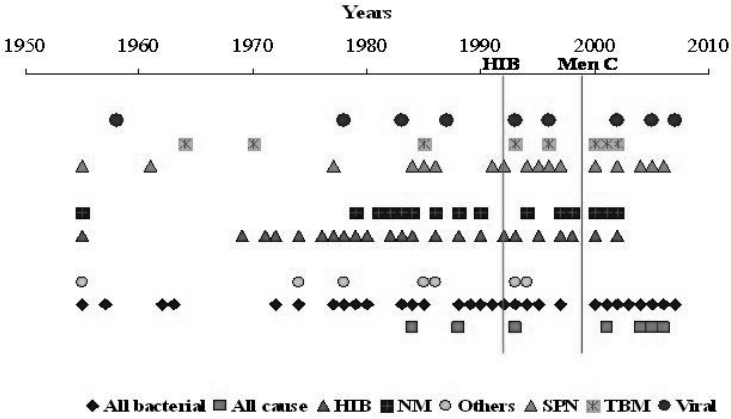
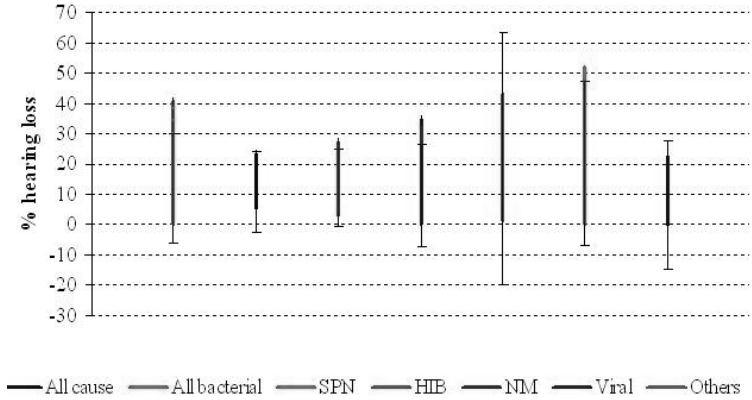


Figure 2: Prevalence of hearing loss by organisms* (*Prevalence within 1 Standard Deviation)



P143

Investigation of variation and selection acting on Factor-H binding protein (fHbp) in *Neisseria meningitidis*

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Background and aims

fHbp is a novel meningococcal vaccine candidate unearthed by genome mining. The protein appears to be expressed in all meningococci, though levels of expression may vary. It has been found to induce bactericidal antibodies and can confer passive protection in the infant rat model. Two different schemes for classifying the protein have been developed according to amino acid sequence. One divides the protein into three variant families i.e. variants 1, 2 and 3 and the other into subfamilies A and B. The aim

of this study was to assess the variation of fHbp in a representative sample of meningococcal isolates, its association with clonal complex and serogroup and to determine the levels of recombination and selection acting on it.

Methods

The fHbp gene was sequenced in the 107 globally representative isolates. Assembled and edited sequences were aligned using Seqlab. The software packages MEGA, ClonalFrame and Paup* were used to produce phylogenetic trees. START2 was used for the Maximum Chi squared test and to estimate the dN/dS ratio. OmegaMap, a program which uses a Bayesian method to estimate the selection parameter ω (dN/dS) from gene sequences, was used to detect selection acting on the gene. The output was used to colour a three-dimensional pdb file of the solution structure of the BC region of a variant 1/subfamily B GNA1870/fHbp protein obtained from the Protein Data Bank.

Results

Protein and nucleotide sequences divided fHbp into two major groups i.e. subfamily A/variant 2 and subfamily B/variant 1 (29% and 64% of isolates respectively). Addition of Genbank sequences to the analysis indicated the presence of a third major group i.e. variant 3. There was an association between clonal complex and subfamily/variant: ST-11 and ST-8 complexes with subfamily A/variant 2; ST-4 and ST-5 (serogroup A-associated) and ST-32 with subfamily B/variant 1 isolates. ST-41/44 complex was more widely distributed through the subfamilies/variants. 55% of subfamily B/variant 1 were serogroup A versus 4% for subfamily A/variant 2. Serogroup C was found in 1.6% of subfamily B/variant 1 versus 35% of subfamily A/variant 2. There were no serogroup W-135, Y or Z subfamily B/variant 1 types while each serogroup accounted for 4% of subfamily A/variant 2 isolates. Serogroup B was more evenly distributed - 50% of subfamily A/variant 2 and 44% of subfamily B/variant 1 isolates. The omegaMap estimation of ω along the codon sequence showed positive values in a region after amino acid 100 with a high probability of positive selection (0.6) between 100 and 160. The omegaMap output was used to colour a pdb file of the protein and highlighted two regions with evidence of positive selection: Ile114-Met123 and Gly147-Arg149 which corresponds to a region previously identified along with an adjacent amino acid (Glu146) as part of a putative bactericidal epitope.

Conclusions

fHbp is a variable protein which subdivides into two or three groups that show associations with clonal complexes and serogroups. Analysis of selection on the gene indicates sites in BC region of the protein undergoing positive selection.

P144

Simultaneous single tube PCR based assay for the direct identification of the five most common meningococcal serogroups from clinical samples

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Objectives

The objectives of the present study was to develop and evaluate a single-tube multiplex PCR assay for the simultaneous detection of serogroups A, B, C and W-135/Y of *N. meningitidis* in clinical samples as a tool for improved non-cultural diagnosis.

Materials and methods

A total of 530 clinical samples obtained from 428 patients (271 blood and 259 CSF) were used for the simultaneous detection of the five most common *Neisseria meningitidis* serogroups (A, B, C, W-135 and Y). DNA was extracted from whole blood samples with the Nucleospin Blood Quickpure kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and from CSF samples as described by Zambarti et al. Specific primers for each of the serogroups A, B and C were used based on those described previously (Tzanakaki et al FEMS Immunol Med Microbiol 39, 2003) for the amplification of specific genetic

loci. In order to improve sensitivity and specificity for serogroup A, a new pair of primers was designed inside the sacB gene (orf2) (GenBank Accession number AF019760) at molecular size of 337 bp (according to the software FastPCR). The methodology of the hotstart stepdown PCR was applied in order to reduce non-specific amplification and to enhance the sensitivity and specificity.

Results

For 295/334 (88.32%) samples, serogroup was identified by the single PCR assay; while 39 samples were non-groupable (NG). In contrast, the multiplex PCR assay identified 93.7% of the clinical samples (313/334) into serogroups; while 21 clinical samples remained non-groupable (21/334). The majority (n=10) of the non-groupable clinical samples-identified by the single PCR were found - by the application of the present multiplex PCR- to belong to serogroup A, while 7 belonged to serogroup B, and 1 to serogroup W-135. The sensitivity and the specificity was calculated to 100%. (PPV 100% (95%, C.I. 99.0%-100%) and NPV 100% (95% C.I. 99.0%-100%)

Conclusions

The high sensitivity and specificity observed suggest that the method is a robust, simple, reliable, easily implemented, rapid and cost-effective way for identification of the most prevalent meningococcal serogroups. The direct application of this assay for assessment of clinical samples could contribute to better monitoring the meningococcal serogroups, especially after the implementation of the monovalent and quadrivalent conjugated vaccines.

P145

Evolution of the clonal complex 41/44

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Objectives

Since 1980, an increase in the incidence of meningococcal disease has taken place in the Netherlands, caused by isolates belonging to clonal complex 41/44 (cc41/44). Isolates of the cc41/44 differ from other meningococcal isolates by the presence of a specific restriction modification system, nmeSI, inserted between NMB2011 and NMB2014 (annotation according that of the MC58 genomic sequence) [1]. In the Netherlands, meningococcal isolates have been collected since 1959. Recently, we started to asses the distribution of MLST types and clonal complexes among meningococci isolated of patients with meningococcal disease from 1960 onwards.

Materials and methods

Isolates were typed according to the standard MLST protocol [2], PorA VR1 and VR2 sequence protocol [3]. In addition, isolates were assessed by the PCR, targeting the region containing restriction modification system NmeSI in clonal complex cc41/44 [1].

Results

Of 39 isolates of patients with meningococcal disease in 1960, 11 (28%) could not be assigned to a clonal complex. Reanalysis of these isolates revealed that 8 (20%) were closely related to each other and clustered with other isolates in the MLST data bases with ST37 as a putative founder into a newly assigend clonal complex, cc37. cc41/44 was the predominant clonal complex among the 39 isolates with 13 (33%) isolates. Of the 39 isolates, only two, both serogroup Y cc167, were positive for the nmeSI locus by PCR. None of the 13 isolates assigned to cc41/44 appeared to harbour the nmeSI locus. Sequence analyses of the locus between NMB2011 and NMB 2014 (MC58 annotation) of two of the cc41/44 isolates revealed sequences, which were previous not seen in Neisseriae. In addition, no hit was found with DNA sequences in the publicly accessible databases. On the protein level, the locus showed highest similarity to two open reading frames in the genome of Psychromonas ingrahamii encoding a MoxR like ATPase and a putative protein containing a von Willebrand factor type A domain. In a maximum parsimonal phylogeny tree of cc41/44, in which time is also considered, ST44 is identified as the founder ST of cc41/44. ST44 evolved

into the hyper virulent ST41, and in that process replaced three of the alleles of the MLST scheme, gained the nmeSI locus as well as a PorA of subtype 7-2,4.

Conclusions

In the Netherlands, isolates of cc41/44 were already present in 1960. Horizontal transfer of nmeSI has occurred most probably from serogroup Y cc167 to cc41/44 between 1960 and 1980. The MLST type 41 has expanded after the acquisition of at least the nmeSI locus and PorA 7-2,4, suggesting that these may have contributed to virulence.

References

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P146

European Meningococcal Epidemiology in Real Time (EMERT)

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Objectives

Over time, the epidemiology of meningococcal disease shows subsequent waves with an increased incidence. These subsequential waves of disease are mainly caused by meningococci belonging to a limited number of clonal complexes. In addition, the isolates causing disease in one wave of increased incidence have usually an antigenic make up that is different from that of the isolates of the previous or next wave of increased incidence. The aim of the development of the EMERT database is to provide real time molecular epidemiology for invasive meningococci circulating in Europe. Data will be important for outbreak management and will enhance the rational selection of meningococcal strains for vaccine development. Participating reference laboratories are requested to submit details of all isolates causing disease enabling data to be shared for comparison.

Methods

EMERT is implemented using the mlstdbNet software that is also used to drive the PubMLST.org MLST website and databases. This is written in Perl and runs on a Linux system, utilizing the PostgreSQL database engine and Apache web server software. The mlstdbNet software is under active development and any new or enhanced functionality introduced for the MLST site will automatically be incorporated into EMERT. EMERT submitters belong to user-groups that reflect the national reference laboratories involved. Access to the entire database is provided to everybody within a user-group, provided someone within that group has submitted data to EMERT within a designated time period, currently 30 days. This time interval exists to encourage active participation and timely submission of data.

Results and conclusions

EMERT is an initiative of the European Monitoring Group on Meningococci (EMGM) Society. Since May, 2007, the EMERT website (http://emgm.eu/emert/) is operational. European Reference Laboratories are being encouraged to submit their data to the web site. Fields collected are: lab sample id, date received, date sampled, serogroup, culture status, PorA VR1/VR2, FetA VR, ST, but can be expanded with information about other typing data like antigens included in newly developed meningococcal vaccines.

This database will help to link European Reference Laboratories, thus providing for the first time a comprehensive overview on circulating strains in Europe in real-time and will help to identify new variants of antigens of interest.

P147

Serogroup C meningococcal invasive disease in Italy: peculiarity of genotypes causing meningitis and septicaemia

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Objectives
In Italy, serogroup C conjugate vaccine is recommended and has been offered in several regions as part of their vaccination policy. Following a peak in 2004 and 2005, the National Reference Centre for bacterial meningitis at the Istituto Superiore di Sanità observed a decline in serogroup C meningococci which totalled 30% of strains circulating in 2007. However, an unexpected outbreak of serogroup C meningococcal disease occurred in a group of adolescents and young adults at the end of 2007. The aim of this study was to characterize the serogroup C isolates to define serotype/serosubtype, minimum inhibitory concentrations (MIC) for penicillin G, presence of hypervirulent clonal complexes as well as Variable-Number Tandem Repeats (VNTR) profiles for short-term epidemiology.
Methods
A total of 160 *N. meningitidis* serogroup C strains, collected from 2005 through the first three months of 2008 were analyzed. Serogroup/serotype and serosubtype were performed by serological methods. MICs for penicillin G were determined using ≥0.094 mg/L as breakpoint to classify strains with decreased susceptibility (PenI). The penA gene of the PenI strains was sequenced and each sequence compared with those deposited in Genbank. Genotyping using Multilocus Sequence Typing and porA VRs were performed as previously described and determined through MLST website (http://neisseria.org). VNTR PCR and data analysis for loci 1, 2, 6 and 8 were also performed, following the procedures described by Yazdankhah et al. 2005.
Results
The results showed that, at the beginning of the study period, group C disease in Italy was caused mainly by C:2b meningococci (57.5% in 2005) which were replaced by C:2a at the end of 2007 (56%). The percentages of fatal cases and septicaemia due to serogroup C were 16 and 41, respectively. Among the serogroup C isolates, 62% showed reduced susceptibility to penicillin G with penA alleles 3, 9 and 12 as the most frequently found. Phenotypic and genotypic results showed that group C strains belonged to six major antigenic combinations and two STs: 2a:P1.5(5,2) and 2a:P1.2 ST-11 complex (cpx) and 2b:P1.5(1.5,2) and 2b:P1.2 ST-8 cpx. In the ST-11 cpx, PorAs have been fairly homogeneous with the most frequent combinations VR1 5 or (5-1) and VR2 2 or (10-8). Strains C:2a:P1.5/ST-11 were responsible for the outbreak among adolescents and young adults at the end of 2007. The VNTR analysis showed the presence of a unique profile, different from the others of the ST-11.
Conclusion
It is important to understand the possible effects of serogroup C conjugate vaccine on the evolution of group C meningococci, especially in the absence of widespread vaccination in the country. The results obtained showed that ST-11 meningococci have overtaken the previously predominant ST-8 strains and represent nowadays the majority of serogroup C circulating in Italy. The emergence in the outbreak of a ST-11 strain with variant VNTRs could be possibly explained by swift changes in cell surface characteristics, suggesting a more rapid evolution of VNTR patterns in comparison to those detected by the other phenotypic and genotypic methods.

P148

Multilocus sequence typing for epidemiological surveillance of meningococcal disease in Moscow

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Objectives
The general incidence of invasive meningococcal disease (IMD) in Moscow has been stable but there has been important changes in its epidemiology: starting in 2002, the number of serogroup A meningococcal disease increased; since 2003 the age-specific rates among persons 15-25 years increased. For epidemiological purposes the analysis A-meningococci isolated from liquor and/or blood of patients in Moscow, which was carried out with the method of multilocus sequence typing (MLST), was presented.
Methods
Individual data about all cases of IMD in Moscow were collected and analyzed. In total 1535 cases of IMD were registered for the period 2001-2006. MLST method was use to characterize serogroup A-meningococci causing invasive disease isolates in Moscow.
Results
The notification annual mean of incidence of IMD in Moscow were: 2001 - 2,21; 2002 - 2,39; 2003 - 3,64; 2004 - 2,48; 2005 - 1,87; 2006 - 2,08 and the average for this period was 2,4 per 100000 population. Important change in the epidemiology of IMD in Moscow were decrease in the proportion of children (0-14 years: 2003 - 49,8%, 2004 - 47,3%, 2005 - 43,4%, 2006 - 37,2%) and increase in the proportion of adult (15 year and more: 2003 - 50,1%, 2004 - 52,7%, 2005 - 56,6%, 2006 - 62,9%. Most important change (increase) was in the proportion of adolescents (15-25 years age: 2003 - 16,7%, 2004 - 18,9%, 2005 - 21,3%, 2006 - 30,3%). Another important change in the epidemiology of IMD in Moscow was increase in the proportion disease caused by serogroup A meningococci (2001 - 30,7%, 2002-2005 - 48,6%, 2006 - 65%) and decrease in the proportion disease caused by serogroup B (2001 - 36,4%, 2002-2005 - 23,8%, 2006 - 18,6%) and serogroup C (2001 - 29,5%, 2002-2005 - 25,2%, 2006 - 13%). At the period 2004-2007 was studied 40 strains of A meningococci with use MLST method. Out all strains 32 (80%) belonging to subgroup X with sequence type (ST) - 3349 (22 strains), ST-75 (7 strains), ST-5805 (2 strains), ST-6346 (1 strains). Other 7 strains belonging to subgroup VI with ST-2 and 1 strain belonging to unknown subgroup with ST-6345.
Conclusion
Although the overall incidence of IMD in Moscow has remained stable it were changes in the epidemiology IMD: 1. decrease in the proportion of children and increase in the proportion of adult; 2. decrease in the proportion disease caused by serogroup B and C meningococci and increase in the proportion disease caused by serogroup A. Genetic variability of circulating A-meningococci at the period 2004-2007 years in Moscow was regarded as characteristic of the non-epidemic period but it is very important to have steady surveillance for timely exposure epidemiological trouble.

P149

Impact of mass vaccination with VA-MENGOC-BC on *Neisseria meningitidis* population structure in Cuba

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Objectives
In response to the epidemic level of serogroup B meningococcal disease in Cuba during the 1980's, the VA-MENGOC-BC® vaccine was developed and introduced into the National Infant Immunization Program in 1991. Since then the incidence of meningococcal disease (MD) in Cuba has returned to pre-epidemic levels. Subtyping of *Neisseria meningitidis* using multilocus sequence typing (MLST) has been widely used for studying the epidemiology of MD and the evolutionary biology of the meningococcus. The aim of this study was to establish a general view of the Cuban meningococcal epidemiology trying to understand the impact of mass vaccination with VA-MENGOC-BC® on *Neisseria meningitidis* population.
Methods
A total of 427 isolates from cases and carriers were included in the current study, all pertaining to the Finlay Institute collection. The isolates were collected between 1983 and 2005 in different regions of Cuba. All strains were preliminarily characterized by slide agglutination with polyclonal antisera for serogroup determination, and were serotyped and subtyped by whole-cell ELISA with reference monoclonal antibodies. The relatedness among isolates was evaluated by MLST. Phylogenetic analysis was performed using Mega 3.1, and tree construction was carried out using Neighbour-Joining. Clonal complex assignation and population snapshot studies were assisted with the use of eBURST. A simple metric of differentiation the classification index D, was employed.
Results
The MLST analysis during these years in Cuba revealed in the studied group 64 sequence types (STs) including 31 new STs. The predominant ST was ST-33 with 213 isolates (49.88%). ST-33 is the main responsible for the low incidence of meningococcal disease that has occurred since the introduction of the VA-MENGOC-BC® vaccine in Cuba. The second ST more frequently found was the ST-53 (19.91%), mainly isolated from carriers. Among the highest representatives clonal complex were the ST-32 complex (57.38%) isolated either from cases and healthy carriers, followed by the ST-53 complex (19.91%). The ST-32 and ST-53 were the predominant clonal complexes obtained from the carriers isolates. In addition, we found isolates from the complexes ST-41/44 (37 strains), ST-103 (11 strains), ST-22 (7 strains), ST-198 and ST-8 (5 strains each), ST-254 and ST-269 (3 strains each), ST-23 and ST-167 (2 strains each) and ST-11 (one strain).
Conclusion
The present analysis suggests that coincidentally with VA-MENGOC-BC® vaccine introduction, a significant change in the clonal complex distribution is recorded, as measured as a significant value of the differentiation index obtained before and after mass vaccination.

P151

Meningococcal meningitis serogroup B epidemiology.

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Objectives
Invasive meningococcal disease (IMD) is caused by a gram-negative diplococcus transmitted via throat secretions and respiratory droplets. Of the 13 known serogroups only groups A,B,C,W135 and Y (and now X) are responsible for most cases. Anticapsular polysaccharide vaccines, conjugated and non-conjugated, are available for serogroups A, C, Y and W135, yet due to poor immunogenicity, not for serogroup B. Current vaccines for serogroup B are outer-membrane vesicle based and are serosubtype specific. A non-capsular, surface protein vaccine is being developed for serogroup B *Neisseria meningitidis*. To better understand the effectiveness and cost-benefit ratio of these vaccines, a more refined knowledge of serogroup B-caused disease epidemiology is necessary. There is no comprehensive review of *N. meningitidis* serogroup B epidemiology.
A systematic review was conducted with the aim of identifying and monitoring of major trends in the epidemiology of IMD caused by serogroup B.
Methods
Information was reviewed through the systematic review of studies and surveillance data on a national or regional level via various search engines. PubMed, regional WHO homepages, websites of disease linked organizations and institutions, as well as individual ministries of health were searched using terms such as: "meningitis", "meningococcal disease", "outbreak", "serogroup B", "incidence" and the name of country or region.
Results
In contrast to epidemic serogroup A meningococcal disease, serogroup B meningococci were initially identified as a cause of sporadic IMD. Since the 1970s, serogroup B meningococcal disease has emerged and is now the most important serogroup in many countries worldwide. Serogroup B predominated from 1977-1998 in 20 different European countries and still causes 2/3 of the meningococcal disease burden in Europe. Endemic or hyperendemic serogroup B disease is reported from Australia and New Zealand, several South American countries, Africa outside the Meningitis Belt and the Near East. In Northern America serogroup B disease has decreased in recent years in favor of serogroup Y and is currently responsible only for a third of all invasive cases.
Major outbreaks caused by serogroup B are widespread. Prolonged outbreaks have been responsible for relatively high mortality and morbidity levels in industrialized countries. Large recent outbreaks occurred in Cuba, Norway and Brazil in the 1980s/90s and in New Zealand since the mid 1990s, with recent outbreaks being in France and the US.
In endemic settings, serogroup B meningococci isolated from meningitis patients are phenotypically and antigenically diverse. During outbreaks a specific serogroup B clone often dominates. Depending on the virulence potential of the dominating clone, serogroup B outbreaks can differ substantially in severity of disease, mortality and sequelae of the affected population.
Conclusion
Despite this panmictic nature, a few serogroup B clones have evolved to cause hyperendemic disease in various parts of the world, which unlike other serogroups, persist over a longer time period. The dynamics of serogroup B outbreaks are distinct and cannot be inferred from the dynamics of other serogroups. Intense and proactive surveillance of serogroup B strains causing outbreaks and epidemics is a necessary part of efforts to implement effective vaccination strategies for the vaccine currently in development.

P152

Primary septic arthritis due to serogroup W-135 *Neisseria meningitidis*

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Background
Meningitis and septicemia are the most common clinical manifestations of infections caused by *Neisseria meningitidis*. In 11% of invasive meningococcal disease (IMD) septic arthritis occurs as a sequelae of meningococcemia. Below we present the uncommon case of primary meningococcal arthritis without concomitant syndromes associated with meningococcemia.
Objective
The aim of the study was to characterize the case of primary septic arthritis due to *N. meningitidis*.
Case report
A 70-years-old woman presented to a hospital with a signs of the right knee septic arthritis. The syndromes included painful and acutely swollen knee and body temperature elevated to 37.8ºC. No other symptoms, such as petechial rush, inflammation of the central nervous system or multi-organ failure were noticed at admission. The patient was directed for the incision and drainage of the affected joint. Synovial fluid was sampled and sent to local microbiological laboratory for further analysis. The patient was given anti-inflammatory treatment, but no antibiotic therapy. The next day patient reported a significant relief and was discharged from a hospital on her own demand. From the synovial fluid a pure culture of Gram-negative diplococci was obtained. Latex agglutination tests performed on the culture gave positive result for *N. meningitidis* serogroup W-135/Y. Identification of the isolate was confirmed by standard biochemical test. The isolate was sent to the National Reference for Bacterial Meningitis in Warsaw for the confirmation and further examination. It was confirmed to be a serogroup W-135 meningococcus by biochemical tests, monoclonal sera and PCR.
Three days later the patient came back to the hospital with the same symptoms of painful and swollen right knee. Since diagnosed as IMD case, she was admitted to ICU. Antibiotic therapy with 2 doses ceftriaxone daily (1 g per dose) was started, and another specimen of the synovial fluid from the affected joint as well as blood were taken for bacteriological examination. Again, the W-135 meningococci were cultured from the joint, but the blood culture remained negative. No other joint of the patient was affected and there were no signs of meningitis or disseminated meningococcal infection. After 6 days in the ICU the patient was transferred to the internal unit of the hospital. After eleven days of treatment with ceftriaxone the patient was discharged from the hospital in a good state, without any symptoms of infection or sequelaes.
Conclusion
The most common agents of septic arthritis are *Staphylococcus aureus* and *Neisseria gonorrhoeae*. *N. meningitidis* is very rarely taken into account in cases of primary arthritis. The case presented above is the first case of isolated meningococcal joint infection identified in Poland. Additionally it is a rare case of meningococcal disease that was not accompanied by any other significant symptoms and did not disseminated into a systemic infection, despite a delay in the introduction of antimicrobial therapy.

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Invasive meningococcal disease in Poland - eleven years of the activity of the National Reference Centre for Bacterial Meningitis

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Background

Neisseria meningitidis is a leading cause of community-acquired bacterial meningitis and/or septicemia worldwide. In recent years an increase of the number of cases of meningococcal infections in Poland (both notified and laboratory-confirmed) was observed leading to the increase of the incidence rate from 0.37/100.000 in 1997 to 1.03/100.000 in 2007.

Objectives

The aim of the study was to analyze the epidemiological situation of IMD in Poland during the eleven years (1997-2007) of the activity of the National Reference Centre for Bacterial Meningitis (NRCBM) in Warsaw.

Methods

The study included all invasive isolates of meningococci collected in the NRCBM during a routine monitoring of IMD in Poland as well as clinical specimens obtained from patients with suspected IMD and sent to the Centre for culture-independent diagnostic procedures. All isolates were reidentified to the species level by standard microbiological methods. Their serogroups were identified by monoclonal sera and/or PCR, and phenotypes - by ELISA method. Selected isolates of meningococci underwent further typing with the use of RAPD, RFLP-PFGE and DNA sequencing methods.

All clinical materials were examined for the presence of specific meningococcal antigens by commercial latex agglutination tests. Additionally they were checked for bacterial DNA by PCR, allowing identification of bacterial species and serogroups of meningococci.

Results

During eleven years of the activity the NRCBM has collected 979 invasive isolates of *N. meningitidis*. Almost 60% of them belong to serogroup B. The most prevalent phenotypes in this group are B:22:P1.14 and B:15:P1.7,16. One third of isolates were characterized as serogroup C, with phenotypes C:NT:P1.3,6 and C:2a:P1.5 most commonly identified. 1.1% of isolates belong to serogroup Y, 2% to serogroup W-135. In almost 4% of culture-confirmed IMD cases isolates could not be serogrouped.

Between 2004 and 2007 the NRCBM collected clinical specimens from 121 patients, in which DNA specific for *N. meningitidis* was identified by culture-independent techniques. Genogroups were obtained for 46 of these (38%): in 15 cases it was group B, in 30 - group C, in one case - Y and one - W135.

During last five years three dominating clonal complexes have been identified among invasive serogroup C meningococci. First, ST-8/A4 Cluster, was frequently isolated between 2003 and 2004. The second, ST-103 CC widely disseminated in Poland in last two years and in 2007 was the most frequent among MenC. The third was ST-11/ET-15 clone, which has spread in 2006 and 2007 and was responsible for all outbreaks IMD identified in Poland.

Conclusion

Invasive meningococcal infections in Poland still remain a serious public health problem. Since 2002 the increase of the number of infections caused by serogroup C meningococci as well as outbreaks caused by this group have been observed. The latest were connected with dissemination of hypervirulent ST-11 strain. The implementation of the culture-independent diagnostic procedures allowed for better recognition of IMD problem, leading to laboratory confirmation of almost 90% of all notified cases in 2007. The incidence rate calculated for IMD in Poland exceeded 1 case per 100.000 in 2007 and has reached the values close to the European average.

P154

Comparison of porB sequence analysis and NG-MAST in identifying circulating Clusters of *Neisseria gonorrhoeae* isolates from Shanghai, China

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Objectives

1) To determine circulating clusters of *Neisseria gonorrhoeae* (Ng) in Shanghai using porB sequence analysis and the Ng Multi-Antigen Sequence Typing (NG-MAST);
2) To determine congruence between epidemiological connections and genetic types of Ng isolates;
3) To compare the NG-MAST with porB sequence analysis alone in molecular epidemiology of Ng.

Methods

Ng isolates (n=199) were collected from 157 male gonorrhoea patients and 42 female sexual partners in Shanghai, China, between November 2004 and May 2005. This comprised 40 pairs and one triplet (one male patient and 2 female partners) from epidemiologically-linked sexual contacts. Gonococcal chromosomal DNA was extracted from these strains for use as templates for PCR amplification of porB and tbpB.. Amplicons were analyzed by DNA sequencing (Hobbs et al 1999, Liao et al 2008, Martin et al 2004). porB sequence analysis covered ˜85% of the nucleotides encoding surface-exposed loops I-VII and interspace regions II-VII (Liao et al 2008). NG-MAST strain types (STs) were assigned as described by Martin et al 2004 (www.ng-mast.net). DNAdist of PHYLIP was used to analyze DNA sequences, and phylogenetic trees were constructed and viewed using TreeView. Simpson's Index of Diversity (ID) was used to determine discrimination powers (Hunter & Gaston 1988). To minimize clonal effects, only sequences of isolates from male patients were used in ID analysis.

Results

The IDs for 174 porB sequences (Liao et al 2008) and NG-MAST were 0.92 and 0.98, respectively. A total of 65 porB alleles was identified while NG-MAST analysis revealed 113 STs. The phylogenetic trees exhibited similar clustering patterns between porB sequence analysis and NG-MAST. Both porB sequence analysis and NG-MAST were congruent with reported sexual links. Two sexual contacts showed different types for both porB sequence type and NG-MAST STs, confirming that the contact pairs were infected with different Ng strains or that the patients had mixed infections. Further analysis of phylogenetic trees revealed that groups of isolates had identical porB sequences or NG-MAST STs, but the patients were not connected in the epidemiological data. Moreover, the majority of isolates having an identical porB sequence exhibited the same ST, suggesting that both analyses are useful in identifying epidemiologically unrevealed sexual networks in gonococcal transmissions.

Conclusions

The porB sequence analysis and NG-MAST typing have sufficient discriminatory powers to distinguish Ng isolates and are effective tools to identify circulating clusters. Both methods reveal sexual connections are congruent with epidemiological findings and reveal sexual links hidden in traditional partner tracing. However, porB sequence analysis costs less, is more rapid, and is easier to perform as compared to NG-MAST typing. Furthermore, porB sequences are associated with serovar types and antimicrobial resistance of Ng isolates. There is an urgent need to establish a publicly accessible and standard database of porB sequences for inter-laboratory comparison and references for molecular epidemiology studies.

P155

A genotypic comparison of invasive and non-invasive meningococci in Scotland 1996 - 2004

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Objectives
To compare *N. meningitidis* strains isolated in Scotland from carriage with strains isolated from cases of invasive disease using Multilocus sequence type (MLST) data over an 8 year period.
Methods
We describe the characterisation of non-invasive meningococci received by the SMPRL between 1996 and 2004. In this study we define "non-invasive"as "throat isolates collected from individuals without clinical manifestations of invasive disease and with no epidemiological link to a clinically or laboratory confirmed invasive meningococcal case". Only isolates from blood or CSF were included in the invasive disease data set. MLST data for 203 carried and 783 invasive isolates was used for comparison. In addition genogrouping PCR to detect serogroups A, B, C, Y, W135, X, Z and 29E and the capsule null locus was performed on all isolates previously described as non-groupable by serogrouping.
Results
During the study period over 90% of invasive disease isolates were serogroup B (58%) or C (34%). In comparison only 37.5% of non-invasive isolates were serogroup B (36%) or C (1.5%). Almost half (48%) of non-invasive meningococci were nongroupable compared with only 1.4% of invasive isolates. Thirty-three percent, 31% and 11.3% of nongroupable isolates were positive for siaD serogroup B, capsule null locus and ctrA serogroup 29E PCR, respectively. A further 21.6% were PCR positive for other serogroups. The major MLST complexes ST-11/ET-37, ST-32/ET-5, ST-41-44/Lineage 3 and ST-269 were identified more often from cases of invasive disease than in carriage. The major MLST complexes ST-213, ST-53, ST-35 and ST-23/Cluster A3 were identified more often in carriage than from cases of invasive disease. The ten most common STs from invasive disease (ST-11, -41, -269, -213, -275, -18, -8, -259, -60 and -22) and the nine most common STs from carriage (ST-53, -213, -22, -41, -269, -750, -1157, -766 and -4221) accounted for just over 62% and 44%, respectively.
Conclusion
In Scotland a considerable amount of work has been, and continues to be, performed on the identification, characterisation and surveillance of *N. meningitidis* strains responsible for causing invasive disease. Retrospective characterisation of non-invasive meningococci in Scotland has recently been undertaken and it is hoped this will aid in our understanding of carried meningococci and how the non-invasive population contributes towards the development of meningococcal disease. The work described here is part of a larger project to characterise non-invasive meningococci over a thirty year period and to subsequently compare them to strains isolated from cases of invasive meningococcal disease.

P156

Neisseria meningitidis carriage by age: a systematic review

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Objectives
- To conduct a systematic review of meningococcal carriage studies in order to estimate the age-specific prevalence of carriage, in settings with comparable meningococcal disease epidemiology and social

structure to the UK,

- To quantify the observed variation in prevalence and to explore reasons for heterogeneity.

Methods

EMBASE, Medline, Web of Science and the Cochrane Library were searched for papers reporting carriage of *Neisseria meningitidis* in defined age groups using the search terms ((Meningitis, Meningococcal or *Neisseria meningitidis* or Meningococcal Infections or Meningitis and Carrier State) or (meningococ* or *Neisseria meningitidis* or meningitis and carrier* or carriage)) not (pneumococ* or Africa* or staphylococ* or haemophilus).

References of relevant papers were checked for additional studies. Papers in any language published after 1969 reporting on at least 100 individuals sampled/swabbed for meningococcal carriage were included. Papers reporting studies from Africa, Asia and Russia, were excluded because the epidemiology of disease is not comparable to the UK (i.e. large proportion of group A disease). Papers were also excluded if they could not be generalised to the broader population, e.g. studies in close contacts of a case, military personnel (with the exception of those sampled on day of entry) and isolated communities. Where carriage prevalence was reported in specified age groups, the group mid-point was used for analysis.

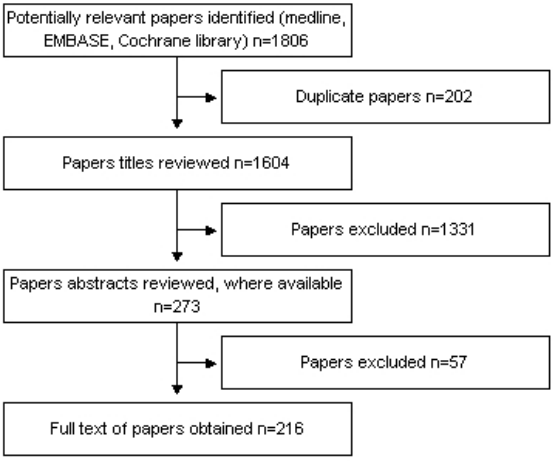
Results

The number of papers identified in different stages of the systematic review is summarised in figure 1. After removing duplicates the search returned 1604 papers; after reviewing titles and abstracts, the full text was obtained for 216 papers. At the time of writing, 150 of these papers (those in English) had been reviewed. Of these, 45 papers satisfied the inclusion criteria. Studies were reported from 20 countries: Australia (1), Belgium (1), Canada (2), the Czech Republic (1), Denmark (2), Germany (1), Greece (4), Lebanon (1), the Netherlands (2), Norway (5), Poland (1), Spain (3), Sweden (1), Turkey (1), the UK (13) and USA (3). Seven papers reported longitudinal studies of carriage, and were analysed separately. A general trend of low carriage prevalence in young children and older adults and higher carriage in teenagers and young adults can be seen; however, there is substantial between-study variation. For example, for a mid-point age of 17 years reported carriage ranged from 3.1% to 24.5%. Further statistical analyses will explore reasons for this heterogeneity.

Conclusion

Carriage of meningococci is often quoted as 10%, however preliminary results from this systematic review show that meningococcal carriage is highly variable by age. It is important to understand the age-specific patterns of carriage and transmission in order to model the potential impact of vaccination programmes. In the absence of a recent large-scale carriage study across all age groups in the UK, this systematic review will allow us to examine age-specific carriage prevalence, quantify the heterogeneity between studies and produce robust age-specific carriage prevalence estimates.

Figure 1: Progress through the stages of the systematic review



P157

Meningococcal B invasive disease in children

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Objective
To study the frequency of meningococcal B invasive disease (MBID) according to age group, sero/subtype, clinical presentation and evolution.
Methods
We prospectively studied 229 children with MBID aged 0 - 14 years (2000 - 2007) at the Infectious Diseases Department, Sor Maria Ludovica Children Hospital, La Plata, Argentina. *N. meningitidis* B strains were isolated from sterile sites at the Microbiology Hospital´s Laboratory, and studied for sero/subtype at the National Institute for Infectious Diseases Carlos G. Malbran, National Administration for Laboratories ant Health Institutes. DifcoTM monoclonal antibodies were used to identify serogroup and enzyme-immune-assay with whole cells and monoclonal antibodies for serosubtyping (Abdillahi and Poolman method).
Results
MBID was more frequent in children ‹ 1 year, 27.9 % (n=64). Those of ‹5 years accounted for 66.8 % (n=153). Mortality rate was 4.7 % and 5.2 % respectively. Global mortality rate was 4.0 %. Frequency / clinical presentation were: purulent meningitis 36.2 % (n=83), sepsis with purulent meningitis 34.9 % (n=80), fulminant meningococcemia 17.5 % (n=40), sepsis with no purulent meningitis 10.0 % (n=23), and bacteriemia 1.3% (n=3). No fatalities occurred in children with bacteriemia or sepsis with no purulent meningitis. The highest fatality rate was observed for fulminant meningoccemia (17.5%), for children aged 1 year (25.0%), and for those ‹5 years (5.2%). Forty-two sero/subtypes were determined in 178 strains (77.7%). The most common were: B:4:P1:NST, 16.9% (n=30); B:15:P1:NST, 11,2% (n=20); B:15:P1.7,16, 8,4% (n=15); B:4:P1.14, B:15:P1.7, B:NT:NST, and B:4:P1.15, 5.6% (n=10) for each one.
The first sero/subtype for sepsis with no purulent meningitis was B:4:P1:NST, and for purulent meningitis B:15:P1.7,16. No related deaths associated with one sero/subtype were observed.
Conclusions
MBID is endemic and epidemic in Argentina. *N. meningitidis* serogroup B was first identify in 1975 during an outbreak of *N. meningitidis* serogroup C disease and it was prevalent from 1984 to 1994, and currently since the year 2000. The most common serotypes were B:15 for Argentina and B:4 in our region. Clinical and microbiological laboratory data are necessary for epidemiological surveillance to detect early changes in frequency, serogroup and sero/subtype incidences. The high frequency of sero/subtypes detected show endemic level even though with changes in frequency of cases. The mechanism by which a strain cause esporadic or epidemic cases and the importance of changing serogroup and serosubtype strains by period remain unknown.

P158

The economic and humanistic burden of meningococcal disease in Europe

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Objectives
The prevention and treatment of meningococcal disease create substantial costs for health systems

worldwide. The objective of this research was to quantify the annual economic and humanistic burden of meningococcal disease in France, Germany, Italy, Spain and the United Kingdom (EU-5).

Methods

Surveillance data from 2006 were combined with data from published epidemiological, economic, and quality of life studies on meningococcal disease as well as public health guidelines to calculate the annual burden of illness. Costs included direct medical costs (vaccination programs, prevention of secondary cases, acute treatment, long-term treatment of sequelae), direct non-medical costs (special education for patients with severe neurological disability), and indirect costs (lost productivity due to acute disease, disease fatalities, and long-term sequelae). The following sequelae were considered: mild or severe hearing loss, amputation, scarring, seizures, and neurological conditions. The estimated humanistic burden of disease was valued in terms of life-years (LY) lost and quality-adjusted life-years (QALYs) lost. QALYs lost were calculated by assigning a utility decrement to each adverse outcome (death or permanent disability), and multiplying this decrement by the country-specific life expectancy. A utility is an index measure of health-related quality of life ranging from 0 to 1, with 1 representing perfect health and 0 death. Costs, LYs, and QALYs were projected to a lifetime horizon based on cases occurring during a single year, and discounted to present values at a rate of 3% per annum.

Results

The projected mean cost per case ranged from € 42,800 in Spain to € 106,200 in France, excluding costs of routine vaccination (see figure 1). The primary cost component was the productivity loss due to fatalities, largely influenced by case fatality rate and labour cost. Each case of meningococcal disease was associated with a projected mean loss of 1.74 to 3.31 LYs, or 1.99 to 3.47 QALYs (see figure 2).

Annual numbers of cases ranged from 179 in Italy to 835 in Germany. The proportion of serogroup B cases was 89% in the UK and ›60% in all countries. The proportion of serogroup C cases was ‹3% in the UK and 20-30% elsewhere. The estimated annual economic burden of illness for the EU-5 countries combined was over € 355 million, including costs of routine vaccination. Indirect costs accounted for 49% of this total and prevention costs for 34%. Direct and indirect costs attributable to sequelae represented 12% of the aggregate cost. The total numbers of LYs and QALYs lost due to meningococcal disease per annual cohort across EU-5 were estimated at 7,277 and 7,928 respectively.

Conclusion

Although meningococcal disease is relatively rare in the EU-5, it is associated with a significant economic and humanistic burden, due to a high cost per case and serious health consequences. These aggregate estimates are likely to be conservative as they are based on confirmed cases of meningococcal disease only, and exclude the potentially substantial costs of controlling outbreaks, due to their unpredictability.

Figure 1: Estimated lifetime cost per case

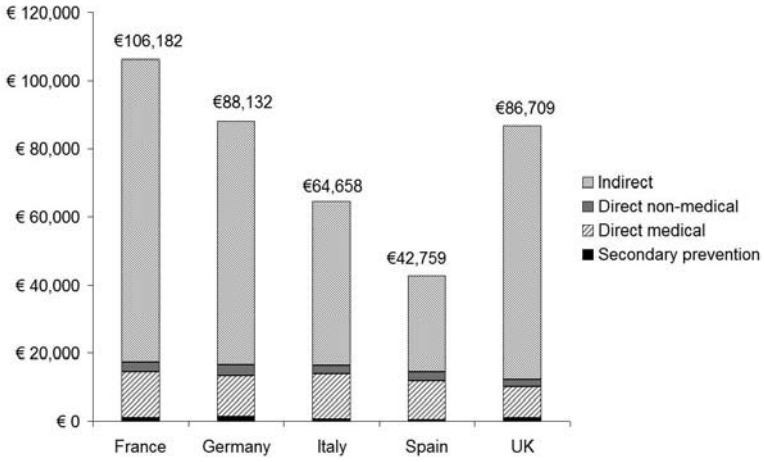

Figure 2: Estimated LYs and QALYs lost per case

	France	Germany	Italy	Spain	United Kingdom
Number of cases	575	835	179	689	697
Average LYs lost per case	3.31	2.42	2.55	1.71	2.51
Average QALYs lost per case	3.47	2.69	2.72	1.99	2.67

P159

The epidemiology of clinically relevant *Neisseria meningitidis* isolated in Scotland between 2003 and 2008

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Objectives

Since the introduction of the MenC vaccine the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) has continued to characterise all clinically relevant *Neisseria meningitidis* isolated in Scotland as well as provide non-culture detection of *N. meningitidis* from clinical specimens. By facilitating enhanced surveillance of this organism we are able to monitor any changes in the epidemiology of meningococcal disease in Scotland, which may or may not, have been prompted by the vaccination campaign. Here we present data obtained between 2003 and 2008.

Methods

N. meningitidis isolates referred from laboratories throughout Scotland where characterised on the basis or their serogroup, porA variable regions (VR1, VR2 and VR3) and Sequence Type (ST), as determined by Multilocus Sequence Typing (MLST). Moreover, molecular determination of serogroup, porA and ST were also attempted on any clinical specimen that was positive for meningococcal DNA, by ctrA PCR.

Results

The number of notified cases of meningococcal disease in Scotland has remained relatively unchanged over the period studied, averaging 149 cases per year (ranging from 139 in 2005, to 155 in 2003 and 2007). Over 60% of notifications were confirmed by laboratory testing, of which approximately a third were detected by either PCR or raised antibody levels.

The majority of disease in Scotland is caused by serogroup B strains. The continued efficacy of the MenC vaccine can be observed by the very low numbers of serogroup C disease reported: 3 cases in 2003, 2004, 2006 and 2007 and 1 case in 2005.

MLST revealed that in each year the two most prevalent clonal complexes were either ST-41/44 or ST-269, which between them accounted for 50% (165/329) of all isolates characterised.

P160

Molecular epidemiology of *Neisseria meningitidis* serogroup B in Brazil using sequence based MLST and OMP analysis

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Objectives
Meningococcal disease (MD) has been observed in Brazil since the early 1970's when outbreak waves of serogroups A, C, B and W135 spread throughout the country. The incidence of MD peaked in 1996 at 7 per 100,000 population, with a predominance of serogroup B disease. The incidence of MD declined to 4 per 100,000 in 2004, with a concomitant increase in the proportion of Group C disease. We analyzed a representative sample of serogroup B isolates from different geographic regions of Brazil to identify circulating clonal complexes and outer membrane protein (OMP) genotypes. We also analyzed isolates from the Sao Paulo (SP) region for 4 time points spanning 18 years to examine evolution of serogroup B.
Methods
A representative sample of 2004 serogroup B disease isolates were analyzed from four major regions of Brazil (n=235) and from four time points for Sao Paolo (SP), the most populous state including 1988 (n=50), 1996 (n=50) and 2006 (n=47). Clonal complexes were determined using multi-locus sequence typing (MLST) analysis of seven housekeeping genes, the genotypes of porA, porB and fetA, and the Neisseria.org database. We report results based on the completed sequence analysis of 287 of the strains (75% of the total).
Results
Five of the 7 hypervirulent lineages that have been circulating worldwide during the last 50 years were detected in Brazil. These 5 lineages were ST-32/ET-5, ST-41/44, ST-8/Cluster A4, ST-11/ET-37 and ST-23/Cluster A3. In 2004, the hypervirulent lineage ST-32/ET-5 was most common in Brazil (166/193, 86%) followed by ST-41/44 (18/193, 9.5%). ST-32 was predominant in all geographic regions, ranging from 82% of isolates from the Southern region to 95% of isolates from the North region. In SP state the proportion of ST-41/44 isolates decreased from 34% (12/35) in 1988 to 8.5% (5/59) 2004. During the same period the proportion of ST-32 clones increased from 57% (20/35) in 1988 to 86% (51/59) in 2004. One strain belonging to the hypervirulent clone ST-23/Cluster A3 was detected in 2004. This clone was previously described in Brazil among serogroups W135 and Y. The distribution of OMP genotypes based on porA, porB and fetA sequencing differed according to the year of isolation and interestingly showed greater diversity in 2004 when MLST type was the least diverse: fetA was the most polymorphic gene with more than 60 different genotypes, while porA and porB showed less polymorphism with 43 and 16 genotypes respectively.
Conclusion
Sequence based analysis of a representative sample of serogroup B meningococcal isolates from the major geographic regions of Brazil and from one region over time reveals complex patterns of clonal lineage and OMP type. A high proportion of strains from 2004 have a distinct clonal complex and genotype across all geographic regions. Recently the proportion of MD caused by serogroup B strains has decreased with an increase in serogroup C. Concurrent with this shift, the serogroup B MD in 2006 appears more genetically diverse than in previous years.

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Genetic diversity of NadA, GNA33 and factor H binding protein (GNA1870/LP2086) in *Neisseria meningitidis* strains isolated in Brazil

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Objectives
Meningococcal disease is a world-wide life-threatening disease that can be prevented by serogroup-specific vaccines. However, there is no broadly effective vaccine against serogroup B strains due the poor immunogenicity of this capsular polysaccharide. Strain-specific vaccines based on outer membrane vesicles have successfully controlled clonal outbreaks. Proteomic and genomic tools are being utilized to identify potential serogroup B vaccine candidates that, ideally, are highly conserved immunogenic proteins exposed on the surface of all meningococci. Two novel proteins under investigation have been functionally characterized. NadA is an exposed adhesin involved in host interaction and is expressed in most pathogenic strains belonging to hypervirulent lineages. GNA1870/LP2086 is a factor H binding lipoprotein that may decrease susceptibility to intrinsic serum bactericidal activity. GNA33/NMB_0033 is a less well characterized lipoprotein associated with membrane architecture. We chose the genes for these three proteins currently under investigation as vaccine candidates to analyze for diversity among a large geographically representative collection of Brazilian serogroup B isolates.
Methods
A representative sample of 2004 serogroup B isolates from four geographic regions of Brazil were obtained for analysis. The selected genes were amplified and sequenced. Nucleotide sequences and predicted protein sequences were aligned and compared in order to determine the genetic diversity within each gene.
Results
All three genes showed low levels of polymorphism with nadA being the most conserved. nadA from 187 Brazilian strains representing X MLST types have an identical nadA gene. When compared with published sequences from other countries, nadA from Brazilian strains contained two previously described polymorphic regions: a 21 nt insertion and a 129 nt deletion. GNA1870 was also highly conserved with all analyzed strains belonging to variant 1 of three published, known variants. GNA33 was the least conserved, but still with a high level of similarity among the strains analyzed. Some regions with non-synonymous nucleotide changes were predicted by computer modeling as possible antigenic sites of the mature protein.
Conclusion
The overall level of similarity for nadA, GNA1870, and GNA33 was more than 95%. The nadA gene was universally detected and showed no genetic diversity in a large collection of Brazilian group B disease isolates. Little genetic diversity was found among GNA1870 and GNA33 genes. Our data suggest limited antigenic diversity among 2004 Brazilian disease isolates for these novel proteins. Studies of phenotypic expression of the proteins encoded by these genes will be important.

P162

Dominance of the Sequence Type (ST-) 5 complex in 5 countries of the African meningitis belt between 2004 and 2007

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Objectives
The WHO Collaborating Center for Reference and Research on Meningococci in Marseilles is involved in the surveillance of meningococcal meningitis in the countries belonging to the African meningitis belt.
Methods
The multilocus sequence typing technique was used for the characterization of 346 meningococci isolated from meningitis cases in 5 countries of the meningitis belt between 2004 and 2007 : Benin (9), Burkina Faso (169), Chad (7), Niger (111), Togo (50).
Results
Among these strains, 90% belonged to 5 STs. The ST-5 complex (cc5) (group A) included 74% of the isolates (26% were ST-7 and 36 % were ST-2859), 8.9% were ST-2881 (group W135), 4.6% were ST-11 (group W135), and 8.3% were ST-181 (group X).
Conclusion
Serogroup A *Neisseria meningitidis* of cc5 was responsible for most of the outbreaks and hundreds of thousands of cases in the African meningitis belt since 1988. Three major STs are included in this complex: ST-5, ST-7 and ST-2859. The pandemic ST-5 and ST-7 emerged first in China and later in Africa. ST-5 was introduced in Africa in 1987 by pilgrims coming back from Saudi Arabia and was responsible for most of the meningitis cases between 1988 and 2001. ST-7 emerged in the region in 1997, and then totally took the place of ST-5. At the opposite, ST-2859 emerged first in Burkina Faso in 2003 and was responsible for the recent epidemics in this country in 2006 and 2007 (18,000 and 26,000 cases). In 2007, this ST was isolated for the first time in Niger and in Togo. Might ST-2859 be at the origin of another epidemic wave in the next years in Togo and in Niger and in the African meningitis belt?
The W135 ST-11 was responsible for the first important epidemic ever notified in the world in Burkina Faso in 2002 (12,000 cases). However, the number of isolates of this complex tends to decrease over the last years and be only responsible for sporadic cases. However, its presence in refugee camps in Chad in 2005 has led to a vaccination campaign organized by Health authorities, WHO and MSF. It was feared that it might be responsible for important epidemics in other countries of the belt; fortunately, it has not happened until now. Group W135 isolates have also been isolated in Benin, Niger, and Togo between 2005 and 2007, but most belonged to ST-2881 that is known to be less epidemic. In 2006 the first important outbreak due to group X ST-181 meningococci in Niger involved approximately 800 cases.
The role of the laboratory network in meningitis outbreak is important in Africa to recommend the appropriate vaccine and appropriate treatment. By performing molecular epidemiology, the WHO collaborating centers can follow the clonal complexes.

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Design and implementation of an external quality assessment (EQA) scheme for *Neisseria meningitidis* characterisation of culture and non-culture material

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An EQA scheme was designed to assess current practice for phenotypic and genotypic strain characterisation to assist in standardisation of European Meningococcal Reference laboratory non-culture detection, to ensure accurate surveillance.
Funded by EU-IBIS (European Union Invasive Bacterial Infections Surveillance) to include all EU member and accession states (eg. 2007 distribution; Austria, Belgium, Czech Republic, Denmark, Estonia, England, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Netherlands, Norway, Poland, Portugal, Scotland, Slovak Republic, Slovenia, Spain and Sweden).
Three panels of *N. meningitidis* isolates and novel simulated septicaemic blood samples were distributed for phenotypic and genotypic (meningococcal nucleic acid detection, serogroup determination, porA sequence typing and MLST) characterisation as performed by the participating laboratories. Isolates were selected to represent epidemiologically important strains in Europe. The simulated septicaemic blood samples comprised "standardised"heat-killed suspensions of organisms diluted in defibrinated horse blood prepared by the MRU. Quantification of viable numbers of organisms were made by the Miles and Misera technique and an assessment of the material in MRU in-house real-time PCR assays was made prior to transfer to eQAD for freeze-drying, safe panel distribution and returned reports analysis.
Each participating laboratory received a report comparing their results to the consensus for each sample characteristic. There was good agreement for the phenotypic characteristics with minor clerical errors in reporting and some differences in nomenclature identified. Access to (or lack of local requirement for) reagents for serogroup A may have caused problems. A poorly expressing serogroup C isolate also challenged some laboratories.
Non-culture detection was generally successful for the increasing numbers attempting it. The maximum number of laboratories was 20 in 2006 for two serogroup B samples. Likewise, for the increasing number of laboratories undertaking genotypic characterisation there was excellent agreement but problems were encountered with the more dilute simulated non-culture samples of approximately x103 organisms/mL; which may indicate a practical limit for successful detection and molecular typing.
Comparison of individuals' laboratory results with the consensus enabled a detailed analysis of some sequencing results to reveal possible base calling errors, clerical or even mis-priming events (exampled by one dilute non-culture sample). The sample panels also included uncommon serogroup C organisms with possible porA deletions or stop codons.
European phenotypic and genotypic strain characterisation including nucleic acid detection and non-culture genotyping was successfully established for EU-IBIS participants. The three EQA distributions demonstrated the utility and improved standardisation obtained by molecular strain characterisation.
The second and third EQA distributions identified areas for improvement primarily concerning low copy number nucleic acid detection for non-culture confirmation of infection and subsequent strain characterisation including serogroup identification. Allowing the designation of clonal complex as opposed to just MLST sequence type accommodated some of the minor errors in characterisation of the dilute non-culture samples. Also highlighted were issues regarding reporting, nomenclature and reagent availability.
Feedback from participants was taken at the EMGM conference in Rome 2007 prior to the final, third distribution. The EU-IBIS EQA project has informed part of the recent successful EMGM led tender for an ECDC funded meningococcal EQA distribution.

P164

Multilocus sequence typing: An approach to the identification of clones within North Indian isolates of *Neisseria meningitidis* serogroup A

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Objective

Neisseria meningitidis serogroup A is the major cause of meningitis outbreaks worldwide, especially in African and Asian countries, including India. Meningococcal disease is endemic in India. A total of 444 meningococcal cases and 62 deaths due to meningococcal meningitis serogroup A were reported in Delhi during April-July 2005. The reappearance of meningococcal cases was reported in Delhi during January-March 2006 (177 meningococcal cases and 17 deaths).The majority of cases and all deaths occurred in young adults between 16 -30 years of age. Multilocus sequence typing is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of (usually) seven house-keeping genes. It has the ability to identify accurately the strains of infectious agents that cause disease is the key of epidemiological surveillance.

The study was designed for the development and validation of mlst for the identification of the virulent lineages of *Neisseria meningitidis*.

Methods

1. CSF from suspected cases were colleted during the outbreak of meningococcal disease in 2005
2. Cultured on chocolate agar plate and kept at 37ºC with 5% CO2
3. Oxidase test was conducted
4. DNA were extracted by boiling method
5. Seven housekeeping genes were amplified from chromosomal DNA of 15 N.meningitidis strains by using PCR.
   abcZ (putative ABC transporter)
   adk (adenylate kinase)
   aroE (shikimate dehydrogenase)
   fumC (fumarate hydratase)
   gdh (glucose-6-phosphate dehydrogenase)
   pdhC (pyruvate dehydrogenase subunit)
   pgm (phosphoglucomutase)
6. Internal fragments of each gene are sequenced by using an automated DNA sequencer ABI PRISM 310. Sequences were submitted to MLST database (http://pubmlst.org/neisseria/)

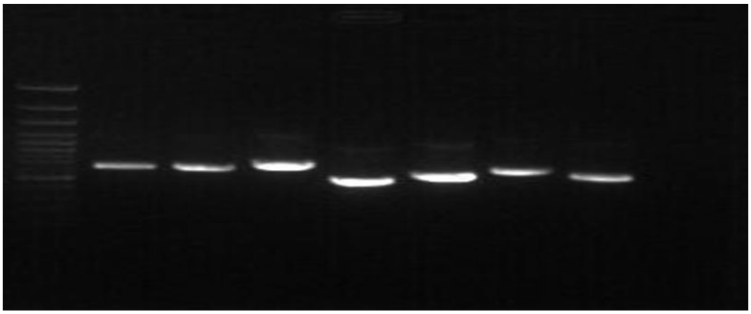
Results

1. All the seven genes matched to existing alleles in the MLST database. No new alleles were found
2. Database confirms all the strains belong to clonal complex ST-5 complex/subgroup III.
3. Sequence type 4789 was confirmed.
4. The MLST database showed the recent outbreaks in Asian countries belonged to clonal complex ST-5

Conclusion

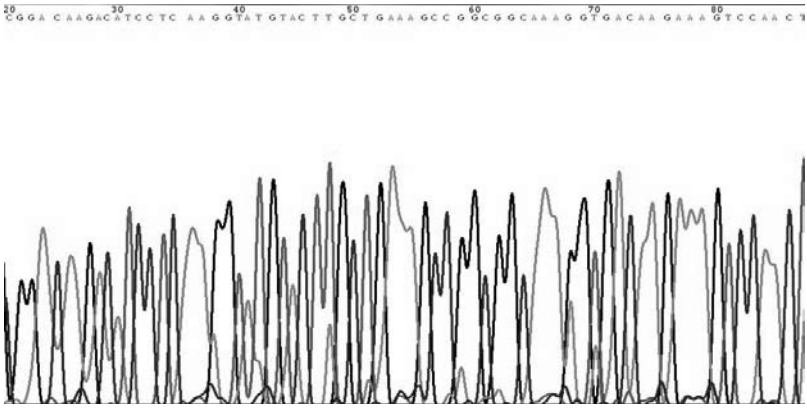
1. Sequence type 4789 was confirmed.
   The MLST database showed the recent outbreaks in Asian countries belonged to clonal complex ST-5
2. MLST is very useful for the identification of the currently circulating hyper-virulent lineages because these are recognized as clusters of isolates with identical, or very similar, multilocus sequence types.

Figure 1: pcr gel picture



From left to right:marker(100bp),**abcZ adk aroE fumC gdh pdhC pgm**
(internal fragments varing from 650 10 450 bp)

Figure 2: chromatogram



P165

Improved Real-time PCR to Detect *N. meningitidis, S. pneumoniae* and *H. influenzae* from international clinical specimens

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Objectives

Real-time PCR assays developed by Mothershed et al have been used to detect ctrA-bearing *N. meningitidis* (Nmen) and serogroups A, B, C, X, Y, and W135. Several changes have been made to the primers, probes, and cycle parameters for Nmen detection from cerebrospinal fluid (CSF). Reported herein

are the modifications of the assays and results from their use to detect Nmen from CSF collected from patients with suspected meningitis in Turkey and Mongolia.

Methods

All probes were synthesized with FAM as the 5' fluorophore. The black hole quencher (BHQ) was moved from the 3' end of the ctrA, sacB, synE (siaD of Nmen C), and synF (siaD of Nmen Y) probes to an interior thymine to reduce the background signal, and SpC6 was added to the 3' end of these probes to prevent priming from that end. The synE forward primer was moved four nucleotides downstream and shortened by one nucleotide. Denaturation and annealing/extension times were decreased. Lower limits of detection (LLDs), the amount of DNA that yields a Ct value equal to the cutoff of 40, were determined for ctrA, sacB, synE, and synF. A modified protocol from that of the QIAamp DNA Mini kit was used to extract the DNA from CSF specimens collected from meningitis patients in Turkey and Mongolia. All specimens came from patients who met the case definition for purulent meningitis [leucocytosis (›100 cells/mm3); and either elevated protein (›100 mg/dl) or decreased glucose (‹40 mg/dl)]. This modification includes the use of mutanolysin and lysozyme to improve detection of the gram-positive meningitis pathogen Streptococcus pneumoniae (SP). This adapted real-time PCR was performed on the extracted CSFs, and this method was compared to latex agglutination or culture for determining bacterial meningitis etiology.

Results

The LLD of our modified assays is approximately 8 meningococcal genomes for ctrA of all serogroups, and in a range of 0.8 to 80 meningococcal genomes for the serogroup-specific genes. PCR efficiency for synE detection is 95.7%.

Sixty-two of 170 (36%) CSFs from Turkey were PCR-positive for Nmen, *H. influenzae* (Hi), or Sp. Of the Nmen PCR-positive specimens, 33% (4/12) were assigned to specific serogroups. Latex agglutination was not performed, and 5/170 (3%) CSFs were culture positive for one of the three agents. Real-time PCR improved detection of Turkish bacterial meningitis agents by 33% compared with culture (p value ‹ 0.0001).

Seventy of 98 (71%) CSFs from Mongolia were PCR-positive for one of the three agents. Of the Nmen PCR-positive specimens, 60% (18/30) were assigned to specific serogroups. Latex agglutination and culture were positive for one of the three agents for 51/98 (52%) and 31/98 (32%) CSFs, respectively. Real-time PCR improved detection of Mongolian bacterial meningitis agents by 19% compared with latex agglutination (p value =0.006) and by 39% compared with culture (p value ‹ 0.0001).

Conclusions

The modified PCR assays exhibit high efficiency and sensitivity sufficient to detect and characterize Nmen, Hi, and Sp in CSF. They significantly improve detection of bacterial meningitis pathogens compared to latex agglutination and culture methods.

P166

A twenty years retrospective analysis of meningitis surveillance data from Burkina Faso, Mali and Niger

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Background

Epidemic meningitis is still a problem of public importance within the African meningitis belt. Long term surveillance data is crucial for understanding the dynamics of the disease and improving the control strategies. Three countries have put in place an extensive surveillance database on cerebrospinal meningitis for the past 20 years (Burkina Faso, Mali and Niger), with a systematic reporting of year-round weekly number of suspected cases and deaths. A detailed analysis of trends and distribution of this data was conducted.

Objectives
Describe dynamics of epidemic meningitis in three hyper endemic countries both at National and district level; Elaborate risk mapping of districts; Make available a data base for operational research purposes and conduct predictive mathematical modeling for forecasting and early warning purposes.
Methods
Data was collected from the Ministries of Health by the Ouagadougou-based WHO Multi Disease Surveillance Centre (MDSC) with support from the Meningitis Vaccine Project (MVP). The databases were standardized, formatted for quality control, cleaned and completed for missing data, double entries, district name labels, and population estimates.
Analysis
The original databases were all converted to Excel format using Stat-transfer software. The descriptive analysis and risk mapping was done using Excel, SPSS, Stata and Health Map. Means incidences and confidence intervals were calculated for selected variables.
Results
Cumulative attack rates per 100 000 inhabitants and case fatality rates (%) for the study period were respectively: Burkina Faso: 684 (14.4%); Mali: 266 (10.2%) and Niger: 1918 (8.2%). The annual average case fatality ratios are higher in non-epidemic years than epidemic years. The epidemic dynamics at district level shows the highest epidemic activity from week 8-20, with a peak at week 13 in Burkina Faso, week 14 in Mali and Niger. The average epidemic duration is 5 weeks in Mali, 7 weeks in Burkina Faso and 10 weeks in Niger. Epidemic waves are observed every 3-4 years with inter epidemic intervals of 3-4 years.
Conclusion
These data provides a comprehensive overview of the seriousness of epidemic meningitis in the African meningitis belt. The data sets provide a sound base to evaluate the impact of the introduction of meningococcal conjugate vaccines.

P167

Association of a bacteriophage with meningococcal disease in young adults

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Objectives
Neisseria meningitidis, an obligate commensal inhabitant of the nasopharynx of humans, occasionally causes severe disease. The phenomenon, of a minority of infections leading to invasion whilst most result in asymptomatic colonisation lasting months or even years, remains poorly understood. It is known that certain phylogenetic groups of meningococci, known as hyperinvasive lineages, are very much more likely to cause disease, but the genetic basis for virulence has not been fully elucidated. A previous genomic comparison of meningococci isolated from disease and carriage in the Czech Republic in 1993 identified a chromosomally-integrated bacteriophage as a putative virulence determinant.
Methods
In this work we have undertaken a survey of the presence of the phage by PCR and DNA-DNA hybridisation in 1288 meningococci isolated from cases of invasive disease and asymptomatic carriage in South East England between 1999 and 2001.
Results
The phage was highly over-represented in disease isolates from young adults, but not in isolates causing disease in infants indicating that the phage contributes significantly to invasion in adults but not infants. After statistical correction for meningococcal population structure, between 20% and 45% of the disease-

causing potential of five common hyperinvasive lineages could be attributed to the presence of the phage or factors associated with the presence of the phage.

Conclusion

The molecular epidemiological strategy, using data from human disease case isolates, permits the investigation of virulence factors in the absence of an animal model of human disease, and will bring to light genetic elements having subtle effects on pathogenic potential which may not be detected in laboratory models. By this methodology we have demonstrated the association of a chromosomally-inserted bacteriophage with disease in a specific age range of the human population. The approach is particularly valuable in the study of exclusively human infection, such as meningococcal disease.

P168

Carriage of *Neisseria meningitidis* in 2-29 years old in Mali, Senegal and The Gambia

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Objectives

A novel tetanus toxoid conjugate *Neisseria meningitidis* serogroup A vaccine (MenAfriVac) is under phase II clinical trials in several African countries. Conjugate vaccines have the ability to reduce carriage and thus, transmission of the bacterium in the population. The aim of this study was to provide preliminary information regarding meningococcal carriage in West Africa and on the effect of MenAfriVac on carriage.

Methods

A total of 900 individuals (300 in Mali, 300 in The Gambia, and 300 in Senegal) from 2 to 19 years old were enrolled as part of the clinical trial and sampled twice, just before vaccination and about one month later in the late summer/fall of 2007. Pharyngeal samples were plated directly on selective medium and incubated for 24-48 hours. Growth was collected from the plate, suspended in Greaves solution and frozen immediately at -20ºC. Samples were sent frozen to Oslo, Norway where meningococci were identified by standard procedures. The strains were serogrouped and analysed by multilocus sequence typing, porA and fetA sequencing as described at http://pubmlst.org/neisseria/.

Results

N. meningitidis was recovered from 57 individuals in Mali (carriage rate 19%), 47 in The Gambia (16%), and 13 in Senegal (4%). In Mali about 50% of the individuals were positive in both samples, while less than a third of the individuals were stable carriers in The Gambia and none were in Senegal. The majority of the isolates were non-serogroupable. Among strains with expressed capsular polysaccharide, serogroup W135 predominated in Mali, while serogroup Y predominated in The Gambia and Senegal. A single serogroup A isolate was identified in a carrier in Mali, but the individual was negative for meningococci in the following sample. Molecular analyses of the isolates will be presented.

Conclusions

Variable carriage rate and serogroup heterogenicity of the *N. mengitidis* isolates are the main findings of this study. The near absence of serogroup A isolates did not allow to draw any conclusion on the impact of MenA conjugate vaccine in this study population.

P169

Significant increase of serogroup W135 invasive *Neisseria meningitidis* strains in Argentina: A new epidemiological feature on the region

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The Reference Laboratory of the National Institute for Infectious Diseases (INEI-ANLIS, "Dr Carlos G Malbrán") in Argentina receive around 50% of meningococcal strains isolated from invasive cases in all the country. The laboratory-based data are supported by a hospital network that is sending isolates in a regular basis. Also the reference laboratory participates in an external quality assurance program coordinated by the Pan American Health Organization (PAHO/WHO). Within this context, the reference laboratory is producing annual reports about serogroup distribution, strains characterization and antimicrobial susceptibility, and the serogroup B has been the most frequent since 2001, and still is. Serogroup C presented an epidemic wave at the end of the 90s years, and it has represented around 20% of the strains until 2005. In 2006, a new situation appeared with a similar proportion (7%) of isolates showing serogroup C, Y or W135, evolving to 11.4%, 6.8% and 13% respectively during 2007. During the first 5 months in 2008 serogroup W135 increased dramatically reaching 27.7% of the received cases. Although in 2007 most of the cases (89%) were isolated in Buenos Aires Province (including Buenos Aires City), seems to be affecting a wider range of territories. Most of the cases (63%) appeared in children under 9 years old, with 28% under 1 year old. The age distribution might be affected because most of the hospitals sending strains to the reference laboratory are in fact paediatric hospitals that might produce some bias in the data. In order to do a preliminary analysis of the new epidemiological situation, 7 strains randomly chosen among those received in 2007 (4 strains) and 2008 (3 strains) have been characterized by MLST, porA and fetA sequence. All the strains belonged to the ST11 (CC ST-11) and showed the same porA (5,2) and fetA (F1-1) alleles. These preliminary results looks like an epidemic situation rather than the results that can be usually found in endemic situations with more heterogeneous strains. A wider study including more than 200 W135 strains is under way not only to analyze the situation in Argentina, but also to compare the W135 invasive cases in Brazil and Chile (with also a slight increase during the last years) and Colombia (with a small proportion of W135 cases).

P170

Interactions of wild-type Neisseria gonorrhoeae and minC, minD and oxyR mutants with HeLa, ME-180 and T-uec cells

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Objective
To assess whether inactivating cell division genes minC, minD and the MinC regulatory gene oxyR of *Neisseria gonorrhoeae* (Ng) affects interactions between Ng and different host cells.
Materials and methods
Individual Ng mutants were created by insertional inactivation of minC (CSRC1 -minC::cat), minD (CJSD1 -minD::cat) and oxyR (KB1 -oxyR::cat) of Ng CH811. The ability of these mutants and Ng CH811 to adhere to and invade HeLa, ME-180 and T-uec cells was evaluated. The MOI was 1:100. Methods to assess adherence and invasion have been described previously (Edwards et al 2000, Infect. Immun. 68: 5354-

5363). Inhibition assays were performed using cytochalasin-D (a microfilament depolymering agent) and wortmannin (a phosphoinositadyle-3-kinase [PI3K] inhibitor). For survival assays, infected cell monolayers were incubated for 24 or 48 hr followed by determination of intracellular Ng cell numbers. Scanning or tansmission electron microscopy (SEM/TEM) of Ng-infected T-uec cells was also performed using standard protocols (Edwards et al 2000, Infect. Immun. 68: 5354-5363).

Results

The Ng mutants CSRC1, CJSD1and KB1 exhibited lowered adherence to T-uec cells, by one log, as compared to Ng CH811, while adherence to HeLa or ME180 cells was not significantly different between the mutants and Ng CH811. The ability of Ng CSRC1 or Ng CJSD1 to invade ME-180 and T-uec cells was significantly lower than Ng CH811. Ng CSRC1 or Ng CJSD1 both exhibited significantly reduced the abilities to invade HeLa cells, while Ng KB1 did not differ significantly from Ng CH811. Although the Ng mutants were able to survive and replicate in T-uec cells, their intracellular cfu were significantly lower than Ng CH811 at 24 and 48 hr. All strains increased their cfu by 2 logs after 24 hr and by a further 1 log up to 48hr. Inhibitory assays demonstrated that both wortmannin and cytochalasin-D prohibited the entry of Ng CH811 by more than 90%. By contrast, there was an approximately 35% decrease in the entry of the three Ng mutants in the presence of these inhibitors. SEM analysis indicated that Ng CH811 aggregated and attached to the T-uec cell surface and induced membrane ruffling, while the mutants exhibited diffuse attachment and failed to induce membrane ruffling. TEM studies revealed that Ng CH811 cells were internalized into T-uec cells as multiple numbers in single vacuoles, whereas the mutant cells were internalized as single cells.

Conclusion

The inactivation of Ng minC, minD and oxyR reduced the abilities of Ng cells to adhere to and invade host cells and this reduction was cell line dependent. The invasion of the Ng mutants was less affected by microfilament depolymerisation and PI3K kinase inhibition in their ability to invade T-uec cells. Coupled with the failure of Ng mutants to induce membrane ruffling in T-uec cells, these data suggest that the interaction of these mutants with host cell is not associated with triggering mechanisms.

P171

Opa expression retards transcytosis of gonococci across polarized epithelial cells

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Objectives

Gonococci possess the ability to cross cellular barriers to induce disseminated infections. Several phase variable molecules of the bacteria play different roles in the disease process. While the role of Opa in facilitating entry into host cells has been clearly demonstrated, the elucidation of the overall contributions of Opa in infection has been difficult because populations that have been defined as Opa negative still express low levels of Opa, and individual variants that express Opa readily arise in study populations. This study examines the role of Opa in gonococci-gonococci interaction and gonococcal transcytosis across polarized epithelial cells.

Methods

We establised a derivative of *N. gonorrhoeae* MS11 that had the coding sequence for all Opa proteins deleted. We compared the properties of this strain to an Opa+ and phenotypically Opa- strains by measuring their growth curves and analyzing microscopically the morphology of the bacteria. Fluorophore-conjugated LOS was used to examine the interaction of the bacteria to bacteria through LOS and Opa. The ability of different strains of gonococci to trancscytose across polarized epithelial cells and the interaction of the gonococci with the epithelial cells were analyzed.

Results

While MS11Opa+ and MS11Δopa strains grow in a similar rate, MS11Δopa strain forms much smaller

clumps than MS11Opa+ strain in growth medium. Similarly after incubating with polarized T84 human epithelial cells for 6 hours, MS11Δopa bacteria appeared to form smaller clusters on the epithelial cell surface. In contrast, MS11Opa+ bacteria formed fewer but much larger cluster on the epithelial cell surface. In both cases, the bacteria preferentially distribute to host cell-cell junctions. Fluorophore-conjugated gonococcal LOS bind to MS11 Opa+, but not to MS11Δopa, further confirming the interaction of LOS and Opa. Most strikingly, MS11Δopa readily transcytoses across polarized T84 cells at 4 hours. When MS11Opa+ cells where added to the apical surface, bacteria were recovered in the basolateral media after 6 hours, but these cells were phenotypically Opa-.

Conclusion

Our results showed that lack of Opa expression facilitates gonococcal transcytosis across polarized epithelial cells, but reduces gonococci-gonococci interaction, implicating a negative role for Opa in entry of gonococci into tissue.

P172

An opacity protein-deficient mutant is attenuated during gonococcal genital tract infection of female mice and ovarian hormones affect the recovery of opa variants *in vivo*

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Background

The gonococcal opacity (Opa) proteins are phase variable outer membrane proteins that mediate adherence to and invasion of host cells and protection from complement-mediated killing. During cervical infections, Opa protein expression and culture positivity correlate with the phase of the menstrual cycle. Consistent with these observations from women, we previously reported that selection for Opa-positive variants occurs in the lower genital tract of female mice. Recovery of Opa-positive variants was cyclical and three phases of Opa protein recovery were defined: selection of Opa-positive variants (early phase), recovery of predominantly Opa-negative bacteria (mid phase) and re-emergence of Opa-positive variants (late phase).

Objectives

Here, we hypothesized that Opa proteins provide an advantage *in vivo* and predicted that an Opa-expressing strain would be more fit than an Opa-deficient strain during lower genital tract infection. We also hypothesized that the cyclical recovery pattern of Opa variants has a hormonal basis and that these events may be driven by complement, which is known to fluctuate over the female reproductive cycle.

Methods

To test whether Opa expression provides an advantage *in vivo*, mice were inoculated with an Opa-deficient mutant or a complemented derivative that carries a constitutive opaB gene and the recovery of gonococci was monitored. To investigate the role of gonadal hormones in the cyclical Opa recovery pattern, estradiol-treated intact or ovariectomized (Ov-) mice were inoculated with predominantly Opa-negative wild type bacteria. The Opa phenotype of vaginal isolates was monitored daily by colony suspension immunoblot. Similar studies were performed in mice treated with cobra venom factor to deplete host complement.

Results

Consistent with selection during the early phase, mice inoculated with the constitutively OpaB-expressing strain had a reproducibly higher colonization load on day 1 compared to mice infected with the Opa-deficient strain. During the mid phase both groups demonstrated an overall decrease in colonization. However, recovery from the mid phase was compromised in mice infected with the Opa-deficient strain as evidenced by significantly fewer bacteria recovered and fewer mice colonized compared to the OpaB-expressing strain. In studies with wild type bacteria, estrogen-treated Ov- mice selected for Opa protein expression in the early phase, but in contrast with intact mice, recovery of Opa-positive variants in Ov- mice was not cyclical and there were no corresponding fluctuations in the total number of bacteria recovered.

No changes in Opa phenotype were detected during *in vitro* culture of gonococci with estradiol or progesterone. Complement-depleted mice showed selection for Opa expression and a cyclical recovery pattern similar to that observed in normal mice.

Conclusions

We conclude from these studies that Opa protein expression confers a long-term colonization advantage in the lower genital tract of female mice. As mice lack human carcinoembryonic antigen-related cellular adhesion molecules, the major Opa protein receptors, and complement depletion did not alter the recovery pattern, we have ruled out two factors that were predicted from *in vitro* studies to play a selective role *in vivo*. We are currently investigating potential hormonally regulated host factors that could select for Opa-expression in the female genital tract.

P173

Tetraspanins influence *Neisseria meningitidis* uptake by human macrophages and Opa-dependent Interactions with epithelial cells

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Objectives

Neisseria meningitidis (Nm) is the causative agent of meningococcal disease and meningococcal meningitis. The first step in the pathogenesis of meningococcal disease is colonisation of the human nasopharynx by Nm. To invade the bloodstream, Nm must cross the nasopharyngeal epithelium. The organism can traverse epithelial and endothelial cells via specific adhesins such as type IV pili and Opa proteins. The tetraspanins are a superfamily of transmembrane proteins with a diverse array of functions, which are able to form large microdomains on the cell membrane in conjunction with partner proteins, including the integrins and members of the immunoglobulin superfamily. We examined the direct or indirect effect of tetraspanin function upon Nm binding and internalisation.

Methods

HEC-1-B and DETROIT 562 epithelial cells, as well as human monocyte derived macrophages (MDMs), were treated with a variety of anti-tetraspanin antibodies (anti-CD9, anti-CD63 and anti-CD151), prior to infection with log-phase, wild-type Nm. Binding and internalisation of bacteria was measured by fluorescence microscopy, as was tetraspanin expression. Adhesin variants of strain MC58 were used to determine which adhesins interact with tetraspanin-mediated adherence.

Results

The tetraspanin family is variably expressed on the surface of epithelial cells. Blocking of tetraspanins using anti-tetraspanin antibodies significantly reduced binding of Nm to both epithelial cell lines after one hour co-incubation. Binding of Nm to MDMs was also significantly reduced following pre-treatment with anti-tetraspanin antibody. Combinatorial use of anti-tetraspanin antibodies accentuated the reduction in Nm binding. Opa-deficient variants of acapsulate Nm demonstrated significantly reduced binding to epithelial cells compared to Opa-replete strains. Pre-treatment of epithelial cells with anti-tetraspanin antibody reduced the binding of the Opa-replete strains but not of the Opa-deficient strains.

Conclusions

Binding of Nm to epithelial cells and MDMs is influenced by tetraspanins. This interaction appears to be Opa-dependent.

P174

NadR, a negative transcriptional regulator of the *Neisseria meningitidis* adhesin NadA

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Objectives
Transcriptional regulation is highly important for pathogenic bacteria as it enables them to adapt to host environments. In the human pathogen *Neisseria meningitidis*, NMB1843 is a transcriptional regulator of the MarR-family. We set out to identify genes controlled by this regulator and determine its role in meningococcal pathogenesis.
Methods
Electrophoretic mobility shift assays (EMSAs) were used to identify and characterize the binding site of the regulator. Dot blot assays were performed to analyze the distribution of the regulator gene within the species *N. meningitidis* and infection experiments as well as susceptibility assays were conducted to elucidate the impact of the deletion of the regulator gene on the meningococci.
Results
We showed that the MarR-like regulator NMB1843 negatively regulates the Neisseria adhesin A (NadA) and therefore designated it NadR. In contrast to NadA, which has been shown to be distributed heterogeneously throughout the species *N. meningitidis*, being overrepresented in the hypervirulent lineages (Comanducci et al., 2002); we could show that the regulator NadR is present in all tested strains, suggesting that NadR established control over NadA after the latter was acquired by horizontal gene transfer. Using EMSAs, we proved that NadR specifically binds to the nadA promoter region, indicating direct regulation. The exact binding site was characterized and narrowed to a sixteen base pair palindromic repeat. By means of point mutations in this recognition sequence the single base pairs important for NadR binding were determined and a preliminary consensus sequence was established. This direct regulation of NadA caused a changed phenotype of the nadR mutant strain in infection experiments, allowing a significant higher association of the mutant strain with epithelial cells.
Conclusion
Hence the expression of the adhesin NadA is not only controlled by phase variation due to the microsatellite in its promoter region (Martin et al., 2003), but additionally by the negative transcriptional regulator NadR.
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P175

Adherence of *Neisseria gonorrhoeae* to human cervical cells in competition with different lactobacilli species

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Objectives
Neisseria gonorrhoeae is a human specific pathogenic bacterium that colonises the urogenital tract where it causes gonorrhoea. Lactobacilli are normal inhabitants of our genital microbiota and are known to protect against pathogens. In this study, we analyse the early interaction between piliated *N. gonorrhoeae* MS11 and different Lactobacillus species and their competition for adhering to ME-180 cervical cells.
Methods
We used the piliated *N. gonorrhoeae* MS11 strain together with four different lactobacilli strains isolated from healthy human individuals. Two of the strains, *L. gasseri* and *L. crispatus* are commonly found in the genital tract, but also *L. reuteri* isolates is frequently found. The fourth strain, *L. rhamnosus*, is not a normal genital inhabitant. ME-180 cervical carcinoma cells was used as the target cell for both gonococcal and lactobacilli adherence. We quantified bacterial adhesion and visualised the interplay between *N. gonorrhoeae* and Lactobacillus by live cell imaging microscopy.
Results
We show that the number of adhered lactobacilli does not correlate to the level of protection against gonococcal infection. Protection against gonococcal adherence is different between lactobacillus species. *L. crispatus*, *L. gasseri* and *L. reuteri* were able to reduce gonococcal adherence while *L. rhamnosus* was not. Lactobacilli strains from a vaginal origin had the best capacities to remain attached to the host cell during gonococcal adhesion. Further, we show that gonococci and lactobacilli interact with each other, with lactobacilli being incorporated into the gonococcal microcolony. Gonococci bind to colonised lactobacilli and this complex frequently detached from the epithelial cell surface, resulting in reduced bacterial colonisation. Also, purified gonococcal pili are capable of removing adherent lactobacilli from the cell surface.
Conclusion
We present new revealing data that quantifies and visualizes early interactions between *N. gonorrhoeae* and Lactobacillus during their competition for adherence.

P176

Lactobacilli inhibit meningococcal interaction with host cells

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Objectives
The normal microflora prevents colonization of pathogenic bacteria and represents an important first line of defence. Mechanisms of colonization resistance are attachment interference, production of inhibitory substances and toxic metabolic end products and nutrient depletion. Lactobacilli belong to the normal microflora and colonize various parts of the body. These bacteria are common inhabitants of the mucus membranes in the oral-pharyngeal tract. Lactobacilli play an important role in the protection against many gastrointestinal pathogens. Less is known about the antagonistic capacity of lactobacillus to *N. meningitidis* infection. In this work, we study the impact of lactobacilli on meningococcal interaction with human oropharyngeal epithelial cells.

Methods
We use a set of lactobacilli strains of different origin as well as various meningococcal strains and mutants. Ability to interfere with meningococcal attachment and invasion was evaluated by exclusion, competition and displacement experiments.
Results and conclusions
The capacity to interfere with meningococcal adhesion to host cells varied among different lactobacilli strains commonly found in the oral-pharyngeal cavity. Some strains inhibited adhesion of meningococci to host cells while other strains did not interfere with adhesion at all. We are studying the role of different meningococcal virulence factors and found that the polysaccharide capsule protected meningococci from lactobacillus-mediated inhibition. The molecular mechanisms of the observed results are being analyzed. The composition of the normal microflora is not constant but vary. Factors that influence the composition are health status, medication, hormonal changes and diet among others. The composition of the lactobacilli flora might be important in how efficiently it provides colonization resistance.

P177

Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukocytes

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Symptomatic infection with *Neisseria gonorrhoeae* results in a potent polymorphonuclear leukocyte (PMN)-driven inflammatory response, but the mechanisms by which gonococci withstand PMN attack are poorly defined. We have found that *N. gonorrhoeae* suppresses the ability of PMNs to produce reactive oxygen species and mount the oxidative burst, a central component of the PMN antimicrobial arsenal. Piliated *N. gonorrhoeae* of strains FA1090 and MS11 were grown in liquid culture to mid-exponential phase and incubated with primary human PMNs at a multiplicity of infection of 100 colony-forming units per PMN. The ability of PMNs to generate reactive oxygen species was monitored using luminol-dependent chemiluminescence. Opacity-associated protein (Opa)-negative *N. gonorrhoeae* of strains FA1090 and MS11 were phagocytosed by PMNs, but the PMNs did not generate reactive oxygen species in response to infection, even after bacterial opsonization. This was not due to effects on PMN viability, since there was no increase in lactate dehydrogenase release from PMNs after infection with *N. gonorrhoeae*. FA1090 *N. gonorrhoeae* expressing OpaB, which has been correlated with PMN reactive oxygen species production, elicited a PMN oxidative burst that was minor in comparison to the burst elicited by phorbol ester stimulation. The oxidative burst in response to Opa- and OpaB+ bacteria was markedly enhanced if bacteria were agar-grown or if liquid-grown bacteria were heat killed. Moreover, liquid-grown Opa- *N. gonorrhoeae* inhibited the PMN oxidative burst elicited by isogenic heat-killed bacteria, formylated peptides or *Staphylococcus aureus* in a dose-dependent manner; however, they did not inhibit PMN ROS production by OpaB+ *N. gonorrhoeae* or phorbol esters. Suppression of the oxidative burst required gonococcal-PMN contact and bacterial protein synthesis but not phagocytosis. We conclude that liquid-grown, exponential-phase *N. gonorrhoeae* inhibit the ability of PMNs to generate the oxidative burst and propose that this is accomplished through bacterial interference with PMN signaling pathways required for induction of NADPH oxidase activity. Suppression of the PMN oxidative burst may contribute to bacterial survival during inflammatory stages of gonorrheal disease.

P178

Neisseria meningitidis microcolonies resist mechanical stress by reshaping the host cell plasma membrane

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Bacterial infections targeting the bloodstream lead to a wide array of devastating diseases such as septic shock and meningitis. To study this crucial phase of the infection, its specific environment needs to be taken into account, in particular the mechanical forces generated by the blood flow. In a previous study using *N. meningitidis* as a model we observed using a laminar flow chamber that bacterial microcolonies that form on the endothelial cell surface in the vessel lumen are remarkably resistant to mechanical stress and the present study aims to identify the molecular basis of this resistance. *N. meningitidis* forms aggregates independently of host cells. Yet we demonstrate here using a cone and plate shear device that cohesive forces involved in these bacterial aggregates are not sufficient to explain the stability of colonies on the cells surface. Upon adhesion, bacteria trigger a potent host cell response consisting of cellular projections similar to filopodia that come in close contact with all the bacteria in the microcolony. We then show by genetic and pharmacological approaches that this cellular response confers resistance to mechanical stress to bacterial clusters lodged on host cell surface. We identify the minor pilin PilV as a triggering factor to induce plasma membrane reorganization and found that a mutant deficient for the pilV gene is highly sensitive to shear stress. Pharmacological inhibition of cellular projections by cholesterol depletion with methyl-ß-cyclo dextrin also renders microcolonies sensitive to blood flow generated drag forces. We conclude that *N. meningitidis* reorganization of the host cell plasma membrane generates a local environment where it can resist the harsh mechanical conditions found in the bloodstream.

P179

N. meningitidis induces degradation of brain endothelial tight junction components by a matrix metalloproteinase-dependent mechanism

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Objectives
The present study analyses the physiological and morphological properties of the endothelial cells underlining the blood-brain barrier (BBB) after exposure to *N. meningitidis.* This endothelium differs from the endothelial cells of the leaky peripheral vasculature by the presence of tight junctions. We investigated the effects of *N. meningitidis* on the tight junction proteins occludin, claudin-1 and zonula occludens-1 (ZO-1), which are important molecules involved in cell-cell contact and the maintenance of the integrity and polarity of the endothelium.
Methods
Human brain microvascular endothelial cells (HBMEC) were grown on porous membranes and infected with *N. meningitidis* for 24 h. Flux of FITC-dextrans was measured to determine permeability changes. The amount and distribution of tight junction proteins was analyzed by immunofluorescence microscopy and Western blotting. Inhibitor studies using the broad-spectrum MMP inhibitor GM6001 and the TNF-alpha converting enzyme (TACE) inhibitor TAPI-2 were conducted.
Results
Our findings showed that *N. meningitidis* induced local disappearance of occludin from the cell border to the cytoplasm in immunfluorescence microscopy studies, while ZO-1 and claudin-1 were not affected.

Immunoblot analysis displayed that redistribution of occludin was a result of protein degradation, leading to the formation of a cleavage fragment with an apparent molecular mass of about 50 kDa and a further weak fragment with an apparent molecular mass of about 28 kDa. Protein degradation was dependent on the number of infecting bacteria. The degraded form of occludin peaked at 24h post infection accompanied by an increase of permeability. Degradation of occludin was inhibited by addition of the MMP inhibitors GM6001 and TAPI-2 revealing that degradation of occludin was due to proteolysis dependent on the activity of matrix metalloproteinases (MMPs).

Conclusion

The presented *in vitro* data indicate that the interaction of *N. meningitidis* with endothelial cells directly contributes to loss of tight junction proteins. Furthermore, our results show that MMPs play a crucial role in the initial stage of BBB pathophysiology by increasing permeability through modulation of endothelial junctions. The described data will help to determine the function and dynamics of tight junctions in meningococcal meningitis and illustrate that compounds that protect tight junction proteins from enzymatic cleavage are attractive therapeutical candidates.

P180

Neisseria gonorrhoeae activates ERK in human genital epithelial cells through trans-activation of EGFR

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Objective

In this work we have investigated the upstream signaling events leading to ERK activation. Infection of genital epithelial cells by piliated, Opa-expressing *Neisseria gonorrhoeae* induces activation of ERK (extracellular signal regulated kinase). Gonococcal induced ERK activation has been suggested to account, at least in part, to its anti-apoptotic effect on host cells.

Methods

We followed gonococcal-induced activation of ERK by Western blot. We used kinase inhibitors against ErbB family receptors to determine their relationship with ERK activation. We used real-time PCR to follow the induction of ErbB ligands in response to gonococcal infection of human genital epithelial cells.

Results and discussion

Inhibiting the kinase activities of epidermal growth factor receptor (EGFR, ErbB1) and ErbB2 altered gonococcal-induced ERK activation. The EGFR kinase inhibitor AG1478 completely abrogated gonococcal induced ERK activation, while inhibition of ErbB2 kinase with AG825 enhanced ERK activation. Using a mAb that inhibits ligand binding to EGFR inhibited ERK activation, without altering the ability of the gonococci to adhere to the epithelial cells. RT-PCR of gonococcal infected HEC-1-B cells shows a time-dependent increase in two EGFR ligands, heparin-binding epidermal growth factor-like growth factor and amphiregulin. Our data suggests that gonococcal-induced ERK activation is initiated by EGFR activation. Furthermore gonococci do not appear to directly engage EGFR, but rather induce ERK activation by trans-activation of EGFR.

P181

Opc invasin of *Neisseria meningitidis* requires activated/multimeric form of vitronectin for interactions with human brain microvascular endothelial cells

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Neisseria meningitidis (Nm) interacts with human endothelial cells via the outer membrane protein Opc, utilising serum factors as bridging molecules to anchor to cell surface integrins. This may include one or more serum components and both vitronectin and fibronectin have been implicated. However, there is controversy in the literature as to whether Opc can bind to vitronectin from human serum.

Objectives

To analyse in detail the cell surface molecular requirements for the binding of Opc to human endothelial cells.

Key methods

The binding requirements for Opc using multimeric and monomeric vitronectin isolated from human serum as well as purified fibronectins were assessed by solid and liquid phase assays. Serum fractionation and antibody inhibition studies were used to confirm the results.

Results

In this study, we observed that both vitronectin and fibronectin support adhesion to human brain microvascular endothelial cells (HBMEC). Using purified vitronectin, we demonstrate further that it is the activated/multimeric form of the protein with high affinity for heparin that is required for its efficient utilisation by Opc. This form of vitronectin is present in serum, in plasma and in platelet releasate. Selective adsorption of the activated form of vitronectin from human serum by Opc-expressing meningococci can be demonstrated. In addition, we have identified a site of the protein only exposed in its activated/unfolded form that is involved in binding to Opc.

Conclusions

Opc binds to several serum factors including vitronectin but it has a higher affinity for the activated form of vitronectin. This implies that in conditions and at sites where vitronectin activation occurs, Opc binding to vitronectin and endothelial integrins may be enhanced leading to increased invasion of the cells.

Acknowledgements

HBMEC were a gift from Dr K.S. Kim, John Hopkins University, USA (Stins et al., 1997, J. Neuroimmunol, 76, 81-90).

P182

Integrin directed outcomes of Opc-mediated serum-dependent interactions at brain and dermal vascular endothelial interfaces

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Objectives

Neisseria meningitidis (Nm) interacts with human endothelial cells via its Opc adhesin using serum proteins and endothelial integrins in an RGD-dependent manner. We have examined the interactions of Opc-expressing Nm with brain and dermal vascular endothelial cell lines (HBMEC and HMEC-1) with and without pre-exposure to inflammatory cytokines to assess changes in bacterial interactions that may occur during disease.

Key methods and results
Serum and purified vitronectin preparations supported Nm Opc-mediated interactions with both HBMEC and HMEC-1 efficiently. Blocking antibodies to the three integrins alpha5beta1, alphavbeta3 and alphavbeta5 decreased serum and purified serum protein-mediated adhesion as well as invasion. Individually, anti-alphavbeta5 was the most effective blocking antibody, but a combination of all three antibodies was required for abrogation of invasion suggesting the redundant roles of RGD-recognising integrins in such interactions. There was also evidence of potential co-operation between the integrins in supporting Opc interactions.
We further show that whilst endothelial cells from distinct vascular sites have distinct expression profiles of the above integrins, the levels of their surface expression do not totally determine serum-dependent, Opc-mediated Nm interactions. In addition, different endothelial cells show different responses to cytokine treatments. Changes in the levels of integrins with cytokines were only partly responsible for the modulation of Opc-mediated adhesion and invasion. The studies indicate that integrin activation state may also be responsible for the observed changes in Nm invasion of endothelia. Greater infiltration of brain compared to microvascular endothelial cells was observed in some situations. The use of several pharmacological activators and inhibitors of downstream signalling pathways demonstrated the important role of protein kinase C in cellular invasion.
Conclusions
Endothelial integrin profiles and activation status change with cytokine treatments and these vary between endothelial cells of distinct vascular origins. Greater infiltration of brain compared to microvascular endothelial cells by Opc-expressing Nm observed in some situations has implications in disease progression.
Acknowledgements
HBMEC were a gift from Dr K.S. Kim, John Hopkins
University, USA (Stins et al., 1997, J. Neuroimmunol, 76, 81-90), HMEC-1 were
obtained from CDC, Atlanta (Ades et al.,1992, J. Invest Dermatol, 99, 683-690).

P183

Alpha-actinin is an intracellular target for the Opc invasin of *Neisseria meningitidis*

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Objectives
Neisseria meningitidis (Nm) Opc protein is an effective invasin for human endothelial cells and may play a critical role in penetration of the blood-CSF barrier. In this study, we have investigated novel human endothelial receptors targeted by Opc by using brain, dermal and umbilical vein endothelial cells.
Methods
Endothelial proteins separated by SDS-PAGE were western blotted and overlaid with Opc-expressing Nm. Identification of a novel Opc-binding protein was carried out by MALDI-TOF mass spectrometry. Co-precipitation and antibody inhibition experiments and confocal microscopy were used to confirm the direct interaction of Opc with the endothelial receptor.
Results
We observed that Opc-expressing bacteria interacted with a 100kDa protein in whole cell lysates of human endothelial and also epithelial cell lines. The identity of the protein was established as alpha-actinin by mass spectrometry. The interaction is Opc-driven as Opc-expression was essential for the recognition of alpha-actinin both in its purified form and in cell extracts. This direct interaction is likely to occur once Nm is internalised since there was no demonstrable surface expression of alpha-actinin in the cell lines studied. However, Nm could be shown to co-localise specifically with alpha-actinin especially after a prolonged

period of internalisation.

Conclusions

These studies have identified a novel and an intracellular target for the Opc invasin of Nm. As previous studies have shown that meningococci can traverse human cell monolayers using a transcellular route and since alpha-actinin is a modulator of a variety of signalling pathways and of cytoskeletal functions, the overall implication is that targeting of alpha-actinin may enable meningococci to influence cellular functions to facilitate traversal of human cellular barriers.

P184

IFN-gamma amplifies NFkappaB-dependent *Neisseria meningitidis* invasion of epithelial cells via specific upregulation of CEACAM1

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Several epidemiological studies have reported spatial and temporal association between specific bacterial and viral infections of the human upper respiratory tract. Susceptibility to infection by *Neisseria meningitidis* increases markedly following influenza infections. One mechanism may involve upregulation of specific host receptors targeted by bacteria. Many receptors are normally expressed at low levels and may be upregulated in response to inflammatory cytokines. Such upregulation, by increasing the affinity of bacteria-host interactions, may encourage bacterial infiltration.

Objectives

In order to address how remodelling of target cell surfaces together with key bacterial attributes (pili, Opa, Opc, capsule) may affect breaching of an intact target monolayer by virulent phenotypes, human epithelial cells were treated with cytokines to mimic post-inflammation state and meningococcal adhesion and invasion investigated.

Key methods and results

Human epithelial cells exposed to IFN-gamma but not TNF-alpha or IL1-beta supported increased meningococcal adhesion and invasion. The increase was related to Opa but not Opc or pili expression. De novo synthesis of CEACAM1, a major Opa receptor occurred in epithelial cells exposed to the cytokine, or when infected with Opa-expressing bacteria. Cell line dependent differences in invasion that were observed could be correlated with CEACAM expression levels. There was also evidence for Opa-pili synergism leading to high levels of monolayer infiltration by capsulate bacteria. The use of NFkappaB inhibitors, diferuloylmethane (curcumin) and SN-50, abrogated bacterial infiltration of both untreated and IFN-gamma treated cells.

Conclusions

The studies demonstrate the importance of CEACAM1 as mediators of increased cellular invasion under conditions of inflammation and bring to light the potential role of NFkappaB pathway in Opa-mediated invasion by meningococci. The data imply that cell-surface remodelling by virally-induced cytokines could be one factor that increases host susceptibility to bacterial infection.

P185

Traversal of polarised respiratory epithelia by *Neisseria meningitidis*

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Objective
The study of meningococcal traversal of the nasopharyngeal epithelium is hindered by the lack of appropriate animal models, and the biological complexity of human organ culture models. Our objective was to establish a physiologically relevant cell culture model of the upper respiratory epithelium which could be used to investigate the translocation of *N. meningitidis*.
Methods
The human bronchial epithelial cell line Calu-3 is the principal cell line for studies of respiratory permeability and drug delivery, but has not been widely used to investigate pathogen invasion. We grew polarised monolayers of Calu-3 cells on 1μm pore size cell culture inserts. The route of passage of serogroup B strains of *N. meningitidis* across these layers was investigated by TEM, confocal microscopy and bacterial recovery after infection. Furthermore, competitive infections between wild-type and mutant strains were performed to identify pathogen genes with an important role in traversal. Infection of cells grown on coverslips, followed by fixation, immuno-fluoresence labelling and confocal microscopy allowed characterisation of the intracellular niche occupied by the meningococcus.
Results
Infection of Calu-3 cells by *N. meningitidis* demonstrated that the pattern of infection resembles that seen the in the widely used Chang cell line. A protocol to grow cells to form a differentiated, polarised monolayer was optimised and TEM demonstrated that the morphology of monolayers closely resembles the respiratory epithelium. The integrity of the monolayer was confirmed by: trans-epithelial electrical resistance (TEER) measurement; confocal and electron microscopy showing tight junction formation between cells, and the failure of non-invasive bacteria and fluorescently labelled tracers to penetrate the monolayer. Monolayers retained TEER and ZO-1 staining over the course of a 24 hour infection with *N. meningitidis.* Competitive infection of the monolayer allowed identification of bacterial determinants for successful traversal such as type IV pili and genes previously shown to play a role in intracellular replication and survival such those necessary for capsule biosynthesis. Wild-type bacteria were recovered from within cells of the monolayer after 8 and 24 hours. Interestingly the capsule deficient strain was recovered from cells of the monolayer in significantly lower numbers than wild-type.
Conclusions
Monolayers of Calu-3 cells provide a physiologically relevant model of the upper respiratory epithelium and can be used to investigate the pathway of invasion of the meningococcus. Competitive infection of epithelial monolayers implicated genes known to be required for intracellular survival and replication in also having a role in traversal. In addition lower numbers of these mutants were found within cells of the monolayer after 24 hours. These data suggest that *N. meningitidis* traverses the epithelial monolayers by an intracellular pathway. Immuno-fluoresence labelling and confocal examination is underway to characterise the compartment occupied by the intracellular meningococcus.

P186

The *Neisseria gonorrhoeae* microcolony: retraction-dependent development, cortical plaque dynamics, and a 3-dimensional ultrastructural view of infection

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Objectives
Neisseria gonorrhoeae (GC) adheres to Fallopian tube tissues, primary cells and established cell lines as microcolonies, and cause the elongation and deformation of nearby microvilli. Adherence via retractible Type IV pili (Tfp) induces the accumulation of host cell cytoskeletal and signaling proteins beneath bacteria. These studies, while informative, provide only "snapshots"of the infection process. To understand the role of twitching motility during colonization, a process that is usually viewed as static, we followed infection using live cell imaging and tested the hypothesis that Tfp retraction influences bacteria-bacteria, and bacteria-host interactions. We further examined the 3-dimensional architecture of the GC microcolony on epithelial cells.
Methods
A431 epithelial cells were mock infected or infected with *Neisseria gonorrhoeae* strain MS11 (P+/Opa-) or MS11pilT (P+/Opa-). Live cell imaging of infection was done using a DVRT system (Applied Precision) consisting of an Olympus 1X-71 microscope base, a high precision XYZ stage on an anti-vibration table, and an environmental chamber digitally controlled for heat and adapted for 5% CO2 perfusion. To examine micrcolony ultrastructure, serial ultrathin sections of resin-embedded samples were stained for pili and host cell structures, imaged by transmission electron microscopy (TEM) and analyzed by 3-Dimensional image reconstruction.
Results and conclusions
GC microcolonies are dynamic communities that use Tfp retraction to crawl over the epithelial cell surface and fuse into larger, ordered structures. GC reorganize within newly fused microcolonies, suggesting communication occurs among the bacteria. The cortical plaques that form beneath developing microcolonies are also dynamic structures. Actin accumulates beneath the developing microcolony and migrates with it as it crawls across the cell surface. 3-D reconstruction of TEM images reveal the complexity of the GC microcolony. A brushwork of microvilli are seen at the periphery of, and within the microcolony. Intercalation of microvilli is particularly extensive within the microcolony. Tfp fibers and Tfp bundles interconnect bacteria within the microcolony. They also mediate contact with the host plasma membrane and microvilli. Our findings reveal the elaborate anatomy of the Tfp-expressing microcolony and demonstrate the dynamic relationship between GC and host epithelium during infection.

P187

Laminin receptor mediates contact between meningitis bacteria and the blood brain barrier

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Prions and certain neurotropic viruses bind to laminin receptor (LR) as a determinant of tropism to the blood-brain barrier (BBB) vascular endothelium. Bacterial meningitis in childhood is almost exclusively caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* but the nature

of the tropism for the BBB is not understood. We hypothesized that an interaction with LR might be an important factor underlying this tropism. Using multiple independent methodologies, including *in vivo* imaging, we demonstrated that LR on human and rodent BBB cells binds all three bacteria.
The pneumococcal choline-binding protein A (CbpA) was used as a bait, to isolate potential ligands from murine BBB endothelial cells, rBCEC6. Two independent approaches were adopted: 1) affinity column chromatography; and 2) retagging, a contact-dependent cross-linking method. When bound proteins were eluted, separated and analyzed by MALDI-TOF, the host 37/67-kDa laminin receptor (LR) was identified. This finding was confirmed by reactivity of the band with anti-LR antibody in immunoblot experiments and by co immunoprecipitation of endothelial LR with recombinant CbpA.
To determine whether pneumococci, meningococci and *H. influenzae* bind LR, we measured bacterial binding to immobilized recombinant LR in the presence and absence of soluble LR by ELISA. All three bacteria bound immobilized LR; in each case, binding was drastically reduced in the presence of soluble rLR ($p<0.001$). In contrast, the non-meningeal pathogens *Staphylococcus aureus* and *Escherichia coli* did not bind. Binding to LR was conserved among clinical isolates: 18 of 20 pneumococcal strains, 68 of 70 meningococcal strains and all 38 *H. influenzae* strains bound LR in ELISA.
Adherence of the three pathogens to cerebral endothelial cells was then tested. We detected LR on the surface of mouse, rat and human cerebral endothelial cells and LR expression was upregulated by tumor necrosis factor (TNF), as observed in other model systems. Because murine cells are deficient in CD46, an endothelial receptor targeted by meningococcus, rat brain endothelial cells (rBCEC6) offered the opportunity to study bacterial adherence to LR in the absence of interaction with CD46. Adherence of the bacteria to rBCEC6 cells was reduced by soluble laminin and antibody to the LR C-terminus, although the effect was not significant for the meningococcus. Adherence to human brain microvascular endothelial cells was also inhibited by the laminin receptor peptide 263-282, but not by peptide 161-182 or by a scrambled peptide 263-282.
When LR expression was reduced by transfection of rBCEC6 cells with LR siRNA, pneumococcal binding was reduced to 53% ± 8% of the binding of controls transfected with control RNA; binding of cbpA- bacteria was unaffected. Inhibition of LR expression by siRNA decreased adherence of wild-type meningococcus (67%±5%). LR siRNA also decreased the adherence of wild-type H. influenzae (64%±8%). These findings suggest that disruption or modulation of the interaction of bacteria with LR, might engender broad protection against progression of bacteremia to meningitis.

P188

PorA and PilQ are meningococcal ligands for the human laminin receptor

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In a recent study, using multiple independent methodologies, including *in vivo* imaging, we demonstrated that lamnin receptor (LR) on human and rodent blood-brain-barrier (BBB) cells binds specifically to *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumonia*.
To identify the meningococcal LR-binding ligand(s), we used a contact-dependent retagging method, which identified the major outer membrane porin PorA (NMB1429) and the pilus secretin protein PilQ (NMB1812). Binding was confirmed by using biotin-labeled cross-linked proteins purified with magnetic beads.
Mutational analysis was undertaken to determine whether PorA and PilQ were responsible for binding of LR by intact meningococci. When both the porA and pilQ were inactivated, LR binding was reduced to ‹7% of wild-type in ELISA ($p=0.005$); single mutants bound LR at almost wild-type levels. Similar results were obtained when binding of bacteria by Cy5-LR beads was observed microscopically. Cy5 LR bound strongly to wild-type meningococcus, while pilQ and porA mutants were labeled to a lesser degree and the double

mutant was virtually unlabeled.
LR causes calcium influx into the cell after interaction with its ligands. To determine whether the identified bacterial LR ligands could induce similar cell-signaling events, human brain microvascular endothelial cells were loaded with the intracellular calcium indicator FLUO4 and then exposed to rPorA, rPilQ or rCbpA peptides either in soluble form or as coated beads. Confocal microscopy of these cells confirmed that addition of each of these proteins caused a transient increase in calcium in the endothelial cells. Importantly, the transient Ca2+ wave evoked in response to rPilQ and rPorA proteins could be inhibited by pre-treatment of the cells with rCbpA, providing evidence that the binding of the bacterial ligands was specific and functionally cross-reactive. In conclusion, meningococcal PorA and PilQ are the two specific LR-binding ligands and play key roles in the bacterial-host (cerebrovascular endothelial) interaction.

P190

Neisseria meningitidis GNA2132 antigen is a target of meningococcus and host proteases: functional investigation of this dual processing

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Objectives
The reverse vaccinology approach has been applied to search for novel vaccine candidates in *N. meningitidis* serogroup B (Nm). One of the antigens identified is the Genome-derived Neisseria Antigen 2132 (GNA2132), a surface-exposed lipoprotein, which is able to induce high levels of bactericidal antibodies and represents a top candidate for the development of a new protein-based vaccine against meningococcus.
Sequence analysis of the antigen revealed the presence of an Arginine-rich region highly conserved among a panel of Nm strains. Based on the evidence that human Lactoferrin (Lf) is a serine protease able to cleave target proteins at Arginine-rich sites, we investigated whether Lf is able to cleave the GNA2132 antigen. GNA2132 is a ubiquitous protein expressed by all strains of Nm tested, however we found that in some of them the antigen is cleaved and a fragment is released in the culture supernatant. Here we also investigated this finding in order to evaluate whether the process was due to an autoproteolytic event or as a result of the action of other proteases
Methods
The expression as well as the processing of GNA2132 was analyzed in different genetically diverse Nm strains, knockout and complemented strains expressing homologues and heterologues variants of the antigen. The cleavage of the antigen was evaluated also in the presence of Lf. Biochemical analysis was instrumental to map the cleavage sites both after the processing in meningococcus and after the action of Lf.
Results
We demonstrated that the processing of GNA2132 was strain-dependent and correlate with the expression of NalP, a phase variable autotransporter with serine-protease activity that has been shown to process known Neisseria virulence factors. Moreover, we showed that the human Lf is indeed able to cleave the GNA2132 protein at the Arginine-rich site. Evaluation of the functional role played by the secreted and the cell-associated GNA2132 fragments in the interaction to host structures is currently under investigation.
Conclusion
The GNA2132 surface exposed protein is a target of the meningococcus protease NalP and of the host Lf, both involved in modulating virulence factors Understanding of the biological relevance of the two events is the aim of our studies and could give new insights on the knowledge of meningococcal pathogenesis and immunological mechanisms.

P191

Role of the lipoprotein signal sequence in the secretion and function of the NalP autotransporter of *Neisseria meningitidis*

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Introduction

Autotransporters are modular proteins with an N-terminal signal sequence for transport across the inner membrane via the Sec machinery, a C-terminal translocator domain for transport across the outer membrane and, in between, a secreted passenger domain. The passenger of the autotransporter NalP of *N. meningitidis* contains a subtilisin-like protease domain. Expression of the phase-variable NalP results in the release of extended forms of the passengers of the autotransporters App, IgA protease and AusI [1] (see figure 1).
NalP is remarkable among autotransporters, because it is a lipoprotein. A lipid moiety is attached to the N-terminal cysteine of the passenger, which might tether the NalP passenger close to the cell surface to facilitate the generation of the extended passengers of other autotransporters (figure 1). This hypothesis is tested by producing NalP without lipid moiety.
Lipidation of bacterial proteins requires a lipoprotein signal sequence and a cysteine residue at position +1 of the mature protein. Accordingly, replacing the NalP signal sequence with a non-lipoprotein signal sequence would result in non-lipidated NalP. Furthermore, some autotransporters, including the neisserial autotransporter NhhA, carry a specialized extended signal sequence, which influences the folding and transport of the passenger in the periplasm [2]. We, therefore, decided to also investigate the role of the signal sequence in NalP secretion.

Methods

We substituted the signal sequence of NalP by either the canonical signal sequences of autotransporter IgA protease or the *Escherichia coli* outer membrane protein PhoE, by the extended signal sequence of NhhA, or by the signal sequence of the lipoprotein LbpB. Furthermore, we constructed a NalP mutant with the N-terminal cysteine substituted by Ala. The mutants were expressed in *N. meningitidis* and *E. coli* to test their secretion and function.

Results

The signal sequences of IgA protease and PhoE did not support the secretion of NalP. The proteins were expressed and transported across the inner membrane. However, they were degraded in the periplasm before insertion of the translocator domain into the outer membrane. Apparently, canonical signal sequences are incompatible with NalP secretion. By contrast, the NalP mutant with Cys substituted by Ala was secreted, indicating that lipidation is not required for NalP secretion. Apparently, secretion requires a specialized signal sequence, but lipidation is not necessary. The functionality of the non-lipidated NalP will be established. Furthermore, analysis of the NalP variants with the lipoprotein and extended autotransporter signal sequences will provide a further insight in the role of this domain in the secretion process.

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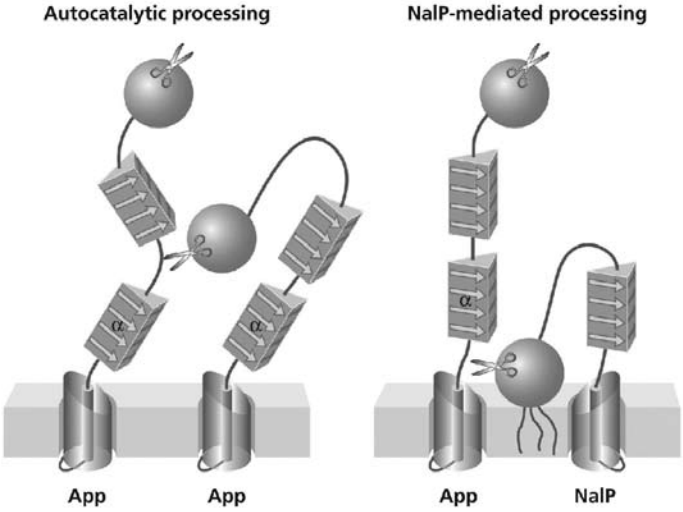
Figure 1: Model for the role of NalP in the proteolytic release of autotransporter passengers



P192

Studies of cellular motility and type IV pili

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Objectives
Neisseria gonorrhoeae and *Neisseria meningitidis* express type IV pilus polymers involved in attachment to host cells and in bacterial motility. These structures also participate in DNA uptake, biofilm formation, and even evoke signalling responses in other cells. Type IV pili mediate twitching motility by cycles of extension and retraction of the pilus filaments. The objective of this study was to determine if localization of pilus proteins in the bacterial cell can be linked to direction of motility, and to monitor *in vivo* expression throughout the course of Neisseria infection.
Methods
We used targeted fluorescence tagging of pilus-associated proteins in order to establish the role of these proteins in bacterial motility. Motility was analyzed by advanced total internal reflection fluorescence (TIRF) microscopy, confocal spinning disc microscopy and time-lapse microscopy in order to dissect critical steps of bacterial migration. Parameters such as randomness, external signalling, and sensing are being evaluated.
Results and conclusions
We have analyzed randomness of gonococcal motility in the presence of excreted host cell factors and on different surfaces. In order to visualize pili *in vivo* throughout the course of infection we are using different approaches to label the pilus filament and analyze its variation over time.

P193

Sequence analysis of the nmb0088 gene: variability, predicted protein topology and antibody response after immunization with the recombinant protein

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Objectives
After several proteomic studies, NMB0088 (or OmpP1) of *Neisseria meningitidis* appears to be abundant in outer membrane vesicle preparations. In addition, we have previously revealed a number of evidences about the use of this protein as a vaccine candidate. In the present work, we propose a structural model for this surface-exposed outer membrane protein and also evaluated if this antigen is subject to diversifying selection. Moreover, a recombinant variant of NMB0088 was evaluated in animal models included in a liposomal formulation.
Methods
Secondary structures predictions were done using ProtScale and algorithms implemented in Jpred. We predicted the 3D structure of NMB0088 using x-ray crystallography or NMR data on structurally related proteins. For the variability analysis, we selected *N. meningitidis* strains from a panel of ›400 isolates representative of the antigenic diversity found in Cuba, and included several reference strains. Forty five DNA sequences were amplified by PCR, from purified chromosomal DNA, using specific primers. In an attempt to induce antibodies directed against native conformational epitopes, a recombinant version of the protein was inserted into liposomes and it was used to immunize mice.
Results
Our analysis suggested that NMB0088 is a beta-barrel protein with 7 exposed loops and 14 membrane spanning segments. Four variants of NMB0088 (≤ 96% similarity) were identified among 45 strains and the variability was confined to three specific segments, designated VR1, VR2 and VR3. The first segment VR was the most variable among strains at the nucleotide and amino acid level. Secondary structure analysis, 3D structural database searches, and homology modeling using FadL of *Escherichia coli*, revealed that almost all the variable regions are located within or near extracellular looping domains. Antisera produced against the recombinant protein were capable of recognize a protein in Western blot analysis of some meningococcal strains from different serogroups and NMB0088 variants. Bactericidal and protective antibodies were detected against the homologous strain, but antisera were not able to kill one heterologous strain with a different variant of the protein.
Conclusion
The antigen NMB0088 could be considered in future works as a vaccine candidate for a subunit recombinant vaccine against the meningococcus. However, the level of diversity of the NMB0088 exposed loops suggests that vaccine preparations based on this antigen should be carefully designed in order to provide broad coverage against *N. meningitidis.*

P194

Genotyping and LOS structural characterization of immunotype L11 *N. meningitidis* strains

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The immunotype L11 LOS is uniquely associated with serogroup A meningococci, however little is known about its chemical structure. In this work we first characterized seven L11 serogroup A strains from the ST-5 complex for the genetic content responsible for LPS α-chain and inner-core decoration biosynthesis. Six of the strains were isolated in Ethiopia (3 of ST-5and 3 of ST-7) and one in Finland (ST-5). The presence of 9 genes of belonging to the *lgt-1*, *lgt-2* and *lgt-3* loci had previously been characterized in these strains by Norheim et al [1]. Here we completed the analysis by: (i) sequencing the *lgtH* gene (region encoding position 147-170 aa) that allows to predict if the gene encode either a β-1,4-galactosyltransferase or a β-1,4-glucosyltransferase (ii) assessing the presence of the *lot-3* gene (responsible for the *O*-acetylation of the *N*-acetylglucosamine (GlcNAc) inner core) (iii) assessing the presence of *lpt-3* and *lpt-6* genes responsible for the addition of phosphoethanolamine groups (PEA) respectively at position 3 and 6 of the heptose II and (iv) establishing the phase status of *lgtG* (responsible for the addition of glucose at position 3 of HeptoseII) and *lot-3* by sequencing their respective homopolymeric tracts. This genetic analysis allowed us to predict a putative L11 structure which thereafter was investigated by physico-chemical analyses.

The LOS from the 7 strains was isolated by the hot phenol-water extraction procedure, and the corresponding oligosaccharides were obtained following mild acid hydrolysis of the LOS. Their structures was determined using glycosyl composition and linkage analyses, NMR spectroscopy and mass spectrometry analyses. The mass of the dephosphorylated oligosaccharides obtained by HF treatment was consistent with the mass of an oligosaccharide being composed by two hexoses, one *N*-acetyl-hexosamine, two heptoses and one 3-deoxy-D-manno-2-octulosonic acid (KDO), as described by Kim *et al.* [2]. The molar composition analysis of the LOS revealed that there were 2 glucose residues present in the structure, but no galactose residues, which was consistent with the sequence of the *lgtH* gene. The proposed structure of the dephosphorylated oligosaccharide is below.

The L11 LOS was *O*-acetylated in all 7 strains. Interestingly, the 4 ST-5 strains were found to attach PEA on the LOS mainly to O-3 of the Hep II, whereas the 3 ST-7 strains attached PEA on LOS both to O-3 and O-6 of the HepII.

References

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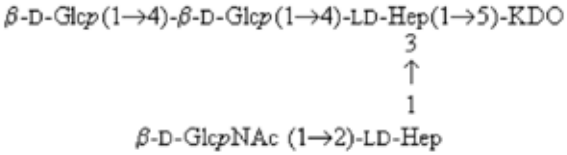
Figure 1:



P195

Profiling structural heterogeneity of native lipooligosaccharides of Neisseria by MALDI mass spectrometry

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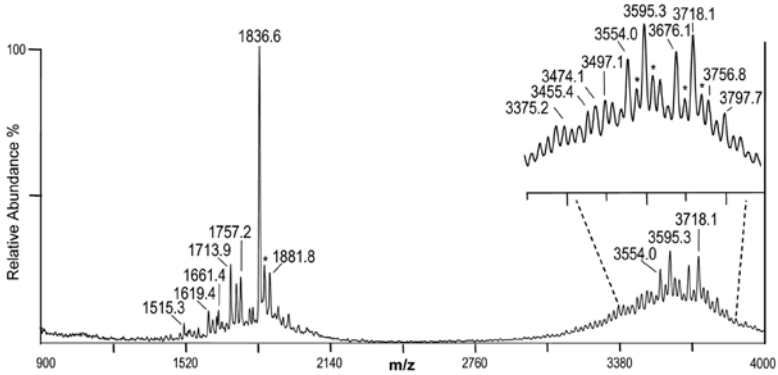
Objective
Fine differences in the phosphorylation and acylation of the lipooligosaccharide (LOS) from Neisseria species are thought to profoundly influence the virulence of the organisms and the innate immune responses of the host such as signaling through TLR4, TREM1 and TREM2. Our current focus is on the heterogeneity of structural variations in Neisserial LOS which result in significant differences in biological potency. In this study, we undertook the characterization of native LOS by MALDI mass spectrometry to determine whether this methodology would provide a more complete analysis of LOS structural variation than previous approaches.
Methods
LOS from *N. gonorrhoeae* strain 1291 and *N. meningitidis* serogroup A strain 7889 were extracted using a modification of the hot phenol-water method. Native (intact) LOS was O-deacylated with anhydrous hydrazine prior to MS analysis. Phosphoesters were removed by 48% hydrogen fluoride (HF) treatment to aid in distinguishing between N-acetylhexosamine (HexNAc; 203 Da), and HPO3 (80 Da) plus PEA (123 Da) substitutions. From 20 to 200 μg of purified, HF-treated, or O-deacylated LOS was suspended (1-2 μg/μl) in a mixture of methanol:water (1:1) containing 5 mM EDTA, desalted with ion exchange beads, and mixed with an equal amount of dibasic ammonium citrate (20 mM) and deposited on the mass spectrometry sample plate on top of a thin layer of matrix composed of 2,4,6-trihydroxyacetophonone in methanol, with nitrocellulose transblot membrane in acetone:isopropanol (1:1 v/v) mixed in a 4:1 v/v ratio. MALDI MS was performed in the linear mode on a Voyager-DE STR model time-of-flight (TOF) instrument equipped with a 337-nm nitrogen laser and delayed extraction. Spectra were obtained in the negative-ion mode.
Results
Prominent peaks were observed corresponding to molecular ions and to fragment ions primarily formed by cleavage between the 3-deoxy-D-manno-oct-2-ulosonic acid and the lipoidal moiety (LM). Analyses of these data and comparison with spectra of the corresponding O-deacylated or HF-treated LOS enabled the detection of novel components that apparently differed by the expression of up to 3 phosphates with one or more phosphoethanolamine groups on the LM. A representative negative ion MALDI TOF mass spectrum of native LOS from N. gonorrhoeae strain 1291 in the linear mode is presented in the figure.
Conclusions
The MALDI methodology presented is relatively simple to perform, enables analysis of the native LOS, and has good sensitivity and reproducibility overall. Analysis of the native LOS along with its chemically-modified forms yields a type of "fingerprinting"that can be used to assess heterogeneity, especially when combined with other techniques for comparative studies of LOS structure. In conclusion, the data obtained suggests that there is heretofore unrealized heterogeneity in the Neisserial LOS. More complete characterization of these molecules is warranted in the context of correlating LOS structure with its biological activity.

Figure 1: MALDI Mass Spectrum of Gonococcal LOS



P196

Processing of the sex pilus protein of the Type IV DNA secretion system of *Neisseria gonorrhoea*

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The versatile family of type IV transporters, one of the seven specialized pathways for secretion across the outer membrane has been extensively studied in recent years (e.g., the F-plasmid conjugation in *Escherichia coli* and the VirD4/B system of *Agrobacterium tumefacience*) but the assembly of the transporter, the mechanism of transport and the energy usage at molecular level is still not well defined. These systems generally consist of a large complex of 12 to 25 proteins and span both the membranes, These systems often extend a pilus structure which is used to attach to a recipient cell. In DNA conjugation systems, a protein, the relaxase, is covalently coupled to the DNA. This protein-DNA complex is transferred via a membrane bound hexameric ATPase, the coupling protein, to the Type IV secretion complex, after which the DNA-protein complex is transported to the receipient cell. Recently, a gonococcal genetic island (GGI) was identified which was present in 78 % of the clincal isolates of *Neisseria gonorrhoeae*. The GGI encodes among others a Type IV secretion system, that was found to secrete DNA directly into the medium.
During our mutational studies of the type IV secretion system, we identified two variants of the pili expressed by the Type IV secretion system among gonococcal clinical isolates. These variants differ by a frame shift mutation (found in 17% of the clinical isolates) which leads to synthesis of a truncated protein without the last 14 amino acids. Remarkably, the strain carrying the truncated form secretes DNA into the medium, whereas the full length protein shows no DNA secretion.
Previously, it has been shown that the pilus proteins of the type IV secretion systems of the RP4 plasmid of *Escherichia coli*, and the Ti plasmid of Agrobacterium tumefaciens are processed to circular proteins. Here we present a study of the *in vitro* membrane insertion and processing of the pilus protein.

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P197

Sequence and antigenic characterization of peptide mimetics of capsular polysaccharide from *Neisseria meningitidis* serogroup B

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Objectives
To characterize antigenically and molecularly four peptide mimetics of capsular polysaccharide (CPS) from *Neisseria meningitidis* serogroup B. The peptides were selected from a phage-displayed peptide library using an anti- CPS B monoclonal antibody (mAb) that specifically recognizes a bactericidal and protective epitope only found in the capsule layer of *Neisseria meningitidis* serogroup B [1].
Methods
Antigenic characterization was conducted by conventional and sandwich ELISA, where the peptides were allowed to react directly with the anti-CPS B mAb, and by inhibition ELISA, where mixtures of the anti-CPS B mAb with the mimetics were allowed to react with purified meningococcal CPS B. The region responsible for mAb binding in one of the peptides was mapped using overlapping 5-mer peptides covering the sequence. Sequences were aligned with previously reported sequences of peptide mimetics of CPS from *N. meningitidis* and other bacteria and the NCBI databases were searched for similarity.
Results
The peptide mimetics bind the anti-CPS B mAb and binding was inhibited by purified CPS B. In one peptide only four consecutive residues are specifically involved in mAb binding. No significant similarity was found in any case with peptide mimetics of CPS from *N. meningitidis* serogroup B or other bacteria, neither with bacterial protein sequences. Sequence analysis revealed the presence of common features with other previously reported carbohydrate-mimicking peptides, such as the presence of aromatic residues that could mimic the carbohydrate moieties due to similarities in cyclic shape and spatial volume, particularly tryptophan residues, rarely represented in peptide libraries, were present in three of the peptides. Also the presence of acid residues guarantees charge similarity to the negatively-charged polysialic acid constituent of meningococcal serogroup B CPS. Proline residues turns in the four peptides allow the voluminous aromatic residues to adopt an adequate spatial disposition. Two peptides contain two consecutive proline residues likely forming a helix structurally similar to CPS B. Arginine residues in three of the peptides may mimic carbohydrate hydroxyl groups.
Conclusions
The peptide mimetics of CPS from *Neisseria meningitidis* serogroup B described here inhibit binding of purified CPS B to an anti-CPS B mAb; their sequences are not similar to any previously reported sequence of peptide mimetics of bacterial polysaccharides. These peptides show features typical of peptide mimetics of carbohydrates. A short sequence of four aminoacid residues in a peptide chain suffices to mimic the meningococcal CPS B epitope. Four different peptides were selected with a single mAb, reflecting the ability of random peptide libraries to provide more than one structural solution for the functional mimicry of an epitope.
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P198

Lactoferrin binding protein B mediates resistance to the antimicrobial activity of lactoferricin

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Objectives
Gram-negative bacterial pathogens such as *Neisseria meningitidis*, *N. gonorrhoeae* and *Moraxella catarrhalis* are capable of utilizing human lactoferrin as a sole source of iron for growth. Acquisition of lactoferrin is mediated by a surface receptor complex comprising of the transmembrane protein LbpA and associated lipoprotein LbpB. The role of LbpA in the uptake of iron has been proven experimentally while that of the LbpB protein has been inferred from its homology to the well studied transferring binding protein TbpB. In addition, the LbpB protein pocesses two unique clusters of anionic residues near the C-terminal end of the C-terminus lobe with unknown function. This study was initiated to test the hypothesis that the presence of this region inhibits the activity of the cationic peptide lactoferricin which is released upon proteolytic digestion of lactoferrin.
Methods
Wild-type, ΔlbpB, and ΔlbpA strains of *N. meningitidis* were subjected to a bacterial killing assay using a pspA mutant of Streptococcus pneumoniae as a control (Shaper et al. 2004. Infection and Immunity. 72: 5031). The assay was performed to determine the susceptibility of the strains to lactoferricin and polymyxin B.
Results
We demonstrated that an insertion mutant of lbpB in *N. meningitidis* showed a significant increase in susceptibility to lactoferricin and polymyxin B compared to the wild-type ΔlbpA strains.
Conclusions
Our results suggest that the unique anionic cluster located on the C-terminus of the LbpB protein of *N. meningitidis* has a role in protection against the antibiotic peptide lactoferricin. Further studies are now underway to determining whether this is a general property of LbpB proteins and whether this protective effect is mediated solely by the C-terminal region of LbpB.

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Accessibility to pili-linked phosphorylcholine of *N. meningitidis* is influenced by phase-variation of the pili-linked glycan

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Pili of pathogenic Neisseria are major virulence factors and are found on the surface of bacteria. Pili are post-translationally modified by several different modifications including the addition of phosphocholine (ChoP) and a trisaccharide. The genes encoding pili-linked ChoP and trisaccharide modifications are phase-variable, which results in the expression of pili variants. ChoP is found on the surface structures of many pathogenic and commensal organisms of the respiratory tract. In *S. pneumoniae*, *H. influenzae*, and commensal Neisseria, ChoP is important for the colonisation of the nasopharynx and invasion of the epithelium. The absence of ChoP is important for survival of the bacteria in the blood.
Objectives
The ChoP and trisaccharide post-translational modifications on pilin are controlled by phase-variable genes.

In this study we investigated a potential role for pilin linked glycan phase variation in modifying accessibility to ChoP.

Methods

Analysis of mutants using gel migration phenotype and immunological analysis has allowed putative functions to be ascribed to the genes involved in post-translation modification. A tryptic cleavage site was introduced to remove the hydrophobic part of peptide of the trisaccharide and ChoP modified peptide. Trypsin digested pilin was then analysed by LC/MS.

Results

From immunological analysis, and peptide modelling, the structural differences of pilin linked glycan caused by phase-variation can affect the exposure of ChoP. Additionally, the putative MS data suggested that majority of *N. meningitidis* pili from strain C311 are post-translationally modified with three ChoP molecules linked to Ser68, Ser69 and Ser70.

Conclusion

Variable exposure of ChoP by variation of the pilin linked glycan may be important in the pathogenesis of *N. meningitidis.* The addition of ChoP to a serine residue close to amino acid 63 of pilin in *N. meningitidis* would be consistent with our observations that changes in the glycosylation of pilin effect ChoP accessibility, as the pilin linked glycan and ChoP are only separated by 5 amino acids. We suggest this close proximity accounts for the glycosylation-dependent variable accessibility.

P200

Structural characterization of the O-antigen polysaccharide repeat produced by *Neisseria sicca*

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Objectives

Neisseria sicca is a commensal organism that rarely causes disease. *Neisseria sicca* 4320 was isolated from a patient who died of bacterial endocarditis. This work was conducted to determine the structure of the LPS molecules found on *N. sicca* 4320.

Methods

We analyzed the glycolipids produced by this strain and found that it expresses lipooligosaccharide (LOS) and lipopolysaccharide (LPS) molecules resulting in a surface not found in pathogenic Neisseria species. Glycolipids from *N. sicca* 4320 were purified by the hot-phenol/lyophilization method. Purified LPS/LOS was subjected to MALDI-TOF mass spectrometry, electrospray mass spectrometry, gas chromatography, and MS n analysis. Purified LPS/LOS was separated by SDS-PAGE, transferred to PVDF membrane, and blotted with lectin GS-II and a polyclonal antibody specific for *N. sicca.* Purified samples of LOS and O-antigen were digested with beta-N-acetyl-hexosaminidase.

Results

The MALDI-TOF and electrospray mass spectrometry data show that the O-antigen of *N. sicca* 4320 is composed of a disaccharide repeat. Gas chromatography of the disaccharide repeat identified the sugars composing the unit as rhamnose and N-acetyl-D-glucosamine. Lectin GS-II, which specifically recognizes terminal N-acetyl-D-glucosamine bound to all oligosaccharide repeat bands. Glycosidase digestion of the LPS was used to confirm the composition and linkage of the terminal sugar of the disaccharide. MSn analysis was also used to verify the linkages within the disaccharide repeat. The combination of these results revealed the disaccharide repeat to be [- 3) b-D-N-acetyl-D-glucosamine (1-4) b-L-Rhamnose (1-] with a N-acetyl-D-glucosamine reducing terminus. This disaccharide was connected to a membrane through an anchor which, while uncharacterized, lacked the traditional lipid A linkage.

Conclusions
Given that all of the pathogenic Neisseria contain the genes necessary to make Rhamnose, yet this compound has never been reported on any biological molecule in the genus, we believe that the ability of *N. sicca* 4320 to produce this novel LPS structure could suggest that other pathogenic strains of Neisseria possess the ability to synthesize LPS, but that its expression is repressed during *in vitro* growth.

P201

Mimicking of the native PorA/PorB interaction using recombinant proteins renatured into liposomes

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Objectives
1. To study the PorA and PorB interaction by reconstitution of recombinant neisserial porins into liposomes and characterisation by 2D-blue native polyacrylamide gel electrophoresis (2D-BNE/SDS-PAGE).
2. To characterise the immune response of mice immunised with the resulting proteoliposomes by serum bactericidal assays.

Methods
- Expression and purification of recombinant porins
  Expression of recombinant PorA and PorB was induced by adding IPTG to a final concentration of 1mM to transformed E.coli cultures. Recombinant porins were purified by affinity chromatography using a Ni-NTA gel matrix under denaturing conditions.
- Incorporation of recombinant porins into liposomes
  L-α-phosphatidilcholines/cholesterol liposomes were prepared using a dialysis-sonication method as previously described by Humphries HE et al (Vaccine, 2006). Multilamellar vesicles were eliminated by filtration (0.2μm) to obtain a homogeneous unilamellar suspension.
- Characterisation by 2D-BN/SDS-PAGE
  Blue native polyacrylamide gel electrophoresis (1D-BNE-PAGE) analyses were carried out following a modification of the protocol described by Schagger H et al (Anal Biochem., 1991). Subunit composition and relative abundance of the different complexes were analysed in a SDS-PAGE second dimension.
- Serum Bactericdal Assays
  Serum bactericidal assays were performed using 25% rabbit complement as exogenous complement source and a 1/8 as final dilution of test sera. Duplicate samples were taken at time 0 and at 30 minutes of incubation. Results are expressed as the percentage of killing (the average number of CFU from the samples taken at time 0 was used as 100% of viability). Sera were considered bactericidal when the percentage of killing was ≥50%.

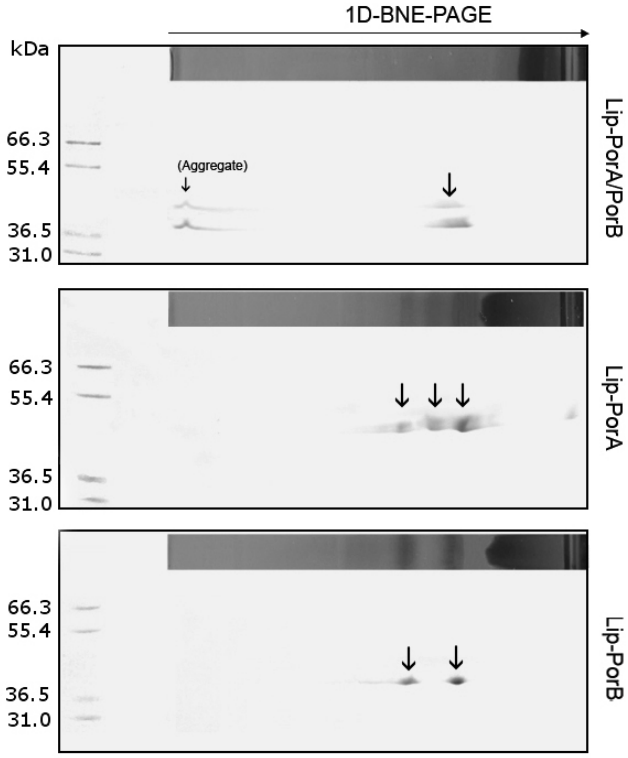
Results
- Characterisation of proteoliposomes by 2D BN/SDS-PAGE is shown in figure 1. PorA/PorB proteoliposomes showed an interaction of both neisserial recombinant porins resulting in a single heterocomplex. PorB only proteoliposomes show two different size homomeric complexes while PorA only proteoliposomes show three different size homomeric complexes.
- Polyclonal sera raised in mice using heteromeric proteoliposomes showed a high mortality against the wild type strain H44/76 but a reduced killing against the isogenic mutant strain lacking PorA and no bactericidal response against the isogenic mutant strain lacking PorB.

Conclusions
- As was previously found in our laboratory analysing native porins in OMVs, this study confirms that

when both neisserial porins are together they interact to form heteromeric complexes. Homomeric complexes were only found when a single recombinant porin was incorporated into the liposomes.

- The negative bactericidal response against the mutant strain lacking PorB suggests that interaction of both porins in the bacterial membrane is crucial for the exposure and/or the correct conformation of bactericidal epitopes in the neisserial porins.

Figure 1: Characterization of porin proteoliposomes by 2D-BN/SDS-PAGE



P202

PorA/PorB heteromers are the basic structures for the constitution of different outer membrane porin complexes through association with other proteins

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Objectives

1. Analysis of outer membrane porin complexes by high resolution Clear Native Electrophoresis (hrCNE).
2. Identification of the componets of complexes by 2-D hrCNE/SDS-PAGE and MALDI -TOF.

Methods

Outer membrane vesicles (OMVs) from the vaccine strain H44/76 and four isogenic knock-out mutant

strains lacking PorA, PorB, RmpM or FetA were used for the experiments. All cultures were grown under iron restriction and OMVs were extracted by using French Press procedure. hrCNE analyses were carried out following a modification of a previously described protocol (Wittig et al, 2007, Mol. Cell. Proteomics). Electrophoretic separation of the complexes was done in 5-15% and 8-12% polyacrylamide gradient gels. Twenty micrograms of protein, solubilised with n-dodecyl-β-D-maltoside (DDM), at a 2:1 DDM/protein ratio (w/w), were loaded in each lane. Anode and cathode buffer were BisTris-HCl 50 mM (pH 7.0) and Tricine 50 mM, BisTris-HCl 15 mM, deoxycholate 0.05%, DDM 0.02%, (pH 7.0), respectively. After 6 hours running at 100V and 4º C, lanes were cut, incubated in SDS sample buffer for 10 min at 95ºC, and placed on top of 12% polyacrylamide gels. Second dimension was done using standard SDS-PAGE settings. All gels were staining with Coomassie Blue G-250.

Results

All strains showed a significant number of bands in the first dimension hrCNE. Eleven of them, showing molecular weights ranging between 150 and 800 kDa, were complexes containing the PorA and/or PorB. The comparative analisys of the 2D complexome maps of the wild type and mutant strain, shows that native porin complexes are formed by heteromers PorA/PorB, or in some case homomers of PorB. RmpM was found associated to not all principle PorA/PorB complexes (Fig. 1 box b), and four of the highest molecular weight ones also showed to associate other proteins (figure 1 box c). The mutant strain lacking PorB was the one showing more revelant differences respect to the wild type strain, with a slower growth rate and a huge alteration of its complex profile showing two relevant PorA homomeric complexes of 700 and 800 kDa respectively. The mutant strain lacking FetA did not present significant differences in its complexome respect to the wild strain.

Conclusions

The presence of PorB in all porin complexes and the aggregation of PorA in the mutant strain lacking PorB suggest a fundamental role of PorB in the structure of pores. Furthermore, we found that the most native porin asociations are heteromeric, recruting some other diferent proteins, probably depending on the specific transport requirements.

Figure 1:

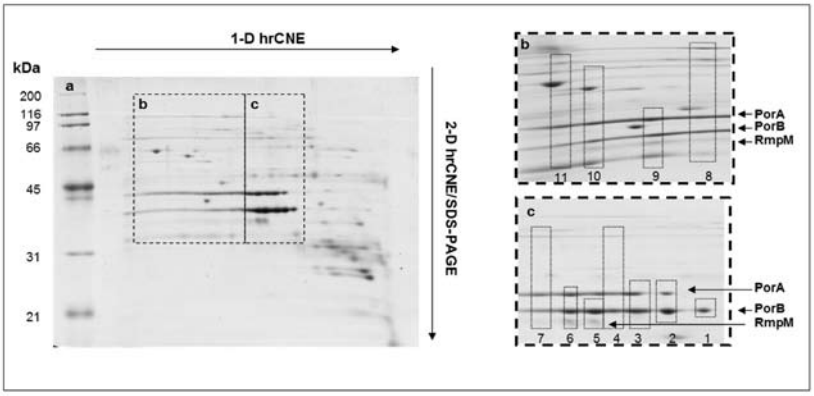

Fig. 1. Coomassie Blue G-250 staining of 2-D hrCNE/SDS-PAGE of OMVs of *Neisseria meningitidis*. First dimension was performed in a 5-15% poliacrilamide gels (**a**) or 8-12% poliacrilamide gels (boxes **b** and **c**). Rectangles include the components of high molecular weight porins complexes (box **b**) and principle porin complexes (box **c**).

P203

Measuring the powers of a microscale superhero

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Objectives
The importance of physical forces in various biological systems is becoming appreciated. Recently, the role of force generation by Type IV pili in the pathogenesis of *Neisseria gonorrhoeae* was demonstrated. It is known that the retraction of a single pilus from *N. gonorrhoeae* can exert forces between 50 to 100 piconewtons (pN). We wanted to know about the forces generated by multiple pili as well as the forces exerted by the bacteria under conditions similar to those during infection.
Methods
We developed a novel assay for measuring forces from a few pN up to a few nanoNewton (nN). This assay consists of an array of microfabricated micropillars constructed using a hydrogel. The deflection of the pillars enables, after proper calibration, the measurements of the forces exerted on them. We also used fluorescent immunostaining and different electron microscopy techniques to visualize the structure of the pili.
Results
In addition to the transient single pilus retraction forces around 50-100 pN previously reported, we measured forces up to a nanoNewton. Not only were these high forces roughly 10 times greater but they can also be maintained for hours. These high force generating pili are in bundles, each consisting of 8-10 pilus filaments. Within a bundle the filaments can retract in a cooperative manner. BSA hindered the bundling of pilus filaments and consequently high force generation. The dramatic enhancement of the interactions between the pili by the addition of Polylysine impeded force generation as well. These results exemplify the need of a fine balance in the interaction of pili in order to form a workable retractable unit of 8-10 pili which is able to exert forces on the nanoNewton range.
Conclusion
We provide information on the intensity and dynamics of the retraction forces exerted by *N. gonorrhoeae* pili under infection-like conditions. We show that *N. gonorrhoeae* Type IV pili can form bundles of up to 8-10 pili that can cooperatively retract to exert forces on the nanoNewton range. Nanonewton forces are sufficient to deform the cortex of most mammalian cells. This study is a first step towards unraveling the intimate physical dance between *N. gonorrheae* and its host.

P204a

Clinical lots physico-chemical comparability: rMenB characterization scheme

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Objectives
This work intends to illustrate the study performed to assess physico-chemical lot-to-lot comparability of a recombinant protein, candidate component for a MenB Vaccine with broad coverage, as our model of recombinant proteins physico-chemical characterization scheme.
The chimera protein GNA2091-1870 was generated by fusion of antigen GNA1870, a surface-exposed lipoprotein discovered by reverse vaccinology [1], with the protein GNA2091, to increase immunogenicity. It was expressed and purified from *E. coli* using conventional centrifugation and column chromatography [2].

Methods and results
Physico-chemical characterization of the protein purified bulk, presented in this work, embraces the multiple structural levels recognized for proteins, from primary to quaternary structure, by means of complementary methodologies such as mass spectrometry, electrophoresis, optical spectroscopy and SEC-MALLS. Additional information on antigen purity have been acquired by RP-HPLC and WB.
Conclusion
In-depth physico-chemical characterization is a fundamental requirement for recombinant vaccines: conservation of the structural organization is important to preserve immunogenicity of the native molecule; structural assessment of the products is necessary along production process development; accurate comprehension of the single Drug Substance components may support vaccine studies at Drug Product level, when the complexity of the matrix restricts possible analytical assessments.
All these reasons explain why efforts are undertaken to obtain the most comprehensive physico-chemical understanding of the purified Drug Substance, using sophisticated techniques that for their intrinsic features are not normally applicable for the routine release testing of such vaccine components.
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P204b

Outer Membrane Vesicles dimensional characterization

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Objectives
The epidemiology of endemic meningococcal disease in many developed country is currently dominated by serogroup B disease [1].
Anti-meningococcus B vaccines based on surface-exposed proteins contained in outer membrane vesicles (OMVs), already available, show good results in term of protection against homologous strains [2], waiting for an MenB vaccine with broad coverage able to overcome failure against heterologous strains, due to sequence and antigenic variability [3].
The specific OMV vaccine consists in a stable colloidal suspension made of small membranous spherical vesicles in which the native complex antigen composition of the subcapsular cell surface of *N. meningitidis* serogroup B is highly preserved.
Due to the inherent complex structure of the OMVs and presence of several antigenic families, all with different highly informational structures, a "tailored made"characterization able to unveil the different aspects of this peculiar Drug Substance material is indispensable.
The characterization strategy set up to monitor OMV dimensional features is here explored.
Methods
Dynamic Light Scattering, SEC-HPLC and Electron Microscopy techniques application to OMV have been described in the present work.
Results
We present data on vesicle sizing obtained along OMV purification process, information on vesicle populations differing in dimensions in the OMV purified bulk, and pictures illustrating vesicles shape,

integrity, and antigen localization.

Conclusion

Other than chemical composition and quantity of the single constituents, vesicle dimensions, morphology and homogeneity may have influence/correlation on industrialization and product immunogenicity, and are important features to monitor for production consistency. The shown results allow to built a better understanding and control of both product and manufacturing process.

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P205

Processing of the Two-partner secretion A (TpsA) proteins of *Neisseria meningitidis* by the autotransporter NalP

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Objectives

The two-partner secretion (TPS) pathway in Gram-negative bacteria is a wide-spread secretion route facilitating the secretion of very large and usually virulence-related proteins. TPS systems consist of a secreted TpsA protein and a TpsB protein involved in TpsA transport across the outer membrane. The secreted TpsA proteins can remain attached to the cell surface or be released into the external milieu. *N. meningitidis* strains can contain a variable number of TpsA- and TpsB-encoding genes that constitute different TPS secretion systems [1]. The autotransporter NalP is a secreted subtilisin-like serine protease, whose expression is phase-variable. It is involved in the proteolytical cleavage of other autotransporters, App and IgA protease [2], thereby modulating the form in which they are secreted. The objective of this study was to investigate whether NalP affects also the processing and release of TpsA proteins.

Methodology and results

A nalP mutant of *N. meningitidis* strain HB-1, which is an unencapsulated derivative of H44/76, showed an altered profile of secreted TpsA proteins. Using rabbit polyclonal antisera raised against the conserved TPS domains of the TpsAs of two different TPS systems, we detected a reduced secretion of a 75-kDa TpsA-derived fragment accompanied by the accumulation of a similarly-sized TpsA fragment in the whole-cell lysates of the nalP mutant. The secretion of this 75-kDa TpsA fragment was restored when the nalP mutant was complemented with a plasmid expressing wild-type NalP, but not when complemented with a plasmid expressing an active-site mutant NalP carrying an S427A substitution, indicating that the protease activity of NalP is required for processing of TpsA. Various tpsA and tpsB genes were inactivated in HB-1 to be able to analyse the processing of specific TpsAs individually in this strain. Analysis of the TpsA secretion profiles in these mutants indicated that NalP affected the individual TpsA proteins. In addition, different levels of NalP-mediated processing of TpsAs were detected when a panel of NalP -expressing *N. meningitidis* strains was screened.

Conclusions
Our data demonstrate that the autotransporter NalP is able to process the secreted TpsA proteins, resulting in the release of shorter TpsA variants. The implication for the function of the different TpsA variants in bacterial virulence requires further study.
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P206

Fructose bisphosphate aldolase is expressed on the surface of *Neisseria meningitidis*

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Objectives
Several glycolytic enzymes have been previously reported on the surface of bacterial pathogens, and shown to possess diverse, non-glycolytic biological functions. Recently, fructose bisphosphate aldolase (FBA; a type II zinc-dependent enzyme involved in glycolysis) was shown to be present on the surface of Streptococcus pneumoniae and to mediate binding to the host cell protein, Cadherin. Furthermore, FBA was demonstrated to be immunogenic in humans and capable of eliciting a protective immune response against *S. pneumoniae*. The objective of this work was to characterise FBA in *N. meningitidis.*
Methods
The gene encoding FBA from *N. meningitidis* serogroup B strain MC58 was cloned and expressed in *E. coli* with an N-terminal His-tag; the ca. 38-kDa recombinant protein was affinity-purified and used to raise polyclonal monospecific rabbit serum. An FBA null-mutant was generated in strain MC58. Subcellular fractions of wild type and mutant cells were probed with the antiserum in immunoblotting experiments.
Results
In *N. meningitidis* and *N. gonorrhoeae*, FBA shows a high degree of sequence conservation at the amino acid level, but only low-level (ca. 30%) homology to the FBA homologue from S. pneumoniae. Despite being predicted to be part of a non-functional metabolic pathway, and lacking a recognisable export signal, FBA was shown to be constitutively expressed, and localised to the meningococcal outer membrane. The FBA null mutant was shown to grow at a similar rate to that of its wild-type parent. Further characterisation of the putative role of FBA in meningococcal pathogenesis will be presented.
Conclusion
FBA is constitutively expressed in *N. meningitidis*, and, despite lacking a signal sequence, is translocated to the outer membrane. This is the first description of the presence of FBA in the outer membrane of a Gram negative organism.

P207

The loop 4 on the meningococcal PorA binds the human laminin receptor

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Our recent *in vivo* and *in vitro* studies have shown that *Neisseria meningitidis* binds to the 37/67 laminin

receptor (LR), and the interaction is mediated by the meningococcal PorA and PilQ proteins. To further localise the LR-binding domain on PorA, overlapping recombinant peptides of the latter protein were examined in enzyme-linked immunosorbant assays. The initial screening assay showed that maximal LR-binding was localized to a peptide containing the variable and surface-exposed loop 4. Peptides containing any of the other predicted PorA loops failed to react with LR. Purified loop 4 peptide inhibited the LR-PorA binding in a dose-dependent manner. Moreover, *N. meningitidis* mutant lacking pilQ and porA's loop 4 exhibited more than 90% reduction in LR-binding activity as compared with the wild type strain. These data indicate that the PorA-LR binding is mediated by the peptide containing loop 4.

P208

The role of LgtE and LgtH in the addition of galactose and glucose to lipopolysaccharide in the meningococcus and gonococcus

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The lipopolysaccharide (LPS) of *Neisseria meningitidis* is well characterised and has been classified into 12 immunotypes, L1-L12. The LPS is based on a di-heptose backbone which can be further extended by oligosaccharide additions (α, β, γ-chains) to the proximal (HepI) or distal (HepII) heptose. The α-chain extension invariably includes a glucose (Glc) attached to HepI and comprises the major outer core structure in most strains. For most LPS immunotypes the second sugar added in the α-chain is a galactose (Gal), however, in the L5 and L10 immunotypes this sugar is a Glc in the same linkage. HepII, which is always substituted by an N-acetylyglucosamine (γ-chain), is the point of synthesis of the β-chain extension which if present invariably includes a Glc as the sugar linked to HepII. α- and β-chain extensions and substitution of HepII by phosphoethanolamaine determine the LPS immunotypes and many of the biological properties of the molecule.
The LPS genes encoding the transferases responsible for the α-chain additions are clustered together in the genome in a region termed Lgt-1. lgtE and lgtH are alternative genes that occupy the same position within this region in different strains in an LPS immunotype independent manner. LgtE and LgtH are responsible for the addition of the second sugar in the α-chain and belong to the GT25 family of glycosyl transferases. Another member of the GT25 family, encoded by the lpsA gene in Haemophilus influenzae, has been shown in different strains to be able to add either a Gal or a Glc dependent on a single specific amino acid in the peptide sequence. An alignment of all available LgtE and LgtH sequences showed similarly that in a minority of *N. meningitidis* strains known to add a Glc, a methionine residue was present instead of the threonine found when the more common Gal is added. Allelic replacement of the lgtE gene from an L3 strain (Gal specific) by an lgtE gene from an L5 strain (Glc specific) and an lgtH gene from an L10 strain (Glc specific) was carried out. The resulting LPS from both allelic exchange strains was truncated compared to that of the parent strain and structural analysis identified that both strains now elaborated a Glc-Glc extension from HepI.
The LPS of *N. gonorrhoeae* is highly similar to that of *N. meningitidis*, however a Gal-Glc β-chain extension has been identified by structural analysis in some strains. All *N. gonorrhoeae* strains possess a lgtE gene that is predicted to be bifunctional and able to add this β-chain Gal. Purified gonococcal LgtE expressed in *E.coli* has been shown *in vitro*, using purified LPS from *N. sublflava*, to be able to add a Gal to a Glc acceptor on both HepI and HepII. Transfer of a gonococcal lgtE gene to replace the equivalent gene in a meningococcal strain resulted in strain expressing LPS that contained a Gal added to the initial Glc of both the α- and β-chain extensions.

P209

Epitope mapping of anti-human transferrin monoclonal antibodies: potential uses for transferrin-transferrin receptor interaction studies in bacteria and humans

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Objectives
Human transferrin (hTf) is a glycoprotein involved in iron transport from the absorption sites to the sites of storage and utilization that also plays a relevant role as a bacteriostatic agent preventing uncontrolled bacterial growth in the host. As an adaptative advantage, some bacteria like meningococcus, developed receptors as a mechanism for iron uptaking. In this work we describe a well-characterized Mabs panel in terms of precise epitope localization and estimate affinity for the two major hTf isoforms.
Methods
A panel of seven Mabs against human transferrin was used in this study. To determine the number of antigenic regions recognized by them, we performed sequential binding experiments on Biacore. Indirect ELISA were carried out to study the reactivity of the generated anti-hTf Mabs against transferrins of different species. Epitope mapping was done using an overlapping synthetic peptide library spanning the entire sequence of the mature human protein. Additional studies to identify conformational epitopes recognized by the Mabs were performed using a random phage-display peptide library. A molecular model of the 3D structure of holo-hTf was constructed using the Modeller program to map the identified antigenic regions and corresponding hTF interacting residues. To determine the affinity of anti-hTf Mabs against apo-hTf and holo-hTf, the equilibrium dissociation constant of the interaction were estimated using surface plasmon resonance.
Results
We found at least four antigenic regions in the hTf molecule, narrowed down the interacting antigen residues within three of such regions, and located them on a molecular model of hTf. Two of the antigenic regions partially overlap with previously described transferrin-binding sites for both human receptor and bacterial receptors from two pathogenic species, including the meningococcus. Hence, such monoclonal antibodies (Mabs) could be used as an additional tool for conformational studies and/or the characterization of the interaction between hTf and both types of receptor molecules.
Conclusion
Although several groups have described the generation of Mabs against human transferrin, only a few works have mapped the antigenic determinants recognized by them. Mabs panel described in this poster could be of great value to those scientists working in the field of transferrin research, particularly to those aimed to reveal the exciting features of human transferrin-transferrin receptor interactions, also considering that such an iron source constitute a key factor for bacterial virulence.

P210

Development and qualification of serum bactericidal assays for *Neisseria meningitidis* serogroup B

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Objective
A candidate vaccine based on *Neisseria meningitidis* serogroup B (MnB) outer membrane liproproteins designated LP2086, also known as factor H binding protein (fHBP), is in clinical trials. To support these

human studies by providing evidence for a functional immunological response in vaccinees, serum bactericidal assays (SBAs) for six MnB strains were developed and qualified in accordance with ICH guidelines.

Methods

The specificity, linearity, precision and robustness of the SBAs for six divergent MnB strains were investigated using sera from rabbits immunized with either a monovalent or bivalent experimental recombinant LP2086 (rLP2086) vaccine. Additional linearity and precision experiments were performed with human sera from individuals immunized with a bivalent formulation. Exogenous human complement was used in all.

Results

rLP2086 was able to inhibit greater than 80% of the bactericidal activity present in sera from rabbits immunized with a monovalent formulation. Thus, the SBAs were specific and capable of measuring anti-LP2086 antibodies in the presence of other serum components. The SBAs were demonstrated to be linear over a range of five or six 2-fold dilutions. Assay precision was assessed based on assay day and analyst. The CVs for each of the six strains were often ‹50%. Various robustness parameters also were investigated. In general, assays could be incubated for 30 to 40 minutes, test sera could go through five freeze-thaw cycles, freshly grown bacterial cultures and freshly thawed human complement could be held for 15 min at room temperature before use in the assay without adversely affecting titers. Investigations regarding the effect of different human complement sources on the SBA titers demonstrated the requirement to screen carefully any complement in order to minimize assay variation when new reagents are introduced.

Conclusions

The SBA performed well when examined by ICH guidance parameters, including specificity, precision, linearity, accuracy, and robustness. Six strains differing in LP2086 subfamily, protein variant, and expression level were qualified for use in clinical studies in support of vaccine development and licensure.

P211

A phase II clinical study to evaluate the safety, immunogenicity and immunological memory a new group A meningococcal conjugate vaccine in healthy African children

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Introduction

The Meningitis Vaccine Project (MVP) was funded in 2001 as a partnership between WHO and PATH to eliminate meningococcal epidemics in Sub-Saharan Africa through accelerated development and introduction of an affordable meningococcal group A (MenA) conjugate vaccine.2,3,4 A new MenA conjugate vaccine (MenAfriVac ™), manufactured by the Serum Institute of India Ltd SIIL (PsA-TT vaccine; 0.5 ml contains 10μg Ps, 10-20μg TT, and adjuvant [AlPO4]) was found safe and immunogenic with durable immunity when tested in a Phase I study in Indian adults.5 In a phase II study performed in The Gambia and in Mali in African toddlers aged 12 -23 months, the MenA conjugate vaccine elicited 20-fold higher serum bactericidal antibody assay (rSBA) titres than a polysaccharide vaccine. 6,7 We report here the results of the booster study performed 10 months later in the same subjects.

Methods

A total of 601 subjects aged 22-33 months who had received 10 month earlier either the MenA conjugate

vaccine (201), meningococcal polysaccharide vaccine (GSK Mencevax ACWY®) or *Haemophilus influenzae* type b vaccine (GSK Hiberix®), were randomised by each vaccination group to receive a single intramuscular injection of either MenA conjugate vaccine (PsA-TT), 1/5 dose of polysaccharide vaccine (PsACWY) or *Haemophilus influenzae* type b vaccine (Hib). Blood samples were obtained just prior to immunization, 7 days and 4 weeks later for rSBA and anti-polysaccharide group A (anti-PsA) IgG levels. Participants were followed for safety evaluation for 4 weeks after the booster dose.

Results

PsA-TT vaccine showed a safety and reactogenicity profile similar to Hib and PsACWY vaccines when administered as a booster dose. At 10 months after the primary immunization (just prior to the booster immunization), persisting antibody levels were significantly higher in the PsA-TT vaccine group (rSBA GMTs), than in the two other vaccine groups. Response to the boost immunization with the reduced dosage of PsACWY (1/5th of the recommended dose to mimic natural exposure) was significantly higher at both 7 and 28 days in the group who had initially received the MenA conjugate vaccine (PsA-TT/1/5thPsACWY) than in the group who had initially received the polysaccharide vaccine (PsACWY/1/5thPsACWY) when all predefined rSBA and ELISA endpoints were considered.. Response to the conjugate vaccine booster at 28 days was very impressive in the PsA-TT/PsA-TT vaccine group as well as in the PsACWY/PsA-TT vaccine group. The vaccine was also highly immunogenic in naive children age 22 to 33 months receiving the conjugate vaccine for the first time (Hib-TT/ PsA-TT vaccine group).

Conclusion

This study showed that one dose of the new Men A conjugate vaccine administered in the second year of life effectively primes for immunological memory. Antibody persistence and boostable responses elicited by the new vaccine are in accordance with the characteristic features of a conjugate vaccine. It is expected that the widespread use of this vaccine in 1-29 years old will eliminate group A meningitis epidemics form Sub-Saharan Africa through both direct protection of vaccinees and herd immunity effect.

P212

A randomized, observer-blinded, active control, phase 1 trial of meningococcal serogroup B rLP2086 vaccine in healthy children and adolescents aged 8 to 14 years

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Background

Neisseria meningitidis is a leading cause of meningitis and septicemia worldwide. Conjugate vaccines for serogroups A, C, Y, and W135 have been developed; however, a vaccine against endemic serogroup B disease is not available. Recombinant lipoprotein 2086 (rLP2086), a conserved outer membrane protein, is a novel vaccine candidate for broad protection against serogroup B meningococci. The rLP2086 vaccine consists of equal amounts of rLP2086 subfamily A and B protein. The vaccine has previously been shown to be safe and immunogenic in healthy young adults.

Methods

One hundred twenty-seven subjects aged 8 to 14 years were randomized in a 2:2:2:1 ratio to receive an intramuscular injection of 20, 60, or 200 μg rLP2086 or active control (TWINRIX Junior®) in a 0-, 1-, and 6-month schedule. Sera were collected at baseline and 1 month after each dose of vaccine for assessment of rLP2086-specific immunoglobulin G (IgG) response and serum bactericidal activity. Seroresponse was defined as a 4-fold increase in the serum bactericidal assay (SBA) titer post-dose 3 over baseline. SBA response was measured against a panel of 4 diverse serogroup B strains.

Results
The vaccine showed a dose-dependent reactogenicity. Most subjects reported only mild or moderate self-limiting adverse events. Transient severe local reactions were recorded in some subjects, including redness and pain, and fever of 39-40ºC tended to occur more frequently than in young adults (18-25 years of age). IgG geometric mean titers (GMTs) are shown in Table 1.
The SBA responses in children and adolescents are to be presented at the session.
Conclusions
rLP2086 vaccine exhibited a dose-dependent reactogenicity which was generally well tolerated in older children and adolescents. Overall a robust dose-dependent IgG response was demonstrated to subfamily A and B rLP2086. These results support the continued evaluation of rLP2086 as a viable vaccine candidate for endemic serotype B meningococcal disease.

Table 1:

		Pre-dose 1			Post-dose 2			Post-dose 3		
Sub-family	Vaccine Group	n	GMC	95% CI	n	GMC	95% CI	n	GMC	95% CI
A	TWINRIX	21	5.6	2.5, 12.6	20	4.9	2.4, 9.9	19	6.8	2.3, 20.4
	20 µg rLP2086	16	4.9	1.9, 13.0	16	279.6	197.7, 395.5	16	1272.5	786.3, 2059.3
	60 µg rLP2086	44	6.5	4.2, 10.2	44	567.2	423.2, 760.3	42	1422.6	1037.4, 1950.8
	200 µg rLP2086	45	4.1	2.5, 6.9	43	861.5	618.0 1201.0	43	2347.7	1911.3, 2883.8
B	TWINRIX	21	3.8	1.5, 9.4	20	3.5	1.4, 8.8	19	9.2	3.5, 24.5
	20 µg rLP2086	16	3.3	1.0, 10.9	16	281.8	205.6, 386.2	16	1052.3	614.7, 1801.4
	60 µg rLP2086	44	6.0	3.7, 9.6	44	724.2	579.6, 904.8	40	1131.3	842.5, 1519.0
	200 µg rLP2086	45	3.4	2.0, 5.7	43	891.7	671.4, 1184.4	43	1884.7	1449.5, 2450.6

P213

A randomized, placebo-controlled, double-blind, phase 1 trial of ascending doses of meningococcal group B rLP2086 vaccine in healthy adults

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Objectives
Neisseria meningitidis is a leading cause of meningitis and septicemia worldwide. Conjugate vaccines for serogroups A, C, Y, and W135 have been developed; however, a vaccine against serogroup B disease is not available. Recombinant lipoprotein 2086 (rLP2086), a conserved outer membrane protein, is a novel

vaccine candidate for broad protection against serogroup B meningococci. The aim of this study was to assess the safety, tolerability, and immunogenicity of the vaccine in healthy young adults.

Methods

One hundred three subjects aged 18 to 25 years were recruited sequentially into 3 ascending dose cohorts of 20, 60, and 200 μg of rLP2086, respectively. Each cohort was randomized in a 2:1 ratio to receive intramuscular (IM) rLP2086 or placebo in a 0, 1, 6 month schedule. Sera for assessment of serum bactericidal activity against a panel of 6 diverse serogroup B strains were collected at baseline and 1 month after each dose. Seroresponse was defined as a 4-fold increase in serum bactericidal assay (SBA) titer post-dose 3 over baseline.

Results

Most subjects reported only mild or moderate self-limiting adverse events. The SBA responses to the 6 strains were dose dependent, varying from 22.2-83.3% for the 20 μg, 55-95% for the 60 μg, and 50-100% for the 200 μg level. At the highest dose, ≥87.5% of subjects responded against 5/6 serogroup B strains, and geometric mean titers (GMTs) postdose 3 were ›60 against all strains and ›100 against 4 strains.

Conclusions

As a candidate serogroup B meningococcal vaccine, rLP2086 demonstrates a favorable safety and dose-dependent immunogenicity profile in young adults.

P214

MenACWY-CRM, a novel quadrivalent meningococcal conjugate vaccine, is well tolerated and immunogenic in infants through adolescents

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Background

Meningococcal disease affects all age groups, causing significant morbidity and mortality, with the highest incidence in infants and adolescents. Disease epidemiology is dynamic and variable geographically and temporally; thus the optimal control strategy is to vaccinate against the broadest range of meningococcal serogroups. Currently, no vaccine protects all age groups against serogroups A, C, W-135, and Y. Here we present data from an ongoing extensive clinical trial program of a novel glycoconjugate vaccine (MenACWY-CRM, Novartis Vaccines) against serogroups A, C, W-135, and Y.

Methods

Tolerability and immunogenicity of MenACWY-CRM was investigated in five Phase II/III clinical trials which included individuals (n=4364) from 2 months to 18 years of age. Comparator vaccines included a licensed quadrivalent (ACWY) meningococcal polysaccharide vaccine (MPSV4; Menomune™, sanofi pasteur), a licensed meningococcal serogroup C conjugate vaccine (MenC; Menjugate®, Novartis Vaccines), and the licensed quadrivalent (ACWY) meningococcal conjugate vaccine (Menactra®, sanofi pasteur). Immunogenicity, against serogroups A, C, W-135, and Y, was assessed by serum bactericidal activity using exogenous human complement (hSBA).

Results

MenACWY-CRM was well tolerated in all age groups; adverse events were similar to the control vaccines and typically limited to injection-site reactions and mild fever. Immunogenicity was demonstrated in all ages. In infants from 2 months of age, MenACWY-CRM was immunogenic 1 month after a primary vaccination schedule of two- or three-doses (hSBA≥1:4, 50-99%, all serogroups). For serogroup C a similar proportion of MenACWY-CRM and MenC recipients achieved an hSBA titer ≥1:4. Similar results were observed following a booster dose of MenACWY-CRM at 12 months of age (hSBA≥1:4, 79-100%, all serogroups). A separate trial demonstrated that MenACWY-CRM is immunogenic as a single dose given at 12 months of age. Immunogenicity has been demonstrated in toddlers (1-2 years of age) given one dose of MenACWY-CRM alone or concomitantly with Prevnar® or DTaP (hSBA≥1:4; 53-96%, all serogroups). In

children (2-10 years of age) one dose of MenACWY-CRM was significantly more immunogenic (the lower limit [LL] of the 95% CI around the difference between vaccines ›0%) than a licensed MPSV4 at 1-month post-vaccination (hSBA≥1:4, 82-95% vs 45-70%, respectively) and 12 months post-vaccination (hSBA≥1:4, 28-93% vs 19-53%, respectively). In adolescent subjects (11-18 years of age) significantly more MenACWY-CRM recipients than MPSV4 recipients achieved an hSBA titer ≥1:4 following primary vaccination for serogroups A, C, and Y (84-96% vs 46-84%, respectively, p‹0.01) and showed good antibody persistence for serogroups C, W-135, and Y 12-months post-vaccination. A comparative trial in adolescents demonstrated that more MenACWY-CRM than Menactra recipients achieved an hSBA titer ≥1:8 for serogroups A, W-135, and Y (LL of 95% CI ≥3%), with non-inferiority for C one month post-vaccination. For all serogroups, 1-month post-vaccination GMTs were significantly greater with MenACWY-CRM than Menactra vaccination (LL of 95% CI ›1).

Conclusion

MenACWY-CRM is well tolerated, immunogenic, and is the first single vaccine formulation with potential to provide broad protection for all age groups against meningococcal serogroups A, C, W-135, and Y.

P215

A phase 1 study of the safety and immunogenicity of an intramuscular group B meningococcal NOMV vaccine made from an lpxL2 and synX deleted strain

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Objectives

The objective of this study was to assess the safety and immunogenicity of a native outer membrane vesicle (NOMV) vaccine prepared from an lpxL2(-) synX(-) mutant of group B meningococcus. By disrupting the synX gene, capsule synthesis and sialylation of outer membrane lipooligosaccharide (LOS) is prevented, which could otherwise decrease immunogenicity due to mimicry of host structures. By disrupting the lpxL2 gene, the endotoxic activity of LOS is reduced. Bactericidal depletion assays were performed on high titer sera in order to characterize the protective antigens.

Methods

34 volunteers were recruited for this study and assigned to one of three dose groups: (1) 25 mcg, (2) 25 mcg plus aluminum hydroxide adjuvant, (3) 50mcg. All three groups were vaccinated intramuscularly at 0, 6 and 24 weeks. Subjects receiving 25mcg and clinicians were blinded as to whether they received adjuvant or not. The 50mcg dose group was not blinded. Specific local and systemic adverse events were solicited in the form of a diary filled out by volunteers and a questionnaire filled out at follow-up visits on days 1, 2, 7, and 14 after each vaccination. Blood chemistries, complete blood count, and coagulation studies were measured on each vaccination day and again two days later. Blood for serum bactericidal assays was drawn two weeks after each vaccination and at the end of the study (day 210).
Bactericidal antibody tests were done by colony counts of meningococci surviving one hour incubation in human serum with human complement as compared to incubation with complement alone. Depletion assays were done similarly but used sera from which specific antibodies were depleted by pre-incubation with specific NOMV or antigens (vaccine NOMV as a positive control; parent strain NOMV expressing a different porA; purified L3,7 LOS; purified Opc protein; and NOMV from an unrelated strain expressing L1, known to cross-react poorly with L7 or L3,7, as a negative control).

Results

Local and systemic solicited adverse events were mild and transient, with no significant differences between dose groups. No serious adverse events developed. Seroconversion rates were comparable, with 6⁄10, 5⁄9, and 6⁄10 volunteers developing a fourfold or greater increase in serum bactericidal antibodies against the vaccine strain in the 25mcg, 25mcg+adjuvant, and 50mcg groups respectively.
Depletion assays showed contributions to overall bactericidal activity from porA, LOS, and Opc.

Conclusion
The lpxL2 mutant NOMV vaccine was well tolerated. The incidence of seroconversion, defined as a fourfold increase in serum bactericidal antibodies, was not significantly enhanced by doubling the dose or by adding aluminum hydroxide adjuvant. The overall rate of seroconversion was about 60%. Depletion assays suggest that porA, LOS, and conserved outer membrane proteins all contributed to the protective response.

P216

Comparison and correlation of *Neisseria meningitidis* Serogroup A immunologic assay results following one dose of Meningococcal serogroup A conjugate vaccine in healthy Indian adults

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Objectives
A double-blind randomised controlled phase I study to assess safety, immunogenicity and antibody persistence of a new serogroup A conjugate vaccine (PsA-TT) in healthy volunteers aged 18-35 years was recently performed[1]. Subjects received either the PsA-TT vaccine or meningococcal A & C polysaccharide vaccine (PsA&C). Blood samples were taken on the day of immunisation and 4, 24 and 48 weeks later. Assessment by standardised ELISA for anticapsular total IgG and serum bactericidal antibody (SBA) assay using rabbit complement (rSBA) showed the vaccine to be immunogenic and able to elicit persistent functional antibody titres[1]. Sera from this study was analysed by additional immunological assays, data from which have been used to investigate the relationship between different serogroup A immunologic assays.
Methods
Six immunologic assays were used to analyse the sera; the SBA assay was performed using both rabbit and human complement, determination of anticapsular total IgG by ELISA and multiplexed bead assay and opsonophagocytic antibody (OPA) determined using two different methodologies. Briefly, one OPA assay (OPA1) utilised HL60 cells chemically induced into monocytes, baby rabbit complement and serogroup A polysaccharide conjugated fluorescent beads as targets, whereas an alternative OPA assay (OPA2) measured the respiratory burst using human freshly drawn PMNs, human complement and live meningococci. Geometric mean concentrations (GMC) or geometric mean titres (GMT) were determined for each assay at each visit for each vaccine group. The hSBA and OPA2 assays were used to analyse pre- and 4 weeks post-vaccination sera only. Pearsons correlation coefficients were used to assess the relationship between the six assays using data from all available visits.
Results
Pearson correlation coefficients are shown for the six assays for both the PsA-TT and Ps A&C vaccine groups in the table 1. The PsA-TT are those with white shading and PsA&C are those with grey shading. Geometric means titres/concentrations for the PsA-TT group are depicted in figure 1. Of note the decline in OPA1 is more pronounced from week 4 to 24 and 24 to 48 weeks compared to the other assays.
Conclusion
For both the PsA-TT and PsA&C groups, serogroup A-specific anticapsular IgG measured by ELISA correlated strongly with those determined by the multiplexed bead assay. Strong correlations were seen between the ELISA, multiplexed bead assay and hSBA for the PsA-TT group. Weak correlations were observed between both OPA assays and all other assays.

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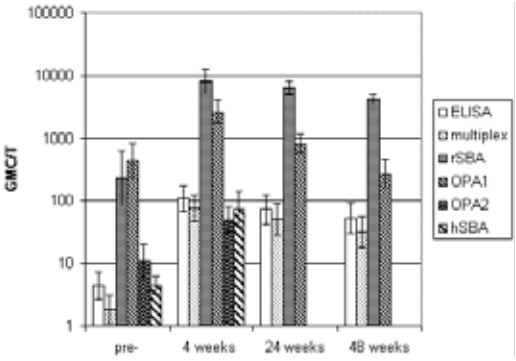
Table 1: Pearson correlation coefficients

	ELISA	MULTIPLEX	hSBA	OPA1	OPA2	rSBA
ELISA		0.894 (n=96)	0.818 (n=45)	0.248 (n=96)	0.433 (n=48)	0.538 (n=96)
MULTIPLEX	0.900 (n=98)		0.780 (n=45)	0.289 96	0.522 (n=48)	0.557 (n=96)
hSBA	0.448 (n=47)	0.499 (n=46)		0.422 (n=46)	0.482 (n=45)	0.569 (n=45)
OPA1	0.090 (n=100)	0.092 (n=98)	0.450 (n=47)		0.514 (n=48)	0.368 (n=96)
OPA2	0.194 50	0.268 (n=49)	0.417 (n=47)	0.156 (n=50)		0.621 (n=48)
rSBA	0.548 (n=100)	0.527 (n=98)	0.357 (n=47)	0.323 (n=100)	0.280 (n=50)	

Figure 1: Geometric mean titres/concentrations



P217

Safety of a Nonavalent Meningococcal serogroup B vaccine in healthy adult volunteers in a randomised, controlled, single blind study

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Objective

NVI developed a nonavalent Outer membrane vaccine (OMV) (NonaMen) containing nine different PorA molecules. This vaccine was based on the hexavalent OMV vaccine Hexamen including six different PorA molecules, which has been tested in clinical studies in adults, schoolchildren, toddlers and infants (see figure 1). In order to further increase the potential immunization coverage of MenB disease NonaMen has

been developed.
The aim of the current phase I trial was to assess safety of NonaMen in 60 healthy adult volunteers following a parallel, randomised, controlled, single blind study design.

Method

The participants were divided over 5 groups of 12 subjects, including two different doses of NonaMen:
Group 1: NonaMen 15 (9x15 μg of total Por A protein)
Group 2: NonaMen 15 (9x15 μg of total Por A protein) with DT vaccine
Group 3: NonaMen 7.5 (9x7.5 μg of total Por A protein)
Group 4: Hexavalent MenB vaccine
Group 5: Placebo
All subjects received a single vaccination after which sollicited local vaccination site and systemic adverse events were collected.

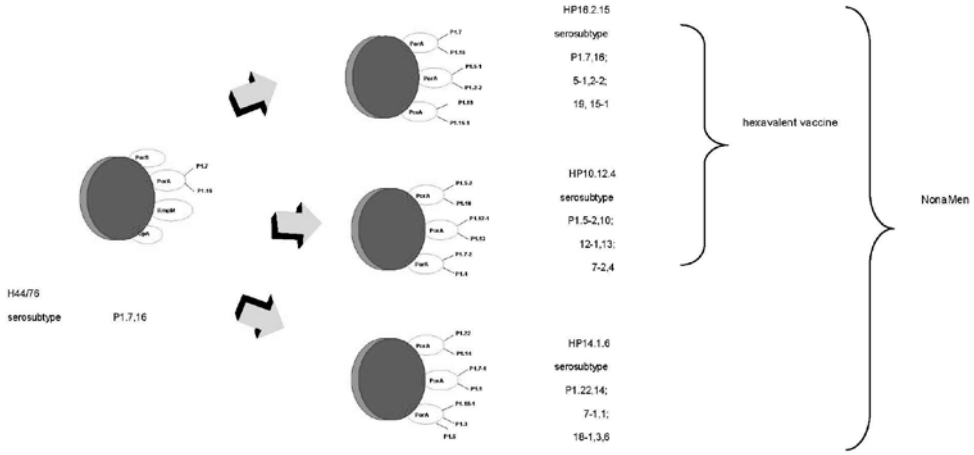
Results

- All treatments applied in this study were well tolerated and no systemic effects were found.
- Injection site pain was the predominant reaction that occurred after vaccination. All active treatment groups scored comparable on injection site pain.
- The NonaMen 15 vaccine given without DT is associated with slightly higher local reaction frequency scores for erythema, induration and swelling than NonaMen 7.5.
- The combination of NonaMen 15 with DT vaccine does not worsen the tolerability profile of NonaMen 15.
- The NonaMen vaccines are associated with slightly higher local reaction frequency scores than HexaValent vaccine.

Conclusion

General conclusion is that no major safety concerns arised during this study.

Figure 1: PorA serosubtypes included in NonaMen



P218

Development of *Neisseria lactamica* OMV ELISAs to support a phase I clinical Trial of a novel meningococcal B vaccine: from research to GxP

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Objective
The aim of this work was to develop and validate ELISA assays required to take a meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles (OMVs) through preclinical studies and a phase I safety and immunogenicity study in adult volunteers. An assay to measure the serological responses in mice immunised with preclinical and clinical batches of the vaccine was developed to good manufacturing practice (GMP) regulations as part of the quality control batch release and stability tests for the vaccine. A good laboratory practice (GLP) compliant assay was required as part of a preclinical toxicology study to measure serological responses resulting from up to four intramuscular injection of the vaccine in rabbits. Finally, a good clinical (laboratory) practice (GC(L)P) compliant assay was required to measure the serological response in the phase I clinical trial.
Methods
All the assays were designed to measure levels of serum IgG specific for *N. lactamica* OMVs relative to a suitable reference serum using a sandwich ELISA format. Assay conditions were examined using checkerboard-style experiments to find those resulting in optimum performance. This included examination of antigen coating concentration, test serum and reference serum starting dilutions and dilution series, conjugate working dilution and buffer formulations. Suitable reference sera were selected for each assay based on their concentration range, degree of parallelism with intended test samples and availability of sufficient volume to conduct all the developmental work, validation work, GxP study testing. Assays were formally validated following ICH guidelines by addressing accuracy, precision, intermediate precision, specificity, linearity, limit of detection, limit of quantification and range.
Results
All the assays were developed, optimised and formal validation showed that their performance was good and they were suitable for their intended purpose. The mean recovery for accuracy of the mouse GMP assay was 92% with a range of 76-109% which is within the range of 75-125% set for acceptance. The mean precision of the rabbit GLP assay as measured by % relative standard deviation (%RSD) was 12.6 with a range of 1.7-26.4. For the human serum ELISA used in the phase I trial, of the 28 tests for precison, 27 had %RSD ‹10. All of the samples tested for precision met the acceptance range of ≤ 30%. The assays were shown to have good linearity over their defined ranges and were shown to be specific for antibodies to *N. lactamica* OMV antigens.
Conclusion
Taking an assay used for research purposes and developing it for use in a regulated study is a lengthy process which must include optimisation of the assay methodology and data analysis method and validation of the assay and equipment used. All of these aspects are necessary to achieve data of high integrity to support the clinical trial. All three assays were successfully developed, optimised and formally validated to provide evidence that they were suitable for their intended purpose of generating data for preclinical studies, batch release and stability of the *N. lactamica* OMV Vaccine and the phase I safety and immunogenicity study.

P219

Opsonophagocytic responses elicited by either three or four doses of the meningococcal serogroup B outer membrane vesicle vaccine MeNZB administered to adult volunteers

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Objectives
The MeNZB outer membrane vesicle vaccine was developed in response to a clonal B:4:P1.7-2,4 epidemic in New Zealand. In a UK-based phase II study, fifty university students aged between 18 and 40 were vaccinated with either a three dose (0, 12 and 60 weeks) or four dose (0, 6, 12, and 60 weeks) schedule. Blood samples were taken at 0, 6, 12, 15, 60 and 63 weeks (prior to any vaccination). This clinical trial was undertaken to evaluate the safety and immunogenicity of the MeNZB vaccine, and to compare the immunogenicity of the three dose or four dose schedules. In this study the opsonophagocytic responses elicited by either the three or four dose schedules have been determined.
Methods
The antibody-mediated opsonophagocytic (OPA) response was determined using meningococci labelled internally with the fluorescent dye BCECF and DMF-differentiated granulocytic HL60 cells as phagocytic effectors, with IgG depleted baby rabbit complement as a complement source. This was performed using the homologous strain (NZ98/254), 44/76-SL and 5 strains isolated in the UK.
Results
Geometric mean OPA activity against NZ98/254 increased following each vaccine dose with the 4 dose schedule giving rise to significantly higher activity at 63 weeks compared with the three dose schedule. A Z-test was used to determine those individuals who mounted a significant rise in OPA activity from 0 to 15 weeks or from 0 to 63 weeks. At 15 weeks, 52% of individuals who received 2 doses and 75% of individuals who received 3 doses showed a significant rise in OPA against NZ98/254. This rose to 69% (3 doses) and 94% (4 doses) at 63 weeks. The percentage of individuals who showed a significant rises against the heterologous strains was lower and at 15 weeks ranged from 21-52% (2 doses) to 30-75% (3 doses). At 63 weeks significant rises against heterologous strains were 38-69% (3 doses) and 15-95% (4 doses). The data was also analysed for average fold changes in OPA response. This showed there were no significant differences in the average fold change between the two dose schedules against any of the strains tested.
Conclusions
The vaccine produced a clear rise in geometric mean OPA activity against the homologous meningococcal strain with 95% of vaccinees showing a significant increase in activity after 4 doses. In addition, the majority of vaccinees demonstrated significant rises in OPA against 4 of the heterologous strains. The results confirm that this OMV vaccine produces antibodies that mediate opsonophagocytosis against the homologous and heterologous strains which may be important in providing protection from meningococcal disease.

P220

The meningococcal antigen NadA induces cross-reactive bactericidal antibodies in human clinical trial subjects

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Objectives
NadA is a promising vaccine candidate and is part of a 5-component recombinant MenB vaccine (5CVMB) that is currently being evaluated in human clinical trials. The NadA gene is present in approximately 50 % of *Neisseria meningitidis* strains and sequence analysis of multiple strains revealed the existence of three well-conserved alleles of NadA. We demonstrated the ability of each allele to induce cross-reactive bactericidal antibodies in mice. Here we investigated this feature in humans. Sera from clinical trial subjects immunized with the 5CVMB containing NadA allele 3, were analyzed against strains carrying different NadA alleles.
Methods
Sera were obtained from adult human subjects one month following immunization with three vaccine doses. Control sera were obtained before the first immunization. Sera were tested for bactericidal activity with human complement against selected MenB strains that carry different NadA alleles and are mismatched to the vaccine for other antigens. For this purpose we chose strains that naturally expressed NadA alleles 2 and 3, and a recombinant strain that originally carried the NadA gene allele 3 and was genetically manipulated to express allele 1 of NadA.
Results
Human subjects immunized with 5CVMB showed bactericidal antibodies against strains carrying all 3 NadA alleles, either naturally or as the result of transformation to express a different NadA protein. Moreover, absorption with purified recombinant NadA allele 3 protein removed bactericidal activity against the complemented strain and against selected isolates naturally expressing the different NadA alleles.
Conclusion
Recombinant NadA protein elicits bactericidal antibodies in human subjects immunized with a 5-component vaccine against MenB. The antibodies are bactericidal not only against homologous strains but also against strains expressing different NadA alleles.

P221

In vitro studies of the biologic activity of components of a recombinant vaccine (MRB197) against *N. meningitidis* group B

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Objectives
A candidate global *N. meningitidis* group B (MenB) vaccine comprised of three recombinant proteins with or without an outer membrane vesicle component (MRB197) is being studied for protection of humans from invasive MenB disease caused by diverse pathogenic strains. We sought to determine whether the recombinant protein components, alone or combined with OMV, had any direct effects on coagulation of human plasma *in vitro*. We also sought to determine the spectrum of cytokines induced *in vitro* in human peripheral blood by exposure to the MenB vaccine components and to compare the cytokine response to the MenB vaccine with that to other commercially available control vaccines.
Methods
PTT and APTT were determined using the Coag-a-Mate MTXII (BioMerieux) for normal human plasma, to

which we added MenB vaccine components in the concentration range 0.1-100 mcg/mL or, as controls, the vaccines Act-HIB® and Pneumovax23®, and E. coli LPS. Cytokine responses were determined by culturing whole blood from four different donors for 6 hours with MenB vaccine components in the concentration range 0.01-10 mcg/mL or with the vaccines Act-HIB®, Prevnar®, Pneumovax23®, and Infanrix®, or with *E. coli* LPS as controls. The cytokines IL-1beta, IL-6, IL-8, IL-10, IL-12p70, IFN-gamma, and TNF-alpha were assayed in culture supernatants using the 96-well multi-spot assay kit (Meso Scale discovery)

Results

No effects on APTT or PT in normal human plasma were seen when any of the vaccine components or LPS were added in the concentration ranges tested. The mixture of three recombinant MenB proteins induced comparable increases in IL-1, IL-6, IL-10, IL-12, IFN-gamma, and TNF-alpha production (relative to unstimulated cultures) to those seen when commercially purchased licensed vaccines (ActHib®, Pneumovax 23®, and Infanrix®) were added to the cultures. These three licensed vaccines induced higher levels of IL-8 than did the recombinant MenB proteins. Prevnar® induced lower increases in cytokine levels than did the other licensed vaccines. The increase in IL-8 secretion induced by the recombinant MenB proteins was similar to that induced by Prevnar®.

Conclusion

We conclude that neither the recombinant MenB proteins nor the control preparations have a clinically significant effect on PT or APTT *in vitro* at the concentrations tested. E. coli LPS induced a strong, dose-dependent increase in the mean levels of IL-1, IL-6 and TNF-alpha. Recombinant MenB proteins alone and licensed control vaccines induced similar increases in IL-1, IL-6, IL-8 and TNF. Incubation of OMV in whole blood cultures induced increased levels of IL-1, IL-6, TNF-alpha, and also of IL-8 and IFN-gamma. When recombinant MenB proteins were added in combination with OMV, the resulting cytokine profiles were similar to those induced by OMV alone, suggesting that the cytokine response to the OMV predominates. The biological activity of OMV may be important to their effectiveness as vaccines.

P222

Opsonophagocytic responses elicited by a *Neisseria lactamica* OMV vaccine administered to adult volunteers

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Objectives

Immunological and epidemiological evidence suggests that carriage of *Neisseria lactamica* is involved in the development of natural immunity against meningococcal disease. We have shown that immunisation with *N. lactamica* outer membrane vesicles can protect from lethal challenge in a mouse model of meningococcal septicaemia. Thus we have developed an OMV vaccine based on *N. lactamica* and assessed this in a phase I safety and immunogenicity in adult volunteers. In this study the antibody-dependent opsonophagocytic responses in volunteer sera have been determined.

Methods

A double-blind, placebo control study in adult male volunteers was carried out at the University of Sheffield Clinical trials Unit with vaccine manufactured at the HPA Centre for Emergency Preparedness and Response. 26 volunteers received three doses of OMV vaccine (25μg protein) at 0, 6 and 12 weeks. 18 of these volunteers received a fourth OMV vaccine dose 6 months later. 29 volunteers received three doses of an Alhydrogel-only placebo. Sera obtained from each volunteer was used in a high-throughput flow cytometry-based opsonophagocytosis assay (OPA) using BCECF-labelled target *N. meningitidis*, IgG-depleted baby rabbit complement and a DMF-differentiated HL60 granulocytic cell line as the effector cell source. Sera from pre 1st dose (day 0), post 3rd dose (day 105) and post boost (day 287) were analysed using a panel of six representative UK *N. meningitidis* strains. The OPA activity was calculated by multiplying

the percentage of HL60 cells phagocytosing bacteria by the average fluorescence of these cells, giving a fluorescence index. The fluorescence index obtained for each test serum was expressed as a ratio over the fluorescence index of the complement-only, no-antibody control.

Results

Between day 0 and day 105 (post 3rd dose) geometric mean fold-rises in OPA activity were seen in the vaccine group against all six bacterial strains, while geometric mean fold-decreases in OPA activity were observed against all 6 strains for the placebo group. The OPA activity observed for the vaccine group increased further after the booster dose. The percentage of volunteers with a significant increase in opsonophagocytic response (z-test, $p<0.05$) was higher in the vaccine group against all 6 strains, with a range of 28% to 39% dependent on strain, compared with 3% to 31% for placebo group. There was a further rise in the percentage of volunteers showing significant rises in OPA activity following the booster dose (between 39% and 56%).

Conclusions

The vaccine produced statistically significant rises in opsonophagocytic activity against some or all of the six meningococcal strains tested, in a greater proportion of volunteers than the placebo group. This activity was further increased in those volunteers who received a booster dose. The results confirm that this *N. lactamica* OMV vaccine induces antibodies that mediate opsonophagocytosis against six divergent and representative UK meningococcal strains and may be important in providing protection from meningococcal disease.

P223

Human complement bactericidal activity following vaccination in A phase 2 safety and immunogenicity study of a new meningococcal A conjugate vaccine in healthy African toddlers

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Background

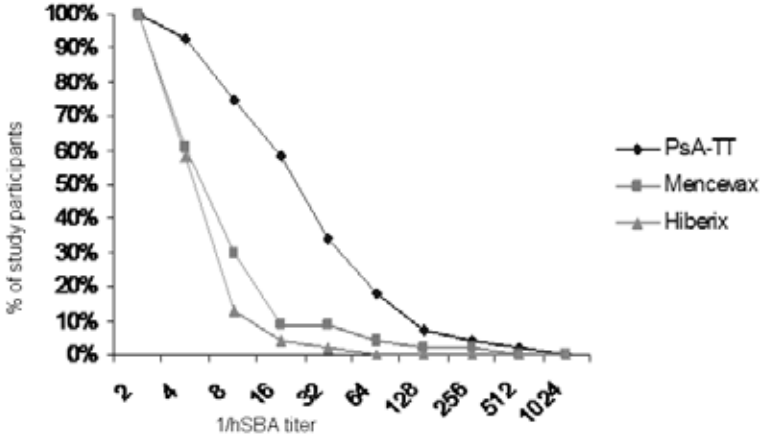
Group A meningococci (MenA) continue to cause devastating epidemics of meningococcal disease in sub-Saharan Africa. The Meningitis Vaccine Project (MVP), a Gates Foundation funded partnership between WHO and PATH to accelerate the development and introduction of affordable meningococcal conjugate vaccines for Africa, has coordinated the development of new vaccine, PsA-TT (SIIL). Following a Phase I study in India, a pivotal Phase II, observer-blind, randomized, controlled study to assess safety, immunogenicity, induction of memory, and antibody persistence after PsA-TT in African toddlers is underway. 601 participants received a single intramuscular injection of PsA-TT, *Haemophilus influenzae* type b (Hib-TT) or meningococcal polysaccharide (PsACYW, Mencevax) vaccine. Blood samples were obtained prior to and 4 weeks after immunization. Human complement serum bactericidal antibody (hSBA) titers were determined for a subset of study participants.

Methods

Four week post immunization sera from a representative subset of 180 participants (60 per study arm) were tested for bactericidal activity in a human complement serum bactericidal antibody (hSBA) assay using pooled human complement. The proportion with SBA titers ≥1:8 and geometric mean titers (GMT) were determined by vaccine group.

Results
Among the subset of African toddlers 12-23 months of age, four week post immunization hSBA titers ≥1:8 were detected in 75% (95% CI 61%-85%) who received PsA-TT, 30% (95% CI 18%-44%) who received PsACYW and 13% (95% CI 6%-25%) who received Hib-TT vaccines. The GMTs were 15 (95% CI 11-21), 5 (95% CI 3-6) and 3 (95% CI 3-4) for the PsA-TT, PsACWY, and Hib-TT groups respectively. The reverse cumulative distribution curves are shown below.
Conclusions
Human complement SBA responses in this Phase II clinical trial indicate that PsA-TT vaccine induced strong functional immune responses in a target population of 12 to 23 month old Malian and Gambian children. These data are highly encouraging as MVP and partners continue working towards the goal of eliminating epidemic meningitis in sub-Saharan Africa.

Figure 1: Reverse Cumulative Distribution hSBA titer



P224

Meningococcal NspA protein is highly immunogenic in mice, but not in humans

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Objectives
Neisserial surface protein A (NspA) is a highly conserved, surface-exposed outer membrane protein present in all serogroups of *Neisseria meningitides*. NspA has been shown to induce immune response in mice, and monoclonal antibodies against NspA were bactericidal and passively protected mice in an experimental model of meningococcal infection. These characteristics make NspA a potential candidate for a broadly effective meningococcal vaccine. However, no bactericidal antibodies were detected when a recombinant NspA vaccine was used in a clinical trial (Halperin et al. 2006). The aim of the present study was to further investigate the immunogenicity of NspA in mice and humans.

Methods
An outer membrane vesicle (OMV) vaccine (MeNZB) was produced at NIPH from a typical B:4:P1.7-2,4 meningococcal strain from New Zealand (NZ98/254). In addition to the major outer membrane proteins, significant amounts of an unidentified protein (MW~15-20 kDa) was detected by SDS-PAGE. The OMV proteins were electrotransferred to a PVDF membrane and a strip with the unknown protein was cut out for N-terminal sequence analysis.
The vaccine was tested in preclinical trials in mice and in a clinical phase I/II trial in New Zealand (V60P1). In the preclinical trials, outbred and inbred mice were immunized twice with 1-5 μg doses of the vaccine. In the clinical trial in New Zealand the same vaccine batch was given as three doses of 25 or 50 μg/dose to adult volunteers. Also acute and convalescent phase sera from meningococcal serogroup A (ST-7) patients from Ethiopia were studied.
For detection of immune responses against NspA we have produced native E.coli nOMVs. A plasmid containing the nspA gene was transformed into *E.coli* strain BL21, and nOMVs, with and without NspA expression, were prepared and used as antigens in ELISA. Immunoblotting (IB) with human and mice sera were also performed using OMVs from strains with high NspA expression as antigens.
Results
The unidentified protein band (Mw~15-20 kDa) in the MeNZB OMV vaccine showed full identity with NspA for the first 33 N-terminal amino acids. A new murine monoclonal antibody (Mab) against NspA (236,B-2) was prepared by immunizing with OMVs from a New Zealand strain. This Mab (IgG1) reacted similarly in IB to the previously published anti-NspA Mab Me-7 (Cadieux et al.1999). Whereas mice immunized with MeNZB vaccine showed high IgG responses against NspA in ELISA and IB, no significant response to this antigen was observed in the human vaccinees given the same vaccine. Neither did we see any significant anti-NspA response in serogroup A meningococcal disease patients from Ethiopia.
Conclusion
NspA protein, presented both as an OMV vaccine and in live bacteria, is poorly immunogenic in humans, whereas the same OMV vaccine induced high levels of anti NspA in mice. The importance of NspA as a vaccine component is therefore questionable.

P225

Socio economic study of meningitis epidemic impact in Burkina Faso

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Objectives
To evaluate the socio-economic impact of acute bacterial meningitis epidemics in Burkina Faso, in particular costs for the health sector and household and administrative impact on the health sector.
Methods
A micro economic study was conducted during the 2006-2007 meningitis epidemic season in two Burkina Faso districts (Réo and Kombissiri). Health care centres, district's hospitals, laboratories, health districts, health regions and central level were included as were 116 meningitis case and 116 family careers. Quantitative (costs and financing) and qualitative (impact on health system) evaluations were performed, both from a societal perspective. Data were collected through specific questionnaires and analysis of appropriate local data (budgetary, stock reports, and epidemiological). For the health sector and household we calculated direct medical costs. For the household we also calculated direct non medical costs and indirect costs.

Results

Economic impact on health sector and household

Surveillance and response for the evaluated meningitis epidemic cost US$ 9.428 million with an average cost of US$ 0.69 per inhabitant. One fourth of costs were borne by the household for case management. Reactive immunization campaigns were the most costly activity (65% of total costs; 85% of costs for the health sector). The average cost per person vaccinated against meningitis was US$ 1.45. The average direct medical cost for meningitis case management was US$ 46.7, of which US$ 20.6 was paid by the household, excluding self medication and traditional health care.

The total average cost per household was US$ 90 per meningitis case, including indirect costs (compared to a GNP per inhabitant of US$ 220). Using the WHO definition, this can be considered a catastrophic expense - which can contribute to a cycle of poverty. Of persons with meningitis, 95% paid for all or a part of their meningitis medical care (in the sample surveyed). The cost of a meningitis episode was 2.5 times higher for urban compared to rural household. Meningitis cases with sequelae had to bear an additional cost of US$ 25.4 to US$ 154.4 for rehabilitation.

Meningitis epidemic impact on health sector

According to health system actors, meningitis epidemics lead to a disorganization of all structures at all levels of the sanitary pyramid. Meningitis case management is the main contributor to this disorganization, due to work overload. The main consequences described included delay or cancellation of routine activities.

Conclusion

Meningitis epidemics have high human and economic costs for Burkinabe society, for both the health care system and individual households. In addition to reduction in health outcomes, preventive immunization likely would reduce poverty.

P226

Serological kinetics and immunological memory after *Neisseria meningitidis* serogroup C conjugate vaccination

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Introduction

A single MenC conjugate (MenCC) vaccination was introduced into the National Vaccination Program in the Netherlands at the age of 14 months in 2002. In addition, in a catch-up campaign all persons between the age of 1 and 18 years received one dose of MenCC vaccine. Long term protection is mainly based on persistence of antibodies and cellular memory after vaccination. The cellular and molecular pathways for induction of MenC memory are not entirely clear.

Methods

Volunteers, age 18-55 years, were recruited in a phase 4, open-label, randomized trial. One group (n=7) received a primary MenCC vaccination; two other groups (n=7) received either a MenCC or polysaccharide MenC booster vaccination after 4 years. Blood samples were obtained before and seven time-points after immunization. IgG, IgA and IgM antibody kinetics, avidity and bactericidal activity (SBA) were evaluated. In addition, circulating MenC specific plasma cells were investigated ex-vivo using a MenC specific ELISpot assay.

Results

The first circulating MenC specific plasma cells are detectable in the MenCC booster group and the MenC PS booster group at day 5 after immunization. The peak in plasma cell response is observed at day 7 in all groups. No protective SBA titers (rSBA titer ›8) were observed at day 0 in the primary immunization group. In contrast, in both booster groups all participants had a protective SBA titer at day 0 four years after vaccination. For all antibody types the first clear rise in titers is observed between days 5 and 7. In all three groups there is a significant rise in IgGt, IgG1, IgG2 and IgA titers between day 0 and 25 ($p = \leq 0.005$). MenC specific IgG titers are higher in both booster groups than in the primary immunization group at

day 0 and 25 after immunization (p= ≤0.008). The response in IgG2 and IgM differs between the MenCC booster group and the MenC PS booster group (P = ≤0.03).

Discussion

A single MenCC vaccines seems to induce a long term antibody titer in adults. The level and rise of antibodies in both booster groups indicate that immunological memory was previously induced by a single MenCC vaccination. However, this immunological memory does not seem to have a positive effect on the swiftness of the booster response.

P227

Safety and Immunogenicity of a new Meningococcal A conjugate vaccine in healthy Indian children aged 2-10 years

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Introduction

The Meningitis Vaccine Project (MVP) was funded in 2001 as a partnership between WHO and PATH to develop and introduce an affordable meningococcal conjugate vaccine for elimination of meningococcal epidemics in sub-Saharan Africa. The new conjugate meningococcal A vaccine (PsA-TT), manufactured by Serum Institute of India Ltd Pune - India, has shown to be safe and highly immunogenic in a phase I clinical study conducted in Indian adults. In a phase II study conducted in African children aged 12-23 months, it has been demonstrated that one dose of PsA-TT vaccine is safe, highly immunogenic and able to prime for immunological memory.

Methods

An observer-blind, randomized, controlled study to assess safety, immunogenicity and antibody persistence up to 1 year after vaccination of one dose of PsA-TT vaccine in Indian children aged 2-10 years is underway. A total of 340 subjects were recruited at Vadu Budruk Health Center, Pune, India, and randomized in a 1:1 ratio to receive either a single intramuscular injection of PsA-TT vaccine [0.5 ml contains 10μg Ps, 10-33μg TT and AlPO4 adjuvant], or of a licensed (GSK Mencevax ACWY®) meningococcal ACWY polysaccharide vaccine [0.5 ml contains 50μg Polysaccharide A, C, W and Y]. The primary objective of the study is to evaluate the immunogenicity of a single injection of PsA-TT vaccine during 4 weeks post-vaccination with comparison to the men A component of the tetravalent polysaccharide vaccine. The immunogenicity responses are evaluated in terms of serum bactericidal antibody (rSBA) activity (rabbit complement) and anti-polysaccharide group A (anti-PsA) IgG levels. Safety is assessed through an active and daily follow-up for 4 days after vaccination. All Adverse Events are collected up to 4 weeks after vaccination and Serious Adverse Events are collected for the entire study duration (1 year). The study is conducted according to ICH/GCP guidelines.

Results

Local and systemic post-immunization reactions frequency was not significantly different between the two vaccine groups, except for tenderness (30% in PsA-TT group vs. 12 % in PsACWY group). Proportion of subjects with at least one Adverse Event reported during the 4-weeks study period was similar in both vaccine groups (58% in PsA-TT group vs. 53 % in PsACWY group). The most common AEs were common cold, fever and cough. One Serious Adverse unrelated to vaccine administration was reported in the PsA-TT vaccine group. A significantly higher proportion of subjects in the PsA-TT group had a rSBA 4-fold increase as compared to recipients of the PsACWY vaccine (95% vs. 78%) as well as significantly higher rSBA GMTs titres (11209 vs.

2838). A similar proportion of subjects had total IgG ELISA 4-fold increase (PsA-TT 100% vs. PsACWY 98%). However, total IgG ELISA GMCs were significantly higher in the PsA-TT group than in the PsACWY group (89 vs. 15).

Conclusions

The results of the present study confirms in 2-10 years old Indian children that the new conjugate meningococcal A vaccine -PsA-TT vaccine- is highly immunogenic and is as safe and well tolerated as a licensed widely used polysaccharide vaccine.

P228

Similarity evaluation of meningococcal capsular polysaccharides molecular size using Sepharose CL4B and TSK columns series

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Objective

According to WHO requirements, the molecular size of meningococcal polysaccharides should be evaluated by gel filtration using Sepharose CL4B. Considering that this chromatography media is no longer supplied by the manufacturer; the TSK columns have been suggested to replace it. In this study we have compared the molecular size of Neisseria meningitides capsular polysaccharides using both types of columns in order to confirm the adequacy of Sepharose CL 4B replacement.

Method

The method comparison studies was performed applying Least-Square Analysis, F test and t-test (P=0,05) as statistical parameters. Five dextran standards with different molecular weights were injected in the two columns followed by samples of meningococcal C polysaccharides (PSC). A GE Liquid Chromatograph with refractive index and spectrophotometric detectors (206 nm) was used. The mobile phase was chosen in agreement with WHO requirements, which only specifies its ionic strength. The electrolyte was sodium chloride in the concentration of 0.2M. The molecular size of polysaccharides was calculated as described in the WHO requirements (at least 75% of the eluted fraction Kd≤0.5). The coefficient of distribution - Kd was calculated for dextrans in order to compare the columns.

Results

The elution profile for the dextrans showed a difference of 50% between the columns, Kd4bcl= 0,5 Kd tsk (figure 1). The difference in elution may be due to the exclusion limit for each resin. Figure 2 shows a polysaccharide's confidence interval in the two columns. It is evident that the polysaccharide preparations have a significant differences between the columns. The molecular size analysis showed that 98% of the PSC run through a TSK-4000 column eluted with Kd ‹0.5 as compared to 82% of the in Sepharose CL 4B.

Conclusion

The relationship between the molecular weight and the immunogenicity of these biopolymers reiterate the importance of this control during the production of this antigen for its use in pure or conjugated polysaccharides vaccines. The data suggest that Sepharose CL4B cannot be substituted by TSK column without a revision of the WHO requirements on polysaccharide specifications.

Figure 1:

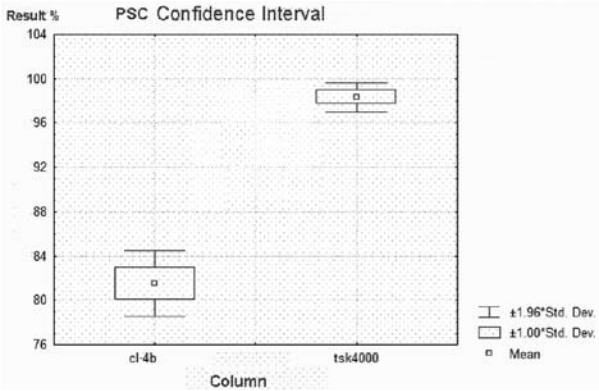
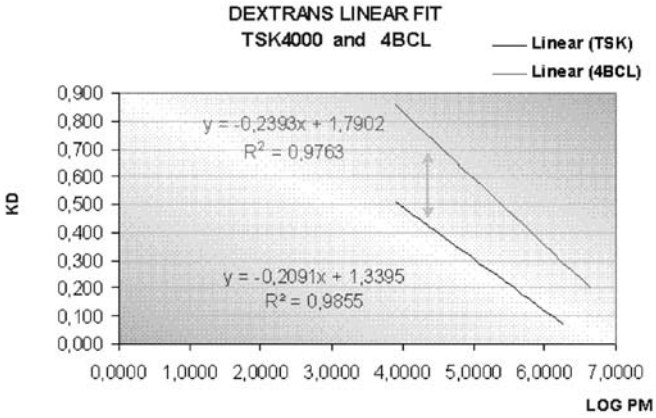


Figure 2:



P229

Approaches to the measurement of human mucosal anti-meningococcal antibodies

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Objectives

We have previously demonstrated an inverse relationship between salivary anti-meningococcal IgA and disease incidence, but no increase in mucosal IgA following systemic vaccination with MenB OMV vaccine. It remains uncertain how best to assess mucosal antibody production both to natural and vaccine challenge. In this study we have sought to further understand the origin and specificity of this mucosal antibody.

Methods
Mucosal B cell memory to *Neisseria meningitidis* was measured by ELISPOT using tonsillar mononuclear cells (TMNC) isolated from children and adults. Salivary meningococcal antibodies were measured by ELISA and Western Blot. Meningococcal antigens (eight purified PorA His-tagged proteins purified from E. coli) coupled to LiquiChip Ni-NTA beads were used to measure specific PorA antibodies using Luminex xMAP technology.
Results
Mucosal B cell memory cell production of IgA mirrors the age related acquisition of salivary antibody to *N. meningitidis*. The saliva of both children and adults frequently contains strong immunoreactivity against PorA as detected by Western blot. The precise specificity of these antibodies remains to be determined.
Conclusion
The tonsil is a common site of colonization by *Neisseria meningitidis* and is an important inductive site for mucosal immunity. We have shown the presence of IgA in the saliva specific for PorA and evidence that this is produced locally in the mucosa. IgA is the most abundant antibody in the nasopharynx and is thought to play an important role in mucosal defence against *N. meningitidis*. Measurement of salivary antibody appears to reflect local production of IgA and therefore represents a reasonable approach to the measurement of anti-meningococcal protein antibodies.

P230

Influence of baseline antibody levels on booster antibody responses in infants after protein-polysaccharide conjugate vaccine

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Objectives
It is widely accepted that there is a negative correlation between baseline antibody levels and the antibody increase after a booster dose of vaccine which arises from an inhibitory effect of antibody on the response to the vaccine antigen.
Methods
The serum IgG antibody responses from four studies of children primed with serogroup C meningococcal conjugate vaccine (MenCV) at 2, 3, 4 months of age and boosted with MenCV or meningococcal A/C polysaccharide vaccine (MenA/C) at 12 months of age were pooled and re-analysed to assess the effect of pre-booster MenC-specific IgG concentrations on the change in IgG concentrations after a booster dose of vaccine using a multilevel modelling technique.
Results
Antibody data were available from 456 babies who had been immunised according to 1 of 5 different vaccine schedules: priming with MenCV or a combined 9 valent pneumococcal and meningococcal serogroup C conjugate vaccine and boosting with either MenCV (with or without MMR) or MenA/C. Pre-booster antibody concentrations did not consistently affect post-booster antibody concentrations. When the plain-polysaccharide vaccine was used as a booster, a consistent positive correlation was observed. By contrast, there were no consistent results when the conjugate vaccine was used for booster.
Conclusion
The relationship between antibodies measured at baseline and the increase in antibody post-booster may be more complex than previously assumed, and may vary with the type of vaccine given.

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Implementation of standard operating procedures for enhanced meningitis surveillance in 13 countries of the African meningitis belt from 2003 to 2008

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Background
Epidemic meningitis continues to be a challenging public health threat in the African meningitis belt, despite intense implementation of control measures developed by WHO/AFRO and partners. The Meningitis Vaccine Project was created in 2001 as a partnership between PATH and WHO after the devastating meningitis epidemic of 1995-1996 that caused more than 250 000 cases and 25 000 deaths, and the emergence of Group W135 *Neisseria meningitidis* following the 2000 pilgrimage in Saudi Arabia with the aim of eliminating meningitis epidemics as a public health importance problem in Africa, through the development, testing and licensure of affordable conjugate meningococcal vaccines. Accurate information was needed on meningitis cases, deaths and bacteriological isolates was necessary for MVP to attain its objectives. Therefore, Standard Operating Procedures were developed in 2001-2002 to codify and enhance meningitis surveillance in the 13 most endemic countries in Africa (Benin, Burkina Faso, Cameroon, central Africa, Cote d'Ivoire, Ethiopia, Ghana, Mali, Niger, Nigeria, RD Congo, Chad and Togo). The results from this enhanced surveillance system are summarized.
Objectives
The goal of enhanced surveillance is to rapidly detect the emergence of meningitis epidemics and identify the causative pathogen in order to undertake appropriate counter measures.
Methods
Standard case definitions, intervention thresholds, and data collection tools were developed for surveillance. Laboratory standards were developed for basic diagnostic tests (Gram stain, antigen detection) at the district level as well as national reference laboratories (culture, antibiotic sensitivity, PCR) and WHO collaborating centers (typing, sequencing). A system for epidemiologic and laboratory data collection, collation, analysis, interpretation and reporting was developed in addition to a feedback bulletin.
Analysis
Weekly data reported by the 13 countries are compiled and analyzed at the WHO-Multi Disease Surveillance Centre (MDSC) in Ouagadougou. Access-based software for data extraction and analysis from the various databases formats used by countries was developed to standardize data into a single database. The results were graphed and mapped using Excel and HealthMapper software.
Results
From 2003 to 2008 (up to week 21) 185 980 meningitis cases and 19 901 deaths (CFR of 10.7%) were reported. 401 alert districts and 338 epidemic districts were mapped. The distribution for the three causal pathogens of bacterial meningitis (*Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*) was determined. A total of 20 669 CSF specimens were collected; among the 7549 positive CSFs, NmA represented 52.5 %, NmW135 6.2 %, Spn 23.7 % and Hib 6.7 %. Other Nm serogroups (mainly X, Y, C) were isolated as well.
Conclusion
The enhanced epidemiologic and laboratory surveillance of meningitis has provided a sound base to better understand meningococcal disease patterns in recent years in Africa, as well as a better appreciation of circulating germs. The success of this effort has encouraged the development of more detailed case-based surveillance in order that the impact of the Men A conjugate vaccine can be accurately monitored and that the effect on circulating strains accurately determined.

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Genotypic characterization of invasive *Neisseria meningitidis* serogroup B (MenB) in Argentina 2006

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Introduction
The annual incidence of meningococcal disease has been estimated to be around 0.6 - 2.9 cases per 100,000 inhabitants in the last fourteen years in Argentina (39 million inhabitants). An active laboratory-based surveillance system for invasive meningococcal infections has been established at the Instituto Nacional de Enfermedades Infecciosas - ANLIS "Dr Carlos G. Malbrán". Isolates are collected from the participating laboratories that are geographically dispersed among the 23 provinces of the country. We report data on the genetic structure of the invasive meningococcal isolates collected during 2006.
Methods
The study was performed on 46/49 serogroup B isolates (MenB) collected during 2006. The relatedness among isolates was evaluated by multilocus sequence typing (MLST) and by sequencing porA gene fragments encoding VR1 and VR2 regions (geno-subtyping).
Results
MenB Isolates were most prevalent and represented 72% of all *N. meningitidis* isolates in 2006. The 46 MenB isolates were clustered in several clonal complexes but 95.6% of the isolates were associated with 4 clonal complexes. The isolated were further grouped into 17 different geno-subtypes on the basis of porA sequencing. The most prevalent geno-subtype was P1.21,16-36 (30.4%), followed by P1.22-1,14 (21.7%), and P1.7,16 (10.9%). The other subtypes were represented by 1-2 isolates each. All of the P1.21,16-36 isolates belonged to the clonal complex ST-865 and 13/14 showed the sequence type ST-3327 of this clonal complex. The P1.22-1,14 isolates belonged to the clonal complex S-T35. The clonal complex ST41/44/ Lineage 3 was represented by 10 isolates with very heterogeneous geno-subtypes.
Conclusions
This study represents the best available resource for obtaining information about clonal distribution and PorA types of MenB strains in Argentina and provides valuable information for considering a probable PorA-based vaccine. However, an Outer Membrane Vesicle (OMV) vaccine should include several PorA types in order to cover most of the current circulating isolates.

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Meningococcal survival on fomites: Exploring a new route of transmission

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Objectives
There seems to be little doubt that the transfer of respiratory droplets shed from the upper respiratory tract is the means by which *Neisseria meningitidis* is spread. However, the exact mechanism is still unclear and, due to the assumption that meningococci are unlikely to survive outside of the human host, the mechanism of fomite transmission has been largely disregarded. The objective of this study was to investigate the ability of *N. meningitidis* to survive outside the human host.

Methods
Initially seven strains representing the three most common capsular groups B, C, and W135, were tested for their ability to survive on glass or plastic (common drinking vessel materials). This range of strains was expanded to increase the validity of the initial study.
A known number of colony forming units (cfu) of each strain were dried in a saliva substitute onto glass and onto plastic and recovered onto CBA. The expression of their capsules and porin proteins upon recovery were checked using sero-grouping and whole-cell ELISA, respectively.
Results
The initial testing found that survival on glass was significantly better than on plastic ($P<0.0001$) with 9-20% of the colony forming units of the group B strains surviving desiccation on glass compared to 5-12% cfu on plastic. Isolates of the New Zealand epidemic strain, B:4:P1.7-2,4, survived better on glass than all other strains tested ($P=0.0013$) with one strain surviving up to 168 hours. All recovered isolates tested still expressed their capsules and outer membrane proteins. All strains survived for at least four hours on glass under standard conditions. Continuing investigations indicate that all meningococci have a low-level ability to survive outside of the host, though the ability varies between different strains. Analyses are on-going.
Conclusion
Low-level environmental survival appears to be a common trait of meningococci. These findings raise the question of whether meningococci can be transferred from person to person via fomites such as drinking vessels contaminated with meningococcal-containing oropharyngeal secretions.
Investigation is now underway into the factors that influence the bacterium's ability to survive.

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Surveillance of meningococcal disease in Burkina Faso

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Objectives
Burkina Faso was disproportionately affected by epidemic meningitis in 2007. Systematically collected surveillance data on suspected cases with rapid laboratory confirmation of causal pathogens is necessary to understand the causes of meningitis epidemics in Burkina Faso, inform epidemic response, and support vaccine effectiveness studies.
Methods
The Burkina Faso Ministry of Health has conducted enhanced, case-based, population-based meningitis surveillance with laboratory confirmation of *Neisseria meningitidis* in all health districts since 2002. Specimens are collected from suspected cases and Gram's stain performed at the district level, with a proportion sent for further confirmatory testing to regional or national reference laboratories. Confirmed cases are defined according to WHO criteria as a person with sudden onset of fever (›38.5ºC rectal or 38.0ºC axillary) and one of the following signs: neck stiffness, altered consciousness or other meningeal signs, and either positive CSF antigen detection for *N. meningitidis* or positive culture of CSF or blood with identification of *N. meningitidis*. Individual testing results are not currently linked to surveillance data with a unique identifying number, thus to assess completeness of laboratory results reporting, counts of laboratory results reported through enhanced surveillance were compared to results reported in the national aggregate laboratory database.
Results
During the 2007 meningitis epidemic in Burkina Faso a total of 9,178 suspected cases with a peak of 1,480 reports in the first week of April (week 12), and 673 deaths were reported with an overall attack rate of

64 cases per 100,000 population and a cumulative case fatality ratio of 7.3%. Of the 55 health districts nationwide, 43 (78%) surpassed an epidemic threshold of 10 cases per 100,000 population. Cases had a median age of 7 years (range: ‹1-87), 6,762 (75%) occurred among children less than 15 years of age, 5,057 (55%) were male, and 2,134 (23%) reported receiving a meningococcal vaccine. Overall, 8,332 (91%) had a lumbar puncture, 2,519 (30%) had gram-negative diplococci, but only 295 (3%) suspect cases were laboratory confirmed. Compared to the national laboratory database, 348 (55%) of 628 latex agglutination test results and 107 (21%) of 520 culture results were recorded in the enhanced surveillance database.

Conclusions

Case-based surveillance in Burkina Faso, while relatively new, is collecting high quality data that are most likely representative of the true disease burden. It could provide valuable data to analyze epidemics, as well as support the Meningitis Vaccine Project in monitoring the effectiveness of the new meningococcal A conjugate vaccine. A significant number of CSF specimens are taken, but the rate of laboratory confirmation by culture is lower than expected.

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Antigenic diversity among strains of *Neisseria meningitidis* isolated before and after mass immunization in Cuba

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Objectives

Analysis of outer member protein (OMP) gene sequences is useful because susceptibility to meningococcal disease is determined, in part, by the serum bactericidal antibodies to these proteins. Two of these OMPs, PorA and PorB, are immunogenic porins that are the basis of the meningococcal serosubtyping and serotyping system respectively. In addition, FetA, an iron-regulated OMP, has immunodominant variable regions (VR) that has also employed as molecular marker. DNA sequences encoding surface-exposed VR of these proteins may provide important information regarding the emergence of meningococcal clones in response to population immunity. The effect of mass immunization with VA-MENGOC-BC® on the evolution of the mentioned antigens and the structure of *N. meningitidis* populations in Cuba was investigated in a study of meningococci isolated from cases and carriers before and after vaccine introduction.

Methods

In the present study 422 strains isolated from cases and carriers that were collected between 1983 and 2005 were genetically characterized by sequencing their correspondent antigen genes porA, porB and fetA. For antigen characterization, the epitopes of the serotype and serosubtype determinants were determined by translation of the porB and porA gene sequences obtained for each isolate, together with the epitope of the FetA protein, which is another component of the OMV vaccine. Antigen gene sequences and MLST alleles were concatenated into unique sequences for each isolate. Sequence alignment was performed with Clustal X and manually corrected with the Bioedit program. Phylogenetic tree inference was performed by using the maximum-likelihood method available in the PAUP* package.

Results

After sequence analysis, variants 3-1 and 3-8 are prevalent for porB; variant F5-1 was the most common for fetA, and variants 19 and 15 were prevalent on regions VR1 and VR2 of porA respectively. These variants match those present in the B4:P1.19,15 vaccine strain. The total of ST-32 complex isolates possessed PorB3 proteins and the most frequent combination among these clonal complex isolates was P1.19,15; F5-1. On the other hand, we found higher variability inside the antigenic variants for other clonal complexes detected in Cuban strains.

Conclusions
The data suggest that the ST-33, B:4:P1.19,15 epidemic strain and novel antigenic variants of that strain are responsible for the extremely low incidence of meningococcal disease that has been occurred since the introduction of the VA-MENGOC-BC® vaccine in Cuba. Evidences of occurrence of small positive selection for antigenic variation, as a mechanism of immune escape, has been found mainly in the porB antigen gene, one of the major component of Cuban vaccine.

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Investigation of a cross-border outbreak of invasive meningococcal disease using a combination of MLVA and MLST

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Objectives
Automated finetype-specific spatiotemporal analyses identified an unusually high concentration of meningococci of the finetype B:P1.7-2,4:F1-5:ST-42:cc41/44 in the region of Greater Aachen comprising a population of 1.1m (Elias et al, EID 12:11 2006). Further analyses confirmed the regional predominance of this finetype (63% compared to 13% in the rest of Germany, 2005) and an approximately threefold (2005) overall incidence of invasive meningococcal disease compared to the rest of Germany. The objective of this study was to elucidate whether this community outbreak represented a spillover of the hyperendemic situation caused by meningococci of the clonal complex ST-41/44 starting in the Netherlands in the 1980ies.
Methods
Serogroup B strains isolated from 2000-2006 in the Netherlands, and in two neighbouring German States, North-Rhine Westphalia and Lower Saxony, were screened for ST-41/44 using a lineage specific PCR (Bart et al, FEMS Microbiol Lett 2000). PCR positive strains were subjected to MLST (Maiden et al. PNAS 1998) and MLVA analysis (Schouls et al. J Clin Microbiol 2006). Results were visualized on geographic maps. Strength of association between MLST and MLVA types was quantified comparing the observed and expected Jaccard Indices for MLST-MLVA combinations as described before (Rhee et al, PLoS Comput Biol 3:5, e87).
Results
MLST revealed an accumulation of ST-42 in counties around Aachen, and in the neighboring Dutch province of Limburg, whereas MLVA suggested increased occurrence of MT-19 in the Aachen area. Only the combined use of MLST and MLVA clearly segregated a clone with MT-19 and ST-42, which accounted for the majority of cases in Greater Aachen, while being rare in the rest of the two German States. Although this clone was more prevalent in Limburg than in the rest of the Netherlands, its marked concentration on the German side of the border suggests its emergence as an independent descendant of the ST-41/44 complex in the Aachen area. Median age of patients infected with this clone was higher than the rest (14.5 vs 3.5 yr), suggesting increased epidemic activity. Of note, all MT-19/ST-42 strains had the finetype B:P1.7-2,4:F1-5 that had caused initial cluster alerts. A positive association was detected for MT-19 and ST-42 (Z=36.5), MT-1 and ST-2713 (Z=12.7), and MT-26 and ST-1194 (Z=11.7). Only the first, however, showed spatial clustering. Among others, MT-18 and ST-41 (Z=-112.6), MT-19 and ST-41 (Z=-50.5), and MT-18 and ST-42 (Z=-9.5) showed negative association. In the prevalent MT-19/ST-42, the VNTR-array of nmb1525 annotated as a virG-related protein gene is out of frame, possibly associated to increased transmission of this clone.
Conclusion
Antigen finetyping (serogrouping and sequence typing of PorA and FetA) was pivotal for the identification of this outbreak. Only the combination of MLST and MLVA, however, successfully delineated the emergence of the outbreak strain. The visualization of bi-lateral geographic data favours the notion that the increase of MT-19/ST-42 strains were due to local emergence in Germany rather than represented a spillover of

disease activity from the Netherlands. The reason for increased transmission of MT-19/ST-42 is unclear: one might speculate that increased VirG-gene inactivation plays a role.

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Comparison of serogroup a *Neisseria meningitidis* strains of sequence type 2859 emerging in Burkina Faso in meningitis seasons 2003 and 2004 with predominating ST-7 strains

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Objectives
Meningitis epidemics in sub-Saharan Africa have been dominated by serogroup A *Neisseria meningitidis*, lately by those of sequence type (ST) 7. The new ST-2859 of serogroup A started to cause disease in Burkina Faso in 2003, and continued into the large 2007 epidemic. ST-2859 has also caused disease in Mali and Niger. We therefore wanted to assess differences between ST-5 and ST-2859 strains in potentially hyper variable loci relevant for immune selection.
Methods
Seven ST-2859 strains and one ST-7 strain isolated in Burkina Faso in 2003-04 were subjected to the phenotypic and genetic analyses. We assessed outer membrane preparations of ST-2859 and ST-7 strains, reaction with monoclonal antibodies in dot-blot and susceptibility to commonly used antibiotics. Thereafter we compared sequence variability in six loci suggested to be hyper variable (opaB, tbpB, fetA, lgtA and gna1870).
Results
All 8 strains were 4/21:P1.9 and L10. Five of seven ST-2859 strains expressed normal levels of PorA, whereas only one strain showed strong expression of NadA. All strains reacted strongly with the L10 Mab, while 6/7 ST-2859 strains also reacted with the L11 MAb. All strains were susceptible to all antibiotics tested except sulphonamide. Alignment of sequences of tbpB, opaB, fetA, lgtA and gna1870 genes from the ST-2859 strains and the ST-7 strain showed that all of them harbored the same allelic variants in the various loci. The allelic variants found were the same as those in Ethiopian ST-7 strains isolated in 2002 to 2003 (Norheim et al. J. Clin. Microbiol. 2006).
Conclusion
The ST-2859 strains from Burkina Faso from 2003 and 2004 were typical of the genocloud 8, ST-5 complex strains in Africa; harboring the pgm19, tbpB55 and opaB92 allele variants, except carrying the adk3 instead of the adk1 allele. Taking into account the significant spread of ST-2859, it may still have acquired novel virulence factors, or surface-exposed antigens escaping immune pressure. Identification of these may be relevant for assessment of epidemic wave potential of future serogroup A clones and for vaccine development.

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Distribution of meningococcal serogroups and fine types according to age, clinical picture, case-fatality and region, Germany, 2002-2007

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Objectives
Results of molecular genetic typing undertaken at the National Reference Cen-tre for Meningococci (NRCM) on samples from 2832 patients with invasive meningococcal disease (IMD) were retrospectively matched to IMD cases statutorily notified to the Robert Koch Institute (RKI) (n=3722) between 2002 and 2007 with the aim of analysing age distribu-tion, clinical picture and case fatality (CF) of distinct fine types.
Methods
Molecular genetic typing of isolates received 2002-2007 to determine fine type (Serogroup:PorA-variable region (VR) 1, PorA-VR2:FetA-VR) and identification of ET-15 was performed as described previously (Elias et al EID 2006; Vogel et al. JCM 2000). NRCM-cases were automatically matched to RKI-cases according to an algorithm requiring identical month and year of birth, county and state of residence, sex, and ≤7 days between date of illness onset and date sample was taken. A manual search was performed for non-matching cases. Possible matches were verified by local health authorities using personalized data. The matched data set was analysed for fine type distribution over time, according to age, clinical picture, case-fatality (CF) and region.
Results
Preliminary analysis of 2392/2832 (84.5%) fully fine typed NRCM-cases (˜200 pos-sible matches pending) matched to RKI cases revealed 548 fine types with the following se-rogroup distribution: B: 66.5%, C: 27.4%, Y: 3.5%, and W135: 2.1. Serogroups A, 29E, and X occurred ‹0.2%. The three most common fine types were B:P1.7-2,4:F1-5 (n=268, 11.2%), C:P1.5,2:F3-3 (n=245, 10.3%), B:P1.7,16:F3-3 (n=171, 7.2%). Compared to the overall age distribution, fine types B:P1.7,16:F3-3 and B:P1.7-2,16:F3-3 caused a higher proportion of disease in adolescents and adults (p Chi2‹0.00001, p=0.002, resp.). Fine types C:P1.5,2:F5-8 (P=0,001), C:P1.5-1,10-8:F3-6 (p=0.002), and Y:P1.5-2,10-1:F4-1 (p‹0.00001) caused dis-proportionately more cases among 1-4 yr old children, younger adults, and older adults, re-spectively.
The highest diversity of fine types was observed in infants with a mean of 2.0 cases/fine type, range 1-34 (386 cases/196 fine types) and the lowest diversity among adolescents aged 15-19 years with a mean of 3.8 cases/fine type, range 1-67 (504 cases/133 fine types, p Chi2=0.02).
In the matched data set, only a few fine types deviated from the overall temporal distribution, e.g. the most common fine type B:P1.7-2,4:F1-5 increased disproportionately in 2004-2005 (p=0.06), comprising 13.9% of all cases and causing a marked increase in IMD incidence in a region in western North Rhine-Westphalia. Logistic regression analysis was performed to identify fine types associated with fatal out-come (9.0% of all cases). Fine types B:P1.7-2,4:F1-5, B:P1.19-15:F5-1, C:P1.5,2:F3-3, C:P1.5-1,10-8:F3-6, C:P1.5-2,F3-6 and C:P1.5-1,10-8:F4-1 were significantly associated with a higher CF. The latter 3 fine types belonged almost exclusively to ET-15, which was also associated with a high CF (15.2% versus 8.7% non-ET-15, p= 0.013). Along with B:P1.7,16:F3-3 and B:P1.19-1,15-11:F5-1, these 3 fine types also significantly increased risk of a septic clinical course.
Conclusions
Linkage of routine surveillance data with molecular genetic NRCM data im-proves data quality and permits correlation of demographic and clinical data with fine types and clonal lineages. High case fatality and more severe disease are associated with a few particular fine types.

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IS1301 insertion in *opaD* of an emergent *Neisseria meningitidis* ET-15 clone

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Background
The increased incidence of serogroup C, ST11 *Neisseria meningitidis* in Maryland during the late 1990s was associated with the emergence of a "late"clone that had undergone antigenic shift at *fetA* and, in selected isolates, deletion of *porA*. Representational difference analysis identified IS1301 sequences only in late clone isolates. Moreover, sequence analysis of the *fumC* gene demonstrated that the serogroup C late clone belonged to the hypervirulent ET-15 clonal complex. A recent study described ten IS1301 insertion sites from an ET-15 clone. However, three IS1301 insertions common to the Maryland late clone and other members of the ET-15 complex have not been characterized.
Objective
The goal of this study was to identify IS1301 insertion sites common to serogroup C, ST-11 *N. meningitidis* late clone and ET-15 isolates which may have contributed to the emergence of this clone in Maryland during the late 1990s.
Methods
Whole genome sequencing of a serogroup C late clone isolate was performed by shot-gun cloning and pyrosequencing of emulsion PCR products on a Roche 454 Genome Sequencer FLX system. Two different assemblies were performed using the DNAstar SeqMan Genome Assembler- a *de novo* assembly and a combination FAM18C-templated assembly with IS1301 plus junkyard sequences. A search of the resulting contigs for IS1301 sequences and associated flanking regions was performed using GeneQuest (DNAstar) software. A BLAST search of IS1301 flanking regions was performed to identify potential genetic disruptions in the meningococcal genome. PCR amplification and sequence analysis was performed with primers that flank one identified insertion site.
Results
The *de novo* assembly of the resulting whole genome sequences identified 4 IS1301 insertions that had been previously described for ET-15. The templated assembly with IS1301 and junkyard sequences identified an IS1301 insertion corresponding to the FAM18C *opaD* pseudogene. Sequence analysis of PCR products from IS1301 and *opaD* specific primers demonstrated that IS1301 is inserted in *opaD* in serogroup C, ST-11 late clone isolates and the ET-15 reference isolate, DE9246.
Conclusions
The serogroup C late clone which emerged in Maryland in the late 1990s is a member of the ET-15 hypervirulent clonal complex. This study demonstrated that IS1301 is inserted in the *opaD* gene of both late clone and ET-15 reference isolates. Disruption of *opaD* expression may contribute to meningococcal antigenic variation and permit clonal emergence. Further studies to investigate the role of IS1301 in *opaD* gene regulation are necessary.

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Disseminated gonococcal infection diagnosed with phenotypic and genotypic methods

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Objectives
Gonorrhoea has been a rare disease in Norway for several decades with only 283 cases reported in 2007. Disseminated gonococcal infection (DGI) has only been reported twice since 1993. This case report of a disseminated gonococcal infection emphasises the use of genotypic methods along with traditional phenotypic methods in improving diagnosis and understanding potential transmission route.
Methods
A 35 year old woman was hospitalized for 2 days with fever, shoulder pain and dysphagia. She received antiflogistica and paracetamol. However, no diagnosis was made. During the following month she had periods with high fever, fluctuating C-reactive protein and migrating joint pain. The patient was then re-hospitalized and transferred to the Department of Rheumatology, University Hospital of North Norway with suspected arthritis, fever and skin rash.
Clinical samples were drawn from the following sites; blood cultures, synovial fluid from the sternoclavicular joint, pharyngeal swab, urine sample, sample from skin rash along with standard blood samples for biochemical analysis.
Upon finding Gram negative diplococci in the blood culture, molecular methods were used to confirm the phenotypic *N. gonorrhoea* diagnosis, and samples were taken from the cervix and urethra.
Results
Microscopy of a positive blood culture revealed Gram negative diplococci and phenotypic (gram staining, biochemical tests, and Phadebact monoclonal GC OMNI test) diagnosis of the positive blood culture confirmed the presence of *N. gonorrhoeae*. The bacterium was also detected in the synovial fluid. An in-house porA pseudogene targeting PCR (Hjelmevoll et al 2006) confirmed these findings. The pharyngeal sample was negative by culturing, but positive with the in house PCR. Both, culture and PCR failed to detect the microbe from the skin rash. The urine sample was lost in internal logistics.
The patient was discharged with Ciprofloxacin 500 mg x 2 for 5 days, after initial treatment with Ceftriaxone 1 gram for 3 days intravenously. However, the isolate was later revealed as Ciprofloxacin resistant and treatment was altered to Amoxicillin 500 mg x 4 a day.
Conclusion
These findings emphasize the need to consider DGI, even in low burden countries like Norway. This case report indicates that genotypic methods can be an important supplement in diagnosing DGI. Culturing is still essential in order to obtain antibiotic susceptibility testing.