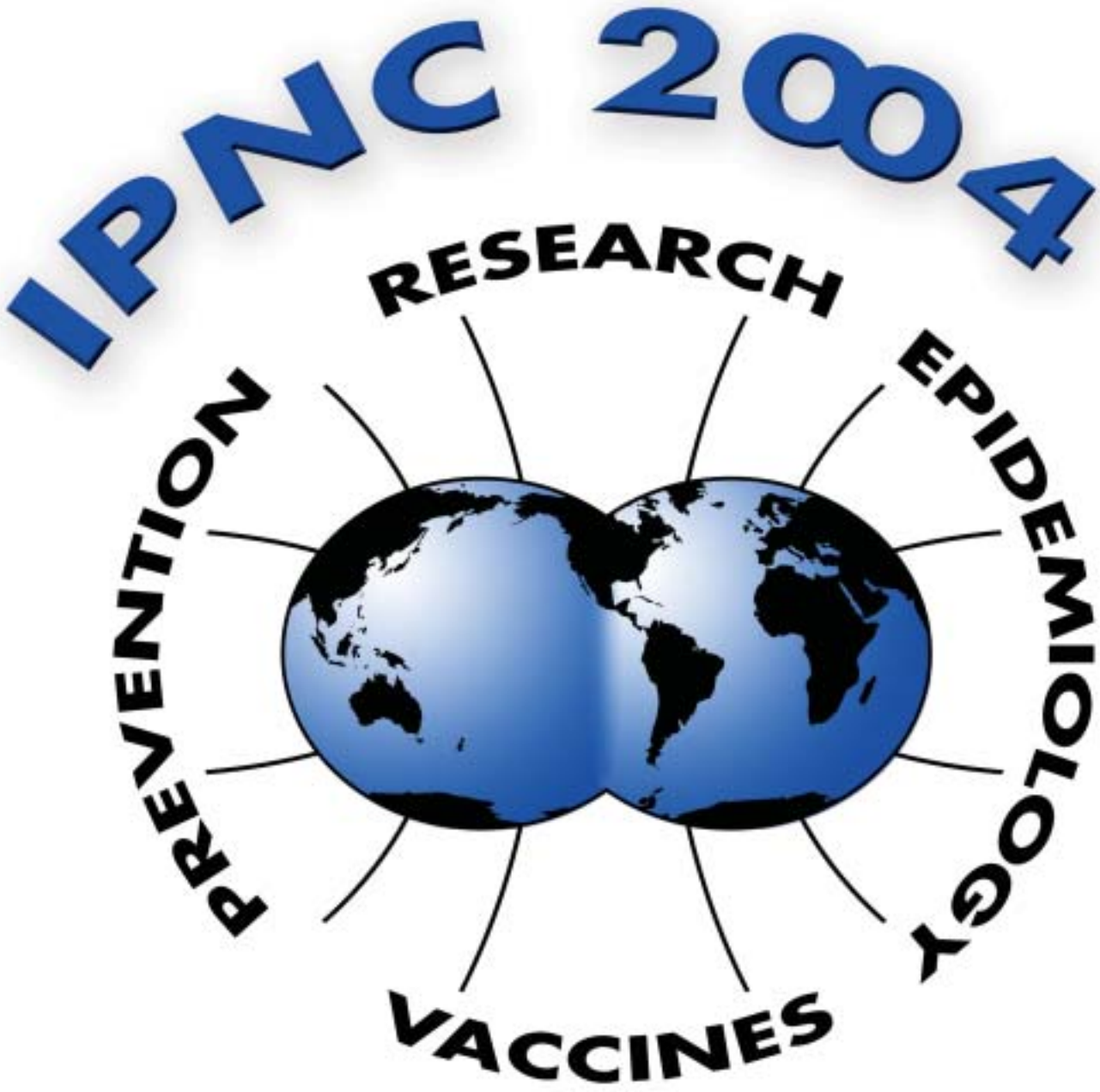

14th International Pathogenic Neisseria Conference

September 5-10, 2004
Milwaukee, Wisconsin USA

ABSTRACTS OF THE FOURTEENTH INTERNATIONAL PATHOGENIC *NEISSERIA* CONFERENCE

IPNC 2004

14th International Pathogenic Neisseria Conference

September 5-10, 2004
Milwaukee, Wisconsin, USA

History of the International Pathogenic Neisseria Conferences

In the 1970s a series of conferences were held dealing with issues of meningococcal epidemiology and vaccination. Some of these conferences were held in Milano, St. Paul de Vence, and Marseille. But the first official conference was held in San Francisco, California, 1978.

First International Pathogenic Neisseria Conference
1978, San Francisco, California, USA
Chair: G.F. Brooks

Second International Pathogenic Neisseria Conference
1980, Hemavan, Sweden
Chairs: S. Normark and D. Danielsson

Third International Pathogenic Neisseria Conference
1982, Montreal, Canada
Chair: I.WDeVoe

Fourth International Pathogenic Neisseria Conference
1984, Asilomar; California, USA
Chair: G.K. Schoolnik

Fifth International Pathogenic Neisseria Conference
1986, Noordwijkerhout, The Netherlands
Chair: J.T. Poolman

Sixth International Pathogenic Neisseria Conference
1988, Pine Mountaine, Georgia, USA
Chair: S.A. Morse

Seventh International Pathogenic Neisseria Conference
1990, Berlin, Germany
Chair: M. Achtman

Eighth International Pathogenic Neisseria Conference
1992, Cuernavaca, Mexico
Chair: C.I. Conde-Glez

Ninth International Pathogenic Neisseria Conference
1994, Winchester, England
Chair: M.C.J. Maiden and I Feavers

Tenth International Pathogenic Neisseria Conference
1996, Baltimore, Maryland, USA
Chair: C.E. Frasch

Eleventh International Pathogenic Neisseria Conference
1998, Nice, France
Chair: X. Nassif

Twelfth International Pathogenic Neisseria Conference
2000, Galveston, Texas, USA
Chair: F. Sparling and P. Rice

Thirteenth International Pathogenic Conference
2002, Oslo, Norway
Chair: E. Wedege

Fourteenth International Pathogenic Conference
2004, Milwaukee, Wisconsin, USA
Co-Conveners: M. A. Apicella and H. Seifert

14th International Pathogenic *Neisseria* Conference

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These organizations are thanked for their generous financial support.

Preface

The meningococcus and gonococcus are closely-related, human-specific pathogens that have been a persistent cause of disease throughout human history. This conference is devoted to the basic biology of these organisms, how they interact with host cells and tissues, how the human host reacts to and combats infection. The meeting will also cover the more clinical areas of how infections spread and interact with human populations and how antimicrobial agents and vaccines can be developed to control these infections. By bringing together researchers from many countries the cutting edge findings and approaches to studying these organisms will be vetted in a collegial atmosphere. Our expectation is that strides can be made to more successfully prevent these infections and improve human health. A total of 61 lectures and over 240 posters will be presented at IPNC 2004. This book contains all the abstracts of these scientific presentations.

We would like to thank the organizing committee and the scientific advisory committee for their advice and help with the program. We greatly the appreciate the assistance of our conference coordinator, Ms. Kristen Weber, and our web master, Mr. Bill Easton. We would also like to express our appreciation to Ms. Linda Johnson of The University of Iowa, who has spent countless hours organizing this abstract book and attending to numerous details of the meeting. Finally, the meeting would not have been possible without generous donations from Aventis, Chiron, GlaxoSmithKline, Wyeth, the Bill and Melinda Gates Foundation, the National Institute of Allergy and Infectious Diseases and the Food and Drug Administration.

Hank Seifert

Mike Apicella

Program Schedule

Monday, September 6, 2004		
Opening Remarks		
8:20-8:30 AM	Apicella, Michael	Opening remarks
Vaccinology		
8:30-11:30 AM	Maiden, Martin C.J.	Effect of conjugate serogroup C-polysaccharide vaccine on meningococcal population structure: herd immunity and vaccine escape.
	Soriano-Gabarro, Montserrat	Effectiveness of a serogroup A/C/W-135 meningococcal polysaccharide vaccine in Burkina Faso, 2003.
	Wong, Sharon H.	Safety and immunogenicity of New Zealand strain meningococcal serogroup B outer membrane vesicle vaccine in healthy 16-24 month old toddlers.
	Welsch, Jo Anne	Naturally-acquired immunity to Neisseria meningitides group C in the absence of bactericidal activity.
	Break	
	Pavliak, Viliam	Neisseria meningitidis LOS conjugate vaccine against meningococcal disease.
	Masignani, Vega	GNA 1870, a novel vaccine candidate of Neisseria meningitidis: immunological and functional properties.
	Heyderman, Rob	Characterisation of naturally acquired immunological memory to Neisseria meningitidis at the mucosal surface.
	Gorringe, Andrew	Neisseria lactamica outer membrane vesicle vaccine for meningococcal disease: towards a clinical trial.
Genomics and Gene Expression		
2:30-5:30 PM	Tzeng, Yih-Ling	Autoregulation of the MisR/MisS two-component signal transduction system in Neisseria meningitidis.
	Martin, Patricia	IHF and potentially fur are involved in the phase variable expression of the nadA gene in Neisseria meningitidis.
	Kahler, Charlene	Regulatory networks controlled by alternative sigma factors in Neisseria gonorrhoeae.
	Claus, Heinke	Genome sequencing of commensal strains of Neisseria meningitidis.
	Break	
	Davies, John	A multicentre microarray-based study of the effects of pilin production in Neisseria gonorrhoeae strain FA1090.
	Arvidson, Cindy G.	Global gene expression analysis of Neisseria gonorrhoeae in response to contact with human epithelial cells.
	Agarwal, Sarika	Expression of iron-regulated and fur-dependent genes during natural gonococcal infection as assessed by microarray analysis.
	Snyder, Lori A.S	Redefining virulence, identification of 'virulence' genes within Neisseria lactamica using the expanded pan-Neisseria microarray.

Monday, September 6, 2004, Continued

Evening Workshop

7:00 PM	Location: First Floor, Lakeshore Rooms A & B

Surrogates of protection against meningococcal disease: Is serum complement-mediated serum bactericidal activity still the "gold standard"?

Moderators:	Dan Granoff and Ray Borrow

1. What do we think we know and what are the questions? (Dan Granoff, Oakland CA)
2. Opsonic anticapsular antibodies. (Joseph Martinez, CDC, Atlanta GA)
3. Effectiveness of group C meningococcal conjugate vaccines in the UK in relation to immunogenicity and ongoing sero-surveillance. (Ray Borrow, Manchester UK)
4. Lessons from the UK experiences with type b *Haemophilus influenzae* conjugate vaccination and recent resurgence of disease. (Elizabeth Miller, Communicable Disease Surveillance Centre, Health Protection Agency, UK)

Tuesday, September 7, 2004		
Miscellaneous		
8:30-10:00 AM	Perea, William	Effective surveillance allows confirmation of epidemic Nm W135 in Africa during 2003.
	Ngampasutadol, Jutamas	Serum resistance of Neisseria gonorrhoeae is restricted to humans; a possible explanation for the species specificity of gonococcal infections.
	Genco, Caroline Attardo	Specificity of binding of the global regulatory protein Fur in the Neisseria meningitidis iron activated secY, nspA, aniA, and norB genes.
	Nicholas, Robert	The penC resistance gene arises from a mutation in PilQ that interferes with oligomer assembly and prevents antibiotic influx through the PilQ complex.
	Nolte, Oliver	Rifampin resistance in Neisseria meningitidis.
Poster I		
10:30-Noon	Poster I	Epidemiology, Host Response, and Vaccinology
Cellular Microbiology		
2:00-5:30 PM	Edwards, Jennifer L.	Gonococcal PLD and signaling events triggered by Neisseria gonorrhoeae infection of primary cervical epithelial cells.
	Schubert-Unkmeir, Alexandra	Interaction of Neisseria meningitidis with human brain microvascular endothelial cells: Role of MAP- and tyrosine kinases in invasion and inflammatory cytokine release.
	Criss, Alison	Pilin antigenic variation during gonococcal infection of model polarized epithelia.
	Duménil, Guillaume	Bacterial Adhesion Under Flow - a real time adhesion assay.
	Break	
	Rudel, Thomas	Modulation of host cell survival by PorB of Neisseria gonorrhoeae .
	Ala'Aldeen, Dlawar	Modulation of host cell apoptosis by meningococcal secreted proteins.
	Christodoulides, Myron	Role of host cell receptors in the inflammatory response of human meningeal cells on interaction with neisseria meningitidis and other bacteria causing meningitis.
	Kirchner, Marieluise	Caveolae are involved in Neisseria-host cell interaction.

Tuesday, September 7, 2004, Continued

Evening Workshop

7:00 PM | **Location: First Floor, Lakeshore Rooms A & B**

Control of Meningococcal disease in Sub-Saharan Africa

Moderators: David Stephens and Kader Konder, WHO/MVP

1. Epidemiology of meningococcal disease in Sub-Saharan Africa (cyclic trends, colonization, risk factors) and why is serogroup A still a problem, and what about serogroup X and W-135? (Nancy Rosenstein, CDC, Atlanta GA)
2. Molecular basis for emergence of serogroup A, X and W-135 in Africa. (David Stephens)
3. Challenges for control of meningococcal epidemics in Sub-Saharan Africa by immunization. (Dan Granoff, Oakland CA)
4. Status of developing a group A conjugate vaccine for Africa. (Marc LaForce, Meningitis Vaccine Project, Ferney-Voltaire, France)

Evening Workshop

7:00 PM | **Location: First Floor, Lakeshore Rooms C**

Antimicrobial Resistance in *Neisseria gonorrhoeae*: Meeting of the International Collaboration on Gonococci

Moderators:

1. Susceptibility testing of *Neisseria gonorrhoeae*:

    - Comparison of agar dilution susceptibility methods
    - Selection of new international panel of strains for quality control of susceptibility testing. (Joan S. Knapp, Atlanta, Georgia)

3. Rapid clonal spread of fluoroquinolone resistant gonococci in men who have sex with men in King County, Washington. (William L. H. Whittington, Seattle, Washington)
4. Antimicrobial resistance in *Neisseria gonorrhoeae*: New Delhi. (Dr. Preena Bhalla, New Delhi, India)

Wednesday, September 8, 2004		
Bacterial Genetics, Physiology and Metabolism		
9:00-10:00 AM	Seifert, Hank	Action of a sequence in the pilE upstream regions followed by branch migration of Holiday Junctions is essential for gonococcal pilin antigenic variation.
	Pelicic, Vladimir	Type IV pilus biogenesis in Neissseria meningitidis. PilW is part of a multi-protein complex in the outer-membrane that stabilizes the fibers and modulates their functionality.
	Cornelissen, Cynthia Nau	Evidence for cooperative interactions between gonococcal transferrin binding proteins.
	Tang, Chrostoph M.	Role of the lactate permease in the pathogenesis of meningococcal infection and as a potential vaccine candidate.
	Break	
	Cole, Jeff	Genomic analysis of the c-type cytochromes of Neisseria gonorrhoeae: mutational and biochemical analysis of cytochrome c', a nitric oxide-binding lipoprotein important for adaptation to micro-aerobic growth and its implications for pathogenicity.
	Owens, Ray	A structural analysis of the DNA-binding regulatory proteins of the pathogenic Neisseria.
	Dillard, Joseph P.	Lytic transglycosylases act in the release of proinflammatory molecules in Neisseria gonorrhoeae.

Thursday, September 9, 2004		
Epidemiology		
8:30-10:00 AM	Tiendrebeogo, Sylvestre M.	Serogroup W-135 meningococcal disease in Burkina Faso, 2002 and 2003.
	Granoff, Dan	Epidemic group A meningococcal disease in the Sudan despite high levels of naturally-acquired serum group A antibodies.
	Harrison, Lee H.	Antigenic shift associated with increased serogroup Y meningococcal infection in Maryland: A population-based, molecular epidemiologic study.
	Jolley, Keith A.	Distributed web-accessible databases developed for meningococcal typing and epidemeology.
	Jansen, Vincent A.A.	Diversity in pathogenicity can cause outbreaks of meningococcal disease.
Poster II		
10:00-Noon	Poster II	Antibiotic Resistance, Bacterial Genetics, Physiology and Metabolism, Cellular Microbiology, Genomics and Gene Expression, and Surface Structures
Host Reponse		
2:00-5:30 PM	Prasad, Alpana	Defining targets for C3 and C4 on pathogenic Neisseria.
	Wooldridge, Karl	The role of meningococcal secreted proteins in host-cell interaction.
	Massari, Paola	Meningococcal porin PorB activity is abrogated in TLR2 ko B cells.
	Jonsson, Ann-Beth	Neisseria-host cell interactions in CD46 transgenic mice.
	Break	
	Singleton, Theresa E.	Neisserial porin induces dendritic cell functional activity, which is MyD88-dependent.
	Zughaier, Susu M.	Meningococcal lipopoly(oligo)saccharide is a potent activator of both the TLR4/MyD88-dependent and -independent signaling pathways.
	Gray-Owen, Scott	CEACAM1-specific Opa proteins suppress dendritic cell maturation in response to Neisseria gonorrhoeae: Implications for gonorrhea and HIV.
	van der Ley, Peter	Lipopolysaccharide-mediated targeting of Neisseria meningitidis to dendritic cells: binding of lgtB LPS to DC-SIGN.
Dinner Dance		
7:00-Midnight	Dinner Dance	

Friday, September 10, 2004		
Surface Structures		
9:00-11:45 AM	Derrick, Jeremy	The three-dimensional structure of the secretin pilq and its interaction with type iv pili.
	Frye, Stephan A.	The meningococcal secretin PilQ: actions and interactions.
	Moe, Gregory A.	A unique capsular epitope recognized by bactericidal, non-autoreactive anti-N-propionyl group B polysaccharide mAbs.
	Jerse, Ann	Selection for opa-positive gonococci occurs during experimental murine genital tract infection in the absence of human CEACAM receptors.
	Break	
	Bos, Martine P.	The outer membrane protein Imp/OstA (NMB0280) is required for lipopolysaccharide transport to the bacterial cell surface.
	Wright, J Claire	lpt6, a gene required for addition of phosphoethanolamine to inner core lipopolysaccharide of Neisseria meningitidis.
	Kahler, Charlene	Lipooligosaccharide (LOS) inner core biosynthesis in Neisseria meningitidis : Identification of the O-6 PEA transferase and role of glycine.
Closing Remarks		


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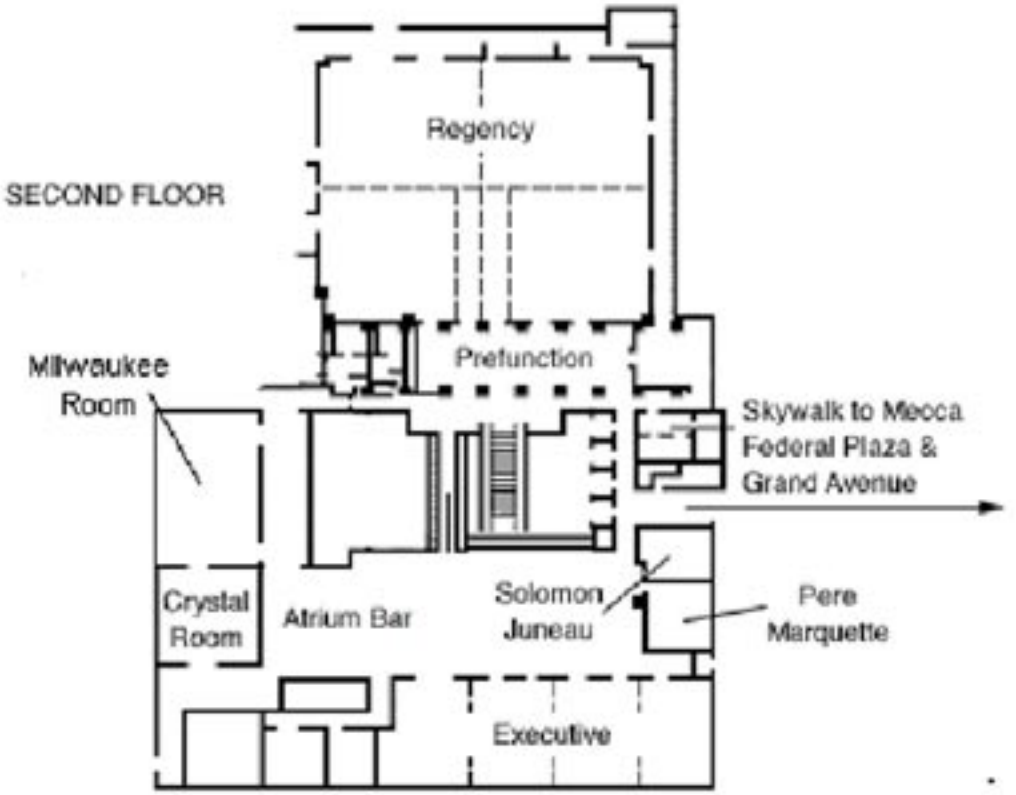
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PLENARY SESSIONS

Session I. Vaccinology

Effect of conjugate serogroup C-polysaccharide vaccine on meningococcal population structure: herd immunity and vaccine escape.

MAIDEN, M.C.J.[1], IBARZ-PAVÓN, A.-B.[1], URWIN, R.[1], MACLENNAN, J.M.[1], BENNETT, J.S.[1], BRAMLEY, J.C.[2], STUART J.M.[3], AND THE MENINGOCOCCAL CARRIAGE GROUP.

1. The Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3SY, United Kingdom. 2. Scottish Centre for Infection and Environmental Health, Clifton House, Clifton Place, Glasgow, G3 7LN, United Kingdom. 3. Health Protection Agency South West, The Wheelhouse, Bond's Mill, Stonehouse, Gloucestershire, GL10 3RF, United Kingdom.

The Meningococcal Carriage Study was initiated in 1999 to measure the effects on meningococcal populations of mass-vaccination with meningococcal serogroup C conjugate (MCC) polysaccharide vaccine. Given the nature of the UK immunisation campaign (initially, all individuals under the age of 18 years were offered the vaccine) and the rates of asymptomatic meningococcal carriage in teenagers, population effects and carriage dynamics could be examined and used to explain any observable change in disease incidence. For example, if transmission of serogroup C meningococci was interrupted by MCC immunisation, the potency of the vaccine might be enhanced, protecting unvaccinated individuals by herd immunity; conversely, vaccine efficacy could be compromised by the emergence of vaccine escape variants or novel pathogenic meningococci spreading as a consequence of the strong selective pressure imposed.

The study was powered to detect the replacement of hyperinvasive serogroup C ST-11 meningococci with ST-11 meningococci of another serogroup. This was anticipated to be the most difficult effect to detect and required a large sample size given the low prevalence of ST-11 meningococci in carriage. Meningococci were isolated from throat swab samples from 15,106 teenagers in November 1999, 18,095 in 2000, and 19,710 in 2001. A total of 8,913 meningococci were collected. The meningococci obtained were characterised phenotypically for serogroup expression, and genotypically for sequence type (ST) and at their capsular operon. This enabled the serogroup, clonal complex, and capsule expression to be determined for each isolate.

Phenotypic results have shown a significant effect on the carriage of meningococci expressing the serogroup C capsule in the two years following immunisation. Genotypic characterisation has now shown that before immunisation the rate of serogroup C capsule expression among carried ST-11 meningococci was approximately 75%, compared to expression rates of ~45% among other serogroup C organisms. Following immunisation, carriage of ST-11 meningococci that possessed the serogroup C capsule gene was significantly reduced. This, however, was not accompanied by a similar reduction in the carriage of other meningococci that possessed the serogroup C capsule gene. These data suggest that herd immunity induced by the MCC vaccine had a highly specific effect on the meningococcal population, substantially reducing the circulation of the epidemic ST-11 strain. It is possible that this strain is more reliant on serogroup C capsule expression for survival and transmission than other non-epidemic strains that possess the serogroup C genotype. These findings have important implications for the design and implementation of future meningocccal immunisation programmes.

Effectiveness of a Serogroup A/C/W-135 Meningococcal Polysaccharide Vaccine in Burkina Faso, 2003

SORIANO-GABARRÓ M[1], TOE L[2], TIENDREBEOGO S[3], NELSON C[4], PLIKAYTIS B[1], ROSENSTEIN N[1], AND THE WHO TRIVALENT VACCINE IMPACT ASSESSMENT STUDY GROUP5[5]
[1]Meningitis and Special Pathogens Branch, CDC, Atlanta, USA
[2]WHO, Multidisease Surveillance Center, Ouagadougou, Burkina Faso
[3]Ministry of Health, Direction de la Lutte Contre les Maladies, Ouagadougou, Burkina Faso
[4]WHO, Department of Immunization, Vaccines and Biologicals (IVB), Geneva, Switzerland.

Background: In 2002, the first major W-135 meningococcal disease epidemic occurred in Burkina Faso (BF). Following this epidemic, a A/C/W-135 meningococcal polysaccharide (trivalent) vaccine (Mencevax ACW™) was produced by GSK Biologicals for evaluation and use in Africa. In 2003, 2 million doses of the newly licensed vaccine were administered to persons 2-29 years, at the start of a new meningitis epidemic in BF (7673 cases, 1146 deaths). Of 368 cerebrospinal fluid (CSF) isolates confirmed as *Neisseria meningitidis* (Nm), 72% and 28% were confirmed as NmA and NmW-135, respectively. We conducted a case-control study to assess vaccine effectiveness (VE) against NmA and NmW-135 meningococcal meningitis.
Methods: Recruitment of cases started in 6 selected epidemic districts (population 1,500,000), 10 days after completion of vaccination campaigns, which had been conducted with 93.5% coverage. Cases were defined as any person 2-29 years with suspected meningitis, for whom NmA or W-135 antigen was detected through latex agglutination of CSF ("probable case"), or isolation from CSF ("definite case"). Three controls were matched to each case by age and neighborhood. Vaccinated persons were defined as any person 2-29 years who reported having received meningococcal vaccine and for whom there was card-confirmation of having received trivalent vaccine ("verified vaccination"), or any person who reported through verbal history having received trivalent vaccine regardless of verification ("reported vaccination"). Univariate and multivariable analysis were conducted using conditional logistic regression. VE was calculated as VE = 1 – odds ratio for vaccination.
Results: From 3/20-5/31/2003, 33 Nm A and 3 NmW-135 meningitis cases were enrolled. Fourteen (40%) cases and 89 (86.4%) controls reported vaccination with the trivalent vaccine in 2003. Nine (25.7%) cases and 53 (51.5%) controls were verified as having received the vaccine. Univariate VE for cases against probable and definite NmA or NmW-135 was 84% (95% confidence interval [CI] 50%-95%) for persons with verified vaccination, and 96% (95% CI 82%-99%) for persons with reported vaccination. VE against probable and definite NmA only was 92% (95% CI 62%-99%) for persons with verified vaccination and 98% (95% CI -0.5%-98%) for persons with reported vaccination. On multivariable analysis, VE for cases with verified vaccination against probable and definite NmA or NmW-135 was 84% (95% CI 38%-97%). VE against probable and definite Nm A alone was 94% (95% CI 59%-99%). Lack of education, preceding respiratory illness, community celebrations attendance, and sharing room with another meningitis case were significantly and independently associated with risk of disease.
Conclusions: We found that the trivalent meningococcal vaccine was highly effective against a primarily serogroup A meningococcal meningitis epidemic in Burkina Faso. Because of low number of cases, VE against NmW-135 only could not be estimated. This vaccine should be considered in the event of new meningococcal disease epidemics.

SAFETY AND IMMUNOGENICITY OF NEW ZEALAND STRAIN MENINGOCOCCAL SEROGROUP B OUTER MEMBRANE VESICLE VACCINE IN HEALTHY 16-24 MONTH OLD TODDLERS.

WONG SH[1], JACKSON CM[1], MARTIN DM[2], O'HALLAHAN JM[3], OSTER P[4], STEWART JM[1], LENNON DR[1].

The University of Auckland, Auckland, New Zealand[1]; Environmental Science & Research (ESR), Wellington, New Zealand[2]; Ministry of Health, Wellington, New Zealand[3]; Chiron Vaccines, Siena, Italy[4].

Background

New Zealand has been experiencing a monoclonal epidemic of *Neisseria meningitidis* B:4:P1.7b,4 which has resulted in more than 5000 cases and over 200 deaths since 1991. Children younger than 5 years are at highest risk.

Objective

To evaluate the safety, reactogenicity and immunogenicity of an outer membrane vesicle (OMV) vaccine specifically developed for the epidemic strain in 16 – 24 month old toddlers.

Methods

A Phase II observer-blind, randomised, controlled, single centre trial of 332 healthy children aged 16 – 24 months. Subjects were randomised in a 4:1 ratio to receive 25mcg New Zealand candidate vaccine (NZ98/254) or 25mcg Norwegian parent vaccine (H44/76). Vaccines were administered at 0, 6 and 12 weeks. Serum bactericidal assay (SBA) was used to measure immune response to candidate and parent vaccine strains at baseline, six weeks post dose 2, and four weeks following dose 3. Local and systemic reactions were monitored for 7 days after vaccination.

Results

Responders were defined as those with a 4-fold or greater rise in SBA antibody titre to NZ98/254 four weeks after the third dose compared to baseline. This was achieved in 75% (95% CI: 69-80%) of subjects receiving the candidate vaccine as compared with 4% (95% CI: 0-13%) of subjects who received Norwegian strain vaccine.

Conclusion

Three doses of New Zealand candidate vaccine (NZ98/254), administered to 16 – 24 month old toddlers, elicit promising bactericidal antibody results against the New Zealand epidemic strain. This study was funded by the New Zealand Ministry of Health and Chiron Vaccines.

Naturally-acquired Immunity to *Neisseria meningitidis* Group C in the Absence of Bactericidal Activity.

WELSCH JA and GRANOFF DM. Children's Hospital Oakland Research Institute, Oakland, CA

The hallmark of protective immunity to group C meningococcal disease is a serum bactericidal titer of • 1:4 measured with human complement. Although a positive titer is accepted as a surrogate of protection, investigators have proposed that this threshold titer may underestimate the true extent of protection. We used an infant rat bacteremia model to measure group C passive protective activity of serum samples from 91 unimmunized adults living in California. The in vivo results were related to group C serum bactericidal titers measured with human complement, and anticapsular antibody concentrations and avidity measured by a radioantigen binding assay. A total of 35 sera (38.5%) had passive protective activity (>2 log decrease in the geometric mean CFU/ml of blood, as compared to that of rats treated with negative control serum). The geometric mean bactericidal titer of the protective sera was 1:8, significantly higher than that of the non-protective sera (1:2, $P<0.0001$). Sera with bactericidal titers of • 1:4 were 3.4-fold more likely to confer passive protection (89%) than non-bactericidal sera (26%, $P<0.0001$). Thus, serum bactericidal titers of • 1:4 are a marker of passive protective activity but this in vitro threshold lacks sensitivity for predicting immunity. We next investigated the 73 sera with bactericidal titers <1:4 to determine the basis of passive protective activity. The 19 sera with protective activity had a higher geometric mean group C anticapsular antibody concentration (0.72 micrograms/ml) than that of the 54 sera that lacked protective activity (0.16 micrograms/ml, $P<0.001$). Sera with group C anticapsular antibody concentrations >0.3 micrograms/ml also were ~6-fold more likely to confer protection than sera with anticapsular antibody concentrations • 0.3 micrograms/ml (12%, $P<0.0001$). Thus protective activity in the absence of bactericidal activity was associated with higher concentrations of serum anticapsular antibody but not all sera with anticapsular antibody concentrations >0.3 micrograms/ml conferred protection. Of the 18 non-bactericidal sera with anticapsular antibody concentrations between 0.31 micrograms/ml and 0.99 micrograms/ml, the 11 that conferred protection had a higher mean anticapsular antibody avidity constant (21.9 nM^{-1}) than that of the 7 non-protective sera (14.6 nM^{-1}, $P<0.03$). Thus, protective sera also have higher avidity group C antibodies than non-protective sera. Recent studies in the U.K. reported high group C conjugate vaccine efficacy in the face of declining or absent serum bactericidal titers (Andrews et al, Clin Diagn Lab Immunol 2003). This observation was interpreted as implying that protection was not dependent on serum bactericidal activity but was conferred by immunologic memory elicited by the conjugate vaccine. Our data suggest that an equally plausible alternative explanation is that protection results from persistence of high avidity group C anticapsular serum antibodies, which are present in insufficient concentrations to elicit complement-mediated bacteriolysis in vitro, but sufficient to confer protection in vivo.

Neisseria meningitidis LOS Conjugate Vaccine against Meningococcal Disease.

PAVLIAK V, FORTUNA-NEVIN M, MONTEIRO M, MASON K, and ZHU D
Wyeth-Research, Pearl River, NY

Meningococcal disease remains a global health problem, especially in infants less than 2 years old and young adults. New glycoconjugate vaccines for serogroups A, C, Y and W-135 hold considerable promise after successful introduction of meningococcal serogroup C conjugate vaccines. The major drawback is the lack an effective vaccine for routine immunization against group B meningococci.

We have evaluated the feasibility of using meningococcal inner core LOS conjugate vaccines to protect against invasive infections caused by *N. meningitidis.* Using a panel of in-house derived mono-and polyclonal antibodies, we have identified 5 patterns of serological reactivity based on inner core epitopes among strains representing 12 immunotypes. This reactivity was dependent on phosphorylation of LOS with PEA. Structural analysis of evaluated LOSs revealed that this new antigenic determinant contained two PEA moieties simultaneously attached to HepII of the inner core. This new antigenic determinant was present on the majority of immunotypes and is serologically distinct from inner core with only one PEA attached at *O*-3 of HepII. Our results show that Meningococcal strains can be characterized as having one of the following five inner core structures: (i) PEA linked to O6 position of HepII; (ii) glucose (Glc) linked to O-3 and PEA linked to O-7 of HepII; (iii) PEA linked to O-3 of HepII; (iv) Glc linked to O-3 of HepII; and (v) the newly identified di-PEA on HepII.

To test feasibility of meningococcal LOS based vaccine, we prepared a tetravalent vaccine composed of four different inner-core glycoforms found among clinical isolates in North America and Europe. To avoid of the presence of epitopes that mimic human blood-group antigens, *galE* mutations were introduced into prototype production strains. Purified LOSs were tested for maintenance of the inner core PEA and Glc substitutions and for the absence of structures that could act as self-antigens. Each LOS glycoform was detoxified by partial removal of fatty acids from Lipid A, then conjugated to CRM 197, and formulated into a tetravalent vaccine. Tetravalent vaccine induced a broadly cross-reactive antibody response in rabbits that recognized LOSs from all 12 immunotypes. The binding of the vaccine-induced antibodies to wild type LOSs was dependent on substitution of inner core with PEA. The functional activity of the vaccine-induced antibodies was investigated in *in vitro* and *in vivo* assays. The results showed that antibodies were bactericidal and opsonic. In addition, the vaccine induced antibodies protected infant rats against bacterial challenge. These findings indicate that meningococcal tetravalent LOS conjugate vaccine is capable of eliciting functional antibodies and has potential to be broadly protective against all disease-causing isolates.

GNA 1870, a novel vaccine candidate of *Neisseria meningitidis*: immunological and functional properties.

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GNA 1870 is a novel surface-exposed lipoprotein of *Neisseria meningitidis* identified by genome analysis of strain MC58, which is able to induce bactericidal antibodies. Sequencing of the gene in 71 strains representative of the genetic and geographic diversity of the *N. meningitidis* population, showed that GNA 1870 can be distinguished into three variants according to its protein sequence. The protein is expressed by all strains of *N. meningitidis*. Antibodies against a recombinant form of the protein elicit complement-mediated bactericidal activity against strains that carry the same variant and induce passive protection in the infant rat model.

The antigenic and immunological properties of GNA 1870 were further studied using mice polyclonal antisera and a monoclonal antibody with bactericidal activity. We were able to map linear and conformational epitopes and found that most of the functional epitopes are located in one region, and that the Arginine 223 is a key residue for a protective epitope. The mapping of novel epitopes that induce bactericidal antibodies against *N. meningitidis* is an important step in the design of novel vaccines against Meningococcus.

Functional studies have also been performed to investigate the biological role of antigen GNA 1870. Preliminary data obtained with global transcriptional pattern studies show that the deletion of the gene in MC58 induces up-regulation of 87 different genes belonging to different functional categories. The genes found particularly up-regulated are involved in the bacterial defense from oxidative stress or from the action of antimicrobial peptides. The bases of this phenotype are currently under investigation.

Characterisation of naturally acquired immunological memory to *Neisseria meningitidis* at the mucosal surface

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Induction mucosal immunity is a key component of the development of natural protection against *Neisseria meningitidis* (Nm). We have previously shown that the magnitude of cellular proliferative responses against Nm outer membrane vesicles (OMV) at the mucosal level is strongly associated with age. The responses were found to be independent of B cells and consisted of both resident Nm-specific memory and naïve T-cells. Since the majority of adults appear to have been primed, presumably through colonisation by *Neisseria sp.*, we propose that it may be possible to boost pre-existing mucosal immunity in early adulthood though exogenous vaccination. In naïve individuals and younger children without pre-existing immunity it may be possible to optimise the mucosal immune response with immunomodulatory agents. However, before this can be achieved in human trials, a better understanding of the regulation of the mucosal immune response and how it can be influenced is required.

In the present study we have sought to determine the nature of this mucosal T-cell memory and investigate whether mucosal immunity can be influenced by systemic vaccination with model antigens. Human mucosal T-cells have been derived from palantine tonsils which are strategically located at the entrance to the upper airways, a common site of meningococcal carriage, a site of intense B-cell maturation and differentiation associated with T-cell activation. We have measured T-cell proliferation and surface expression of IL-18R and CRTH2 in conjunction with intracellular IFN-gamma and IL13 in $CD45RO^+$ (memory T-cells), $CD45RA^+$(largely naïve T-cells) and $CD25^+$ (regulatory T-cells) depleted populations of both tonsillar and peripheral blood mononuclear cells (PBMC). We have also vaccinated a cohort of adults prior to tonsillectomy. We have found evidence of immunological memory to two mucosal pathogens, Nm and influenza virus which have characteristics of both a Th1 and a Th2 profile. Comparisons with the PBMC response to these antigens suggests that the regulation of this immunity is compartmentalised. The mucosal responses to Nm antigens also seem to be influenced $CD25^+CD45RB^{low}$ Treg cells resident within the tonsil. Vaccination with flu-antigens significantly increased T cell proliferation in both tonsils and blood. The PT responses to flu post-vaccination were associated with a shift from a response involving both $CD45RA^+$ and $CD45RO^+$ T cells to an entirely $CD45RO^+$-dependent response. This change was not associated with an altered cytokine profile but vaccinees PT demonstrated reduced IL-10. Nm vaccine studies are underway. We conclude that generation of immunological memory to Nm protein antigens at the mucosal level is feasible, and suggest that parenteral vaccination may influence both mucosal and systemic naturally-acquired T cell immunity to mucosal pathogens.

***Neisseria lactamica* outer membrane vesicle vaccine for meningococcal disease: towards a clinical trial.**

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Vaccines against meningococcal disease have been produced based on outer membrane vesicles (OMVs) from *Neisseria meningitidis* but these have shown variable efficacy in clinical trials, especially in young children. In addition, many meningococcal surface proteins are subject to antigenic variation limiting protection against diverse circulating strains. Protection against meningococcal disease has been associated with the presence of serum antibodies bactericidal for *N. meningitidis*. These antibodies are acquired progressively over the first few years of life, a period when meningococcal carriage rates are low. Thus it has been suggested that acquisition of natural immunity in young children follows colonisation by nonpathogenic *Neisseria* species. *N. lactamica,* a commensal species that does not possess a polysaccharide capsule or PorA but has many common antigens with *N. meningitidis*, may be the most important of these species.

Like *N. meningitidis, N. lactamica* naturally shed OMVs during growth. Thus we have used methods developed for the production of meningococcal OMVs to develop *N. lactamica*-based vaccines. *N. lactamica* OMVs have been prepared with deoxycholate extraction and used to immunise mice by subcutaneous injection. Antisera raised against *N. lactamica* OMVs were cross-reactive against a diverse range of *N. meningitidis* strains. We have assessed the efficacy of an *N. lactamica* OMV vaccine in a mouse model of bacteraemic meningococcal disease and *N. lactamica* OMVs protected mice against lethal challenge with meningococcal isolates from the ET-5, ET-37, lineage III and A4 cluster clonal lineages, irrespective of serogroup. This protection has been observed in the absence of a detectable serum bactericidal antibody response but opsonophagocytic responses have been demonstrated and may be responsible for the observed protection.

The *N. lactamica* OMV vaccine is being extensively characterised by a range of methods, including SELDI-MS. Silver-stained 1D SDS-PAGE gels show 8 major bands, including PorB, RmpM, NspA and LOS. Analysis on 2D gels shows approximately 80 spots and the immunogenicity of these is being examined by western blotting. In addition, we have compared the proteins present in *N. lactamica* and *N. meningitidis* OMVs using a proteomic method. 1D SDS-PAGE gels of either *N. lactamica* or meningococcal OMVs were split into 24 slices, subjected to in-gel tryptic digestion followed by nanocapillary liquid chromatography and tandem mass spectrometry. Proteins were identified from the detected peptides by automated database searching. Approximately 60 meningococcal proteins were identified in the *N. meningitidis* OMVs with 90 orthologues of meningococcal proteins in the *N. lactamica* OMVs.

The essential next step for this vaccine approach is a safety and immunogenicity study in adult volunteers. We have produced an *N. lactamica* OMV vaccine using pharmaceutical manufacturing facilities at Health Protection Agency, Porton Down, which is currently undergoing toxicology and stability studies and will commence a clinical study in 2005.

Session II.
Genomics and Gene Expression

Autoregulation of the MisR/MisS two-component signal transduction system in *Neisseria meningitidis*

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Two-component regulatory systems are prevalent in many prokaryotes, and are involved in processes important for bacterial pathogenesis. A limited number of environmental-sensing two-component regulatory systems have been identified in meningococci. One such regulatory system, named *misR/misS* for meningococcal inner core structure or *phoP*/*phoQ*, has been shown to be involved in the HepII PEA substitution of LOS inner core[1] and causes attenuation of virulence when mutated[2]. Interestingly, microarray analysis revealed the potential regulatory role of this system in the expression of *hmbR*, *bfrAB,* encoding subunits of bacterioferritin, and *NMB1497*, a putative TonB-dependent receptor. Quantitative real time PCR experiments confirmed the altered expression of these iron-related proteins in the *misR* mutant; however, iron availability did not mediate MisR/S regulation. Real time PCR data also revealed a significantly decreased *misR* expression in both the *misR* and *misS* mutants, suggesting an autoregulatory role. In order to biochemically investigate this regulatory mechanism, His-tagged MisR as well as MBP-MisS fusion proteins were overexpressed and purified from *E. coli*. MisR can be phosphorylated by the small molecule phosphate donor, acetyl phosphate. In addition, the MBP-MisS fusion was shown to be autophosphorylated in the presence of ATP, and the phosphoryl group can be subsequently transferred to MisR. The phosphotransfer reaction was halted with a D52A mutation in MisR, while a H246A mutation of MisS prevented autophosphorylation. These data confirm the function of the MisS/MisR as a two-component signal transduction system, and that the two proteins interact with each other. In addition, specific interactions of phosphorylated MisR (MisR~P) and MisR with the *misR*, *hmbR* and the capsule *synA*/*ctrA* promoters were demonstrated by gel mobility shift experiments, and MisR~P exhibits higher affinity than does the non-phosphoylated protein. DNaseI protection assays revealed that MisR interacts with a ~16-bp region upstream of the *misR* transcriptional start site, and that the MisR~P protects a much larger promoter region. A *misR* recognition sequence was identified.

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IHF and potentially Fur are involved in the phase variable expression of the *nadA* gene in *Neisseria meningitidis*

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In *Neisseria meningitidis*, phase variation, i.e. the reversible expression of surface antigens, is associated with changes within repeated simple sequence DNA motifs located in coding or promoter regions of genes involved in the biosynthesis of surface antigens. The phase variable gene *nadA* encodes an adhesin proposed as a vaccine candidate (Comanducci *et al*., 2002). The expression of the *nadA* gene was shown to be regulated at the transcriptional level through a variation of the number of reiterated (TAAA) motifs present in the repeat tract located upstream of the core promoter of the gene (Martin *et al*., 2003).
We identified three statistically distinct levels of *nadA* transcription exhibiting a periodic pattern related to the number of repeated (TAAA) motifs.
An investigation of the likely three-dimensional conformation of the DNA sequence containing the repeat tract and the upstream region when the number of (TAAA) motifs varied revealed that the level of *nadA* transcription depends on the helical facing of the DNA located upstream of the repeat tract with respect to the core promoter of the gene.
Deletion of the DNA sequence located upstream of, or including the repeat tract resulted in an increased level of *nadA* transcription. Two distinctive DNA sequences were shown to be involved in the regulation of *nadA* transcription. One of these DNA sequences contained a putative integration host factor (IHF) binding site and was shown by electrophoretic mobility shift assays (EMSA) to bind purified IHF. The other DNA sequence contained a putative ferric uptake regulator protein (Fur) binding site and is currently being studied in EMSA using purified Fur protein.
Although deletion of each sequence upstream of the core promoter resulted in an increased level of *nadA* transcription, the *nadA* promoter was only partially de-repressed. However, mutant strains presenting $(TAAA)_9$ but deleted for the DNA region upstream of the repeat tract produced fully de-repressed variants having $(TAAA)_8$. Therefore, phase variation can still occur when the DNA sequence located upstream of the repeat tract is deleted.
We showed that a modification of the number of repeated (TAAA) motifs alters the chromosomal conformation of the *nadA* promoter region and consequently affects the interaction between Fur, IHF and the RNA polymerase. This work has therefore identified a novel system of transcriptional regulation in which variations of the hypermutable repeat region play a central role in modulating phenotype through the interplay of mutational (stochastic) and classical gene regulatory (deterministic protein-DNA) interactions.

Regulatory networks controlled by alternative sigma factors in *Neisseria gonorrhoeae*.

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Bacterial sigma factors function as global regulators by conferring promoter specificity to the core RNA polymerase. In *Neisseria* two putative alternative sigma factors belonging to the sigma 70 family, *rpoH* and *ecf*, have been identified. *ecf* is a member of a family of sigma factors that typically respond to extra-cytoplasmic stimuli whereas *rpoH* (sigma 32) activates the stress response of many bacteria. Efforts to insertionally inactivate both genes encoding these sigma factors in *N. gonorrhoeae* revealed that *ecf* was non-essential for growth on laboratory media whereas *rpoH* appears to be essential. To determine the regulatory network specifically controlled by each sigma factor, *rpoH* and *ecf* were independently cloned into the Hermes shuttle vector system and over-expressed in *N. gonorrhoeae*. The transcriptomes of these over-expression strains were analysed and compared to the original strain at mid-log phase using the Pan-Neisseria genome array.

As RpoH acts as an activator, over-expression resulted in the increased transcription of genes encoding factors identified as being involved in the stress response in many bacteria. The *dnaK*, *dnaJ* and *grpE* chaperone system, *groEL*, *clpB* and minor putative chaperones such as *lon* and *ppiB* were significantly up-regulated in the presence of excess RpoH. Activation of these genes appeared to be directly mediated by RpoH as the promoters of these genes contained a consensus RpoH binding site. Only two other genes were up-regulated by the presence of excess RpoH, however, as no RpoH consensus binding sites were present in these promoters it appears that this effect was indirect. The results of these experiments were compared to the heat shock response of *N. gonorrhoeae* exposed to 42^0C for 10 min. As expected, all of the genes controlled by RpoH were up-regulated when the strain was exposed to elevated temperatures. In addition, however, a small cohort of down-regulated genes were also detected suggesting that other regulatory factors are involved in this response.

Microarray analysis of the *ecf* null mutant did not reveal any changes in the transcriptome of this mutant. This result may indicate that under normal growth conditions, this sigma factor is silenced by an anti-sigma factor. To overcome this, *ecf* was over-expressed and transcriptional analysis performed on this strain at mid-log phase. These experiments showed that *pilB*, a conserved hypothetical gene upstream, and a cluster of genes upstream of *ecf* were transcriptionally upregulated. Gonococcal PilB possesses peptide methionine sulfoxide reductase activity which is necessary for the repair of proteins damaged by oxidative stress. Western blotting using anti-PilB antisera confirmed an increased PilB production in the strain in which *ecf* was over-expressed. Thus, *ecf* appears to regulate *pilB* transcription, and hence is probably involved in mediating a protective response to oxidative stress.

Genome sequencing of commensal strains of *Neisseria meningitidis*

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Most carrier isolates of *Neisseria meningitidis* are considered as virtually apathogenic despite of the presence of virulence factors such as the polysaccharide capsule. Only few meningococcal carriage isolates exist which constitutively do not express a capsule, e.g. isolates of the sequence type (ST)-53 (Claus *et al*., 2002). These observations indicate that meningococcal virulence is caused by a complex interplay of several factors, which is only partly understood. Genome sequencing seems to be an appropriate tool to unravel the molecular basis of pathogenicity. Up to now, only pathogenic meningococci have been sequenced, i.e. the serogroup A strain Z2491 (Parkhill *et al*., 2000) and the serogroup B strain MC58 (Tettelin *et al*., 2000).

In this study, four commensal strains of the Bavarian meningococcal carrier strain collection comprising 830 sequence typed isolates were selected for whole genome sequencing for the comparison of pathogenic and apathogenic meningococcal genomes. We selected one capsule null locus (*cnl)* isolate (ST-53), one serogroup B isolate (ST-136), one serogroup W-135 isolate (ST-22), and one serogroup 29E isolate (ST-60). The sequencing of the ST-53 and the ST-136 isolates was finished to gap closure whereas the genomes of ST-22 and ST-60 were sequenced to an 8-fold coverage. The annotation of the ST-53 sequence is almost finished and revealed that genes specific to this ST comprised five restriction-modification systems, one prophage, as well as more than 100 hypothetical genes. As known from recent studies, ST-53 lacked the genes required for the synthesis of a capsule. Furthermore, besides a large number of hypothetical genes, the *opc* gene was lacking in comparison to MC58. The lack of pathogenicity of ST-53 may easily be explained by the capsule null locus found in this strain. Nevertheless, the ongoing sequence analysis of other encapsulated commensal isolates will reveal the importance for pathogenicity of other genes among the large number of differences between ST-53 and pathogenic meningococci. Work is in progress to systematically inactivate those genes in strain MC58 to analyze their impact on virulence. The sequence data of the ST-53 genome were already included into the design of a meningococcal micro-array in collaboration with Qiagen and Julian Parkhill (Sanger Institute). This multi-clonal oligonucleotide micro-array is commercially available.

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A multicentre microarray-based study of the effects of pilin production in *Neisseria gonorrhoeae* strain FA1090.

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Four different laboratories independently compared the transcriptional profiles of gonococcal strain FA1090 *recA6*, and a *pilE* deletion mutant of this strain, using a pan-*Neisseria* microarray.The findings of this study address both important practical aspects of conducting microarray experiments and the consequences of the loss of *pilE* expression.
Practical microarray issues:
The four laboratories, using the same stocks of isolated RNA, but using a range of different labelling and scanning protocols, generated highly consistent and reproducible data, demonstrating that different methodologies produce similar results in microarray analysis using the identical sample. Selected results were verified using RT-PCR and proteomics. In contrast, independently isolated RNA, both within and between the laboratories, produced much more variable results. This finding highlights the essential role of such replicates in microarray experiments to distinguish between consistent changes in gene expression and normal biological variance that occurs within (even very simple) biological systems. The consistency of results generated by biological replicates is of primary importance. Also, for results to be compared and combined the fold changes in gene expression levels must fall within similar dynamic ranges.
The transcriptional consequences of *pilE* inactivation:
A relatively small number of genes were observed to alter expression by more than 2.5 fold up or down. These genes and additionally those with significant ($P<0.001$) lower fold-changes fell into a wide range of functional categories, but a number of patterns were discernable. The absence of pilin results in a cell surface disruption and the increased expression of *mtrR* and *mtrF*. This alteration in cell surface properties is also reflected in the upregulation of genes encoding several ABC transporters and LPS biosynthesis enzymes. Transcriptional units (operons) showing altered mRNA levels often contained genes with diverse functions, and in one case contained a minimal mobile element (MME) region which has been shown previously to contain a wide range of strain-specific genes in different strains. Common alterations in expression of transcriptional units between strains therefore have the potential to generate different phenotypic consequences depending upon the repertoire and locations of these elements. Alterations in the transcription of several restriction-modification system genes were observed. Current models of these systems do not anticipate a role for environmental responsiveness and the relative roles of specific regulators and transcription of co-transcribed genes need to be considered. Decreased expression of *aniA* was observed, raising questions as to whether this gene is only responsive to anaerobic conditions or perhaps responds secondarily to altered pilus expression or functions in the absence of oxygen. Despite the range of transcriptional changes observed, none were obviously involved in pilus-associated phenotypes. The majority of phenotypes associated with piliation can be directly ascribed to the presence and functions of pilin.

Global gene expression analysis of *Neisseria gonorrhoeae* in response to contact with human epithelial cells.

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Neisseria gonorrhoeae, a strictly human pathogen with no reservoir outside of its host, has limited environmental signals to which it might need to respond upon transmission. Since it is primarily a pathogen of the urogenital tract, and transmission is frequently male-to-female-to-male, and so on, signals to which gonococci might be expected to respond include pH, hormone levels, normal microflora, and cells of the epithelial mucosa. A goal of our research is to identify the regulatory phenomena involved in the colonization of a new host, and in this work have examined one facet of colonization, attachment to epithelial cells. While much progress has been made in the area of understanding the events occurring in an epithelial cell upon attachment of gonococci, relatively little is known of the events that occur in the bacterial cell at this step. Our hypothesis is that upon attachment to epithelial cells, signals are transmitted to the bacterium that result in a modulation of the expression of genes necessary for survival and proliferation in the host.

In order to examine global gene expression in gonococci, we have developed DNA microarrays which consist of PCR amplicons representing 2074 ORFs of the *N. gonorrhoeae* genome, 2016 from strain FA1090 (http://www.stdgen.lanl.gov/) and 58 from the MS11 genetic island[1]. An initial validation of the DNA microarrays was done comparing expression in otherwise isogenic piliated (P^+) and non-piliated (P^-) strains of *N. gonorrhoeae* grown in tissue culture medium. As expected, the results of these experiments showed that *pilE* (70-fold) and several *pilS* sequences (7-15 fold) were among the highest differentially expressed, essentially expressed only in the P^+ strain.

We next analyzed global gene expression in gonococci following adherence to epithelial cells in culture. Data from four independent experiments (including one dye-swap) showed that several genes were up- and down-regulated by host cell contact. Since nearly half of the genes in the annotated genome sequence of *N. gonorrhoeae* encode hypothetical proteins of unknown function, it was not surprising that many genes regulated by host cell contact fell into this category. Among the known genes that were up-regulated in adherent bacteria were two putative transcriptional regulators, RpoH and MtrR. Also up-regulated were several genes known or predicted to be regulated by these regulators. Of those that were down-regulated, many appeared to be involved in housekeeping functions such as glycolysis, amino acid synthesis, and nitrogen assimilation, suggesting that there might be a general shift in cellular metabolism in response to host-cell contact. This may be a mechanism for the bacterium to begin to adapt to different nutrient availability in the new host and/or the different environment inside of the host cell.

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Expression of Iron-Regulated and Fur-Dependent Genes During Natural Gonococcal Infection as Assessed by Microarray Analysis. AGARWAL S[1], KING C[1], KLEIN E[1], RICE PA[1,2],WETZLER LM[1,2] and GENCO CA[1,2]. Department of Medicine[1] and Department of Microbiology[2], Boston University School of Medicine, Boston, MA 02118, USA.

Iron is limiting in the human host and bacterial pathogens respond to this environment by regulating gene expression through the ferric uptake regulator protein (Fur). In vitro studies have demonstrated that *Neisseria gonorrhoeae* controls gene expression of several critical genes through an iron and Fur mediated mechanism. While most in vitro experiments usually are designed to determine the response of the gonococcus to a complete deficiency of exogenous iron, these organisms may not be actually exposed in vivo to such severe iron limitation. To determine if *N. gonorrhoeae* expresses iron and Fur regulated genes during human mucosal gonococcal infection in this study, we examined gene expression profiles of specimens obtained from urethral swabs from males and cervical swabs from females with gonococcal infection from the Public Health Clinic's at Boston Medical Center and John Hopkins Medical Institutions. Initially, we utilized RT-PCR analysis to study the expression of iron-regulated genes. However, a major limitation was the limited number of transcripts (*fbpA*, *tbpA*, *tbpB*, *fur* and the constitutively expressed gene, *rmp*) that could be examined due to the small amounts of total RNA isolated from the clinical samples. To overcome this limitation, we designed a custom DNA microarray with over one hundred genes, representing gonococcal iron and Fur regulated genes, including *fbpA*, *tbpA*, *tbpB*, *lbpA*, *lbpB*, *tonB*, *fur*, *secY*, *nspA* and the *opa* family of genes, as well as genes that are known or are hypothesized to play a role in pathogenesis and virulence of *N. gonorrhoeae*. A number of the genes included here were the same as those in a previous meningococcal microarray that we studied, where the expression profile of genes grown in iron-replete and -deplete conditions were analyzed. For the microarray, oligonucleotides spanning 50 bp of conserved region from gonococcal specific iron-regulated genes were spotted on a microarray slide and hybridized with total RNA isolated from gonococcal positive samples. Data from microarray analysis of total RNA isolated from gonorrhea infected clinical specimens confirmed our findings using RT-PCR where the variability of expression of the iron and Fur regulated genes from subjects with uncomplicated gonococcal infection was similar using both methods. Our results indicate that gonococcal specimens obtained directly from subjects with mucosal infection express both Fur-repressed (*fbpA*, *tbpA*, *tbpB*, *tonB, lld*, *fumC*) and Fur-activated (*secY*, *nspA*, *sodB*, *aniA*, *kat*) genes. As a whole, this study demonstrates the effectiveness of DNA microarray analysis in understanding the role of the gonococcal Fur regulon in human mucosal infection.

Redefining virulence, identification of 'virulence' genes within *Neisseria lactamica* using the expanded pan-*Neisseria* microarray.

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Introduction. We have recently expanded the pan-*Neisseria* microarray to include the additional genes identified in *N. meningitidis* strain FAM18, GenBank, and from our own sequencing of Minimal Mobile Elements (MMEs), in addition to those from the 3 previously available genome sequences and additional MS11 genes. This expanded microarray was used to assess the presence of these genes within the genomes of 12 unrelated *Neisseria lactamica* strains using Comparative Genome Hybridization.

Materials & Methods. The expanded pan-*Neisseria* microarray was printed onto Genetix Amine slides using Genetix QArray Mini microarray printers. DNA was extracted from 12 unrelated *N. lactamica* strains that have been assessed by MLST previously. Hybridization results were assessed using tools custom designed for this type of Comparative Genome Hybridization study.

Results & Discussion. Comparisons between related species with commensal and virulent behaviours provide insights into those genes that are central to virulence. The publication of the *N. meningitidis* strain MC58 genome sequence included a description of a set of 96 'virulence' genes based upon roles associated with virulence in this and other species, including genes for iron acquisition, colonization, immune evasion, and toxin production (Tettelin, Science, 2000). The *N. lactamica* strains tested on the expanded pan-*Neisseria* microarray have approximately one half of these genes, with some strain-to-strain variability in presence/absence being evident. This identifies a subset of divergent and absent genes which may be key to virulence while differentiating them from genes that are adaptive to conserved characteristics associated with fitness and common behaviour in both commensals and pathogens. Among the genes present in *N. lactamica* are those involved in LPS biosynthesis, iron acquisition, efflux, and pilus biosynthesis.

These comparisons also reveal a striking similarity in the extent of diversity within the genus. The microarray results with these commensal *Neisseria* strains indicate that they share 92% of the genes present in the pathogens. This figure is similar to that obtained by comparing the genome sequences of any two of the four sequenced pathogenic *Neisseria* strains. Therefore, the key differentiators between pathogens and commensals may be strongly associated with the functions of a relatively small subset of genes that are both species and pathogen defining, and not simply reflecting strain-to-strain variation.

Conclusion. Some genes that classically considered to be 'virulence' determinants are present in strains of the commensal *N. lactamica*, suggesting that these are involved in survival rather than being directly responsible for the virulence of the pathogenic *Neisseria* spp. At the same time a limited number of genes can be identified and associated with gonococcal and meningococcal disease causing potential.

Session III. Miscellaneous

Effective surveillance allows confirmation of epidemic Nm W135 in Africa during 2003.
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Context: *Neisseria meningitidis* (Nm) serogroups A and C are historically responsible for annual epidemics in the African Meningitis Belt, a dry region stretching from Senegal in the west to Eritrea in the east at the southern edge of Sahara dessert and home to 250 million people <30y of age. Other areas in Eastern and Southern Africa can also be affected. The most recent major epidemic of meningococcal disease in the Meningitis Belt occurred during the dry seasons (December-May, typical peak incidence in week 14) of 1996-98 and included at least 250,000 cases. Major epidemics of meningococcal disease occur approximately every 8-12 years.

During a meningitis epidemic case fatality ratios (CFRs) can exceed 50% among untreated cases with CFRs among treated cases remaining high at 7-25%. Among survivors, 10-20% manifest long-term, measurable disability including mental retardation and hearing loss and these cases experience increased mortality.

Nm W135 has been reported in Africa since 1982 and sporadic cases are common in each epidemic season. In 2000, the first large epidemic of Nm W135 disease was reported among Hajj attendees in Saudi Arabia and upon return to their home countries. In 2001, Nm W135 was detected increasingly in West and Central Africa. In 2002, the first large epidemic of Nm W135 disease in Africa was reported in Burkina Faso.

Epidemic preparedness includes surveillance and response activities. Surveillance focuses on outbreak detection and laboratory confirmation of responsible pathogens/serogroups. Response includes proper case management and mass reactive vaccination campaigns. The aim of this presentation is present results of WHO's recent efforts to establish a regional surveillance team, re-enforce surveillance for epidemic meningococcal disease (EMD) in African Meningitis Belt countries and support epidemic response.

Results: In 2003, 23,000 cases of EMD were reported from 8 countries with Niger and Burkina Faso being most affected. Reported CFRs ranged from 7-21%. The highest EMD incidence was reported among age groups <15y. Although Nm A was most prevalent, Nm W135 was detected in every reporting country with a substantial number of cases detected in epidemic districts of Mali, Burkina Faso, Niger and Nigeria.

Two million doses of trivalent Nm ACW vaccine and 1.6m doses of bivalent Nm AC vaccine were used for epidemic response. Small amounts of tetravalent Nm ACWY vaccine were also used.

Conclusions: Although epidemic Nm A continues to be the predominant cause of EMD in the Meningitis Belt, Nm W135 persists as a threat in these populations. Surveillance, including laboratory confirmation of cases, is central to the choice of an appropriate vaccine for epidemic response. The challenge of addressing the future production and availability of the trivalent vaccine has been successfully met through the establishment of a secure vaccine stockpile.

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Serum resistance of *Neisseria gonorrhoeae* is restricted to humans; a possible explanation for the species specificity of gonococcal infections

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Neisseria gonorrhoeae infection is restricted to humans. In order to survive in the human host, the gonococcus must evade the innate immune system. Complement is an important arm of the innate immune system that combats neisserial infections, as evidenced by the clinical and epidemiologic observation that individuals deficient in certain components of the complement system are highly predisposed to recurrent, disseminated gonococcal infections (DGI). An important mechanism by which gonococci resist complement-mediated killing by normal human serum (NHS), is to bind complement regulatory proteins such as factor H and C4b-binding protein (C4bp). We hypothesized that the ability of gonococci to bind human complement regulatory proteins specifically, but not complement regulatory proteins from other species, may confer protection to gonococci in the human host, thereby helping to explain why gonococcal disease is restricted to humans only.

We have shown previously that the porin (Por) molecule of gonococci can bind C4bp and factor H in human serum; gonococci thereby evade complement-mediated killing. A human C4bp-binding gonococcal strain capable of fully resisting killing by NHS (100% survival of gonococci in • 50% concentration of NHS) was killed 100% by normal rat, guinea pig, and rabbit sera at concentrations as low as 5% serum. Addition of purified human C4bp, to these sera at physiologic concentrations fully restored serum resistance. Consistent with the results obtained in these serum killing assays, we found that gonococci did not bind rat, guinea pig, and rabbit C4bp that was present endogenously in their sera. These results illustrate the importance of classical complement pathway regulation in restricting both the deposition of complement on bacterial surfaces and resultant killing.

Similarly, certain gonococcal strains that bear factor H-binding Por molecules are resistant to NHS, but are fully susceptible to normal rat serum (NRS). Such gonococci were rescued from killing by NRS by the addition of purified human factor H. Another mechanism that enables gonococci to resist killing by NHS is sialylation of their lipooligosaccharides (LOSs), which augments binding of human factor H. While NRS readily killed sialylated gonococci, the addition of purified human factor H restored the serum resistant phenotype. Our studies highlight the importance of binding of human complement regulatory proteins in mediating resistance of *N. gonorrhoeae* to human serum killing. Acknowledgement of these differences in non-human sera may prove useful in developing suitable animal models for gonococcal infection.

Specificity of binding of the global regulatory protein Fur in the *Neisseria meningitidis* iron activated *secY*, *nspA, aniA,* and *norB* genes. SEBASTIAN S, SHAIK YB, SZMIGIELSKI B, and GENCO CA. Department of Medicine, Section of Infectious Diseases, and Department of Microbiology Boston University School of Medicine, Boston, Massachusetts 02118, USA.

We recently utilized global transcription profile analysis using microarray technologies to study the regulation of gene transcription in response to iron in *Neisseria meningitidis* MC58. These studies have proved to be extremely valuable since they have established that a remarkably large number of genes are activated during growth in high iron. Interestingly, we also observed that a large percentage of these iron-activated genes were regulated by the transcriptional regulatory protein Fur in support of the proposed dual role of the Fur protein as both a negative and positive regulator of gene transcription. This was established by the presence of Fur-binding consensus sequences in the up-stream proximal promoter regions of the genes and by gel-retardation experiments demonstrating Fur binding to PCR-amplified promoter regions. Among the Fur-dependent, up-regulated genes identified by microarray analysis were the *nspA* and *secY* genes, and the divergently transcribed *aniA* and *norB* genes. While the molecular mechanisms of Fur mediated transcriptional repression have been thoroughly investigated, the mechanism of activation by this global regulator has not been well defined. To further our understanding of the mechanism of activation of Fur in this study we mapped the Fur binding sites in the promoter proximal regions of the *N. meningtidis* MC58 iron-activated *nspA* and *secY* genes and in the intergenic region of the divergently transcribed *aniA* and *norB* genes. The *nspA* Fur binding site as determined by foot printing studies overlapped the computer predicted Fur box and spanned from –69 to –49 of the *nspA* operator sequence. While two putative Fur boxes were identified in the promoter regions of the *secY* operon, Neisseria Fur was shown to bind to only the proximal Fur box sequence of the *secY* operon. Mapping of the Fur binding site in the intergenic region of the divergently transcribed *norB* and *aniA* genes revealed a region of protection which spanned from –115 to –153, and which overlapped the computer predicted Fur box. Analysis of the protected regions of the iron-activated genes revealed perfect hexameric repeats surrounded by repeats with varying degrees of homology and was similar to the Fur box sequence present in genes which are repressed by Fur. Thus it appears that Fur binds to a DNA sequence in iron-activated genes which is similar to that of iron-repressed genes. Our results also suggest that *aniA* and *norB* may undergo at least two levels of positive regulation; one mediated by Fur and another involving the positive regulator Fnr. These results provide evidence for the direct involvement of Fur in regulating the expression of the iron-activated *secY*, *nspA*, *aniA*, and *norB* genes of *N. meningitidis* MC58 and enhance our understanding of the complex iron and oxygen dependent regulatory networks of the pathogenic Neisseria.

The *penC* resistance gene arises from a mutation in PilQ that interferes with oligomer assembly and prevents antibiotic influx through the PilQ complex. Shuqing Zhao[*], Deborah M. Tobiason[#], Mei Hu[*], Hank S. Seifert[#], and Robert A. Nicholas[*]. [*]Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, and [#]Department of Microbiology and Immunology, Northwestern University Medical School, Chicago, Illinois, USA.

The *penC* resistance gene was previously characterized as a spontaneous mutation that increased penicillin and tetracycline resistance by 2-fold each in a third-level resistant FA19 strain (PR100) containing the resistance determinants *penA* (mutations in PBP 2), *mtr* (overexpression of an efflux pump), and *penB* (alterations in PIB porin). Moreover, the presence of *penC* in this third level transformant allowed resistance to be increased by a mutated *ponA* gene encoding an altered form of PBP 1. We now show that the *penC* mutation maps to PilQ and results in alteration of Glu-666-->Lys. Western blots of cell lysates from *penC* strains with a PilQ antibody revealed the complete absence of the characteristic high molecular mass PilQ oligomer and increased levels of the PilQ monomer, strongly suggesting that the mutation interferes with formation of the PilQ mulitmeric complex. Moreover, these data indicated that a significant flux of antibiotics occurs through the PilQ complex in PR100. Consistent with this observation, deletion of PilQ confers the same level of antibiotic resistance to PR100 as the *penC* mutation. Introduction of *penC* or deletion of PilQ increased the MICs for penicillin and tetracycline only in strains containing the *penA*, *mtr*, and *penB* resistance determinants, demonstrating that antibiotic entry through the PilQ complex does not become rate limiting unless influx through other routes (i.e. porins) is decreased. Deletion of PilT in the presence of wild type PilQ had no effect on resistance to penicillin, whereas deletion of PilE caused a small but consistent increase in resistance. To understand the nature of the *penC* mutation, the codon encoding Glu-666 was randomized and mutants conferring increased penicillin resistance were selected by transformation. Multiple uncharged hydrophobic amino acids at this position conferred increased antibiotic resistance by interfering with PilQ oligomerization. Early studies with PilQ demonstrated that the oligomeric PilQ complex could be dissociated into monomers on SDS-PAGE following reduction and alkylation, which suggested the presence of inter-subunit disulfide linkages. Inspection of the primary sequence of FA19 PilQ identified two cysteine residues, both of which were located within 25 amino acids of the site of the *penC* mutation. Mutation of either of these cysteine residues and transformation into PR100 gave rise to an identical phenotype as *penC*, i.e. increased resistance to penicillin and tetracycline and complete loss of the high molecular mass PilQ complex. In summary, our data indicate that the *penC* mutation increases resistance by preventing the formation of the PilQ complex, thereby decreasing the influx of antibiotics into the periplasmic space of the bacteria. Moreover, our mutational data are consistent with the hypothesis that the *penC* mutation exerts its effect by preventing the formation of inter-subunit disulfide bonds that are critical in PilQ oligimerization.

Rifampin resistance in *Neisseria meningitidis*.

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Introduction: Rifampin resistance in *Neisseria meningitidis* is caused by single point mutations in *rpoB,* coding for the second largest subunit of RNA polymerase. Besides this well characterised resistance mechanism alternative mechanisms have been discussed. Transfer of the resistance mechanism from a resistant DNA donor to a susceptible recipient would allow examining the role of the *rpoB* mutations in high level rifampin resistance in more detail. For this purpose we have developed a system allowing transformation of *Neisseria* species with PCR amplified *rpoB* fragments of resistant donors. Further work was spent in generating spontaneous mutants from either susceptible meningococci or commensal *Neisseria* species. The results allowed judging the role of *rpoB* mutants in high level rifampin resistance.
Methods: PCR amplified subgenic DNA fragments of *rpoB* were used to transform meningococci and different commensal *Neisseria* species. Different transformation protocols (regarding the use of detergents or specific nutrients/ions) were tested for efficacy. Transformation was carried out using meningococci as donor and commensal neisserial species as recipient and vice versa.
Results: A 1352 bp *rpoB* fragment harbouring one of the rifampin resistance conferring mutations and the 10bp uptake sequence was used to transform a susceptible meningococcal strain. Transformants were identified unambiguously by private, silent mutations which were present in the donor fragment but not at the homologous site of the recipient. Regardless of the concentration of rifampin in the agar slants used (8 µg/mL, 32 µg/mL, and 256 µg/mL) transformants were found. The PCR fragment was transformed into different commensal *Neisseria* species, too. A high number of silent mutations present at the homologous site of the commensal species allowed the observation that the entire 1352 bp subgenic fragment was not always adopted completely, although the resistance conferring mutation and surrounding DNA sequences were integrated into the genome of the recipient. Rather, partial integration was observed. Following generation of spontaneous mutants from commensal susceptible *Neisseriae*, a resistance conferring 9 bp deletion was found. A PCR fragment harbouring the site of deletion was successfully transformed in a susceptible meningococcal strain, leading to high level resistance in the recipient.
Discussion: Our results demonstrate that high level rifampin resistance of meningococci can be caused by mutations in *rpoB* alone. The transferability from meningococci into commensal *Neisseria* species without the need of cloning provides a safe and effective tool for studying the resistance mechanism in more detail. This may also be true for examination of resistance mechanisms other than that for rifampin.

Session IV. Cellular Microbiology

Gonococcal PLD and Signaling Events Triggered by *Neisseria gonorrhoeae* Infection of Primary Cervical Epithelial Cells

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Complement Receptor 3 (CR3)-mediated endocytosis is a primary mechanism by which *Neisseria gonorrhoeae* elicits membrane ruffling and invades primary cervical epithelium. Gonococci specifically release proteins, including a phospholipase D (NgPLD), upon infection of cervical epithelia that facilitate membrane ruffling. NgPLD augments infection by potentiating cytoskeletal reorganization and recruiting CR3 to the cervical epithelial cell surface. To elucidate the signaling pathways triggered with gonococcal CR3 engagement and the putative function of NgPLD in these events, we performed comparative association and invasion assays in the presence and absence of signal transduction inhibitors. Similar to what is observed in the absence of NgPLD, decreased CR3 recruitment to the cervical cell surface and gonococcal association and invasion of these cells occurs with tyrosine kinase and protein kinase C (PKC) inhibition. Tyrosine kinase activation with pervanadate rescued CR3 recruitment to the cervical cell surface and gonococcal association with these cells but was insufficient to rescue intracellular survival of PLD mutant gonococci. Phorbol ester-induced PKC activation rescued cell surface CR3 recruitment and the association and intracellular survival of mutant gonococci. This indicated that NgPLD plays a role upstream of tyrosine kinase activation in modulating signal transduction events leading to CR3 recruitment to the cervical cell surface and also participates in signaling events downstream of tyrosine kinase activation but upstream of PKC, which are required for intracellular gonococcal survival. p21-activating kinase (PAK) is activated with cervical infection, which is consistent with gonococci-induced vinculin focal complex formation and small G-protein activation. Western blotting using phospho-PAK-specific antibodies revealed that PAK activation kinetically coincides with the onset of gonococcal-induced membrane ruffling, suggesting PAK involvement in these processes. The ability of PAK to activate small G-proteins suggests that this molecule could play a role in Rho-mediated PIP formation. PIP phosphorylation forms PIP2, which, in turn, is phosphorylated to form PIP3. Downstream effector molecules of PIP3 include some PKCs and Akt kinase. Akt is implicated in membrane ruffling and anti-apoptotic events in other cell systems. Association and invasion assays demonstrate that PIP2 and phosphatidic acid are critical mediators of cervical infection, whereas diacylglycerol is not. PIP3 itself is not required for primary cervical cell invasion; however, Akt appears to be crucial to gonococcal invasion. We demonstrate by Western Blot and immunoprecipitation studies that NgPLD directly associates with Akt during cervical infection. This might allow the gonococcus to bypass the transformation of PIP2 to PIP3 and in this manner augment membrane ruffling and promote intracellular gonococcal survival. Collectively, our data indicate that both gonococcal and human constituents contribute to cervical signaling cascades initiated with, and required for, the successful infection of primary cervical epithelia by gonococci.

Interaction of *Neisseria meningitidis* with Human Brain Microvascular Endothelial Cells: Role of MAP- and Tyrosine Kinases in Invasion and Inflammatory Cytokine Release

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Abstract for oral presentation:

Neisseria meningitidis traversal across the blood-cerebrospinal fluid (B-CSF) barrier is an essential step in the pathogenesis of bacterial meningitis. This step is accompanied by increased production of inflammatory cytokines, including interleukin 6 (IL-6), IL-8 and tumor necrosis factor alpha (TNF-alpha). We have previously shown that invasion of cerebral microvascular endothelial cells (HBMEC) by meningococci is mediated by bacterial outer membrane protein Opc that binds fibronectin, thereby anchoring the bacterium to the integrin alpha 5 beta 1 on the endothelial cell surface. However, subsequent signal transduction mechanisms essential for or regulated by *N. meningitidis* adhesion and invasion, or HBMEC responses to *N. meningitidis* are unknown.

In this report we investigated the role of c-Jun-N-terminal kinases 1 and 2 (JNK1 and JNK2), p38 mitogen activated (MAP) kinase and protein tyrosine kinases in endothelial - *N. meningitidis* interaction. Binding of meningococci to HBMEC phosphorylates and activates JNK1 and JNK2 and p38 MAPK as well as their direct substrates c-Jun and MAP kinase activated kinase-2 (MAPKAP-2), respectively. In contrast, no activation of p42/44 MAP kinases was detected. Non-invasive meningococcal strains lacking *opc* gene (*opc* mutants and ST-11 complex meningococci) still activate p38 MAPK, however, fail to activate JNK. Inhibition of JNK1 and JNK2 using the anthrapyrazolone SP600125 significantly reduced internalization of *N. meningitidis* by HBMEC without affecting its adherence. Blocking the endothelial integrin alpha 5 beta 1 also prevents *N. meningitidis*-induced JNK activation in HBMEC. These findings indicate the crucial role of JNK signaling pathway in *N. meningitidis* invasion in HBMEC. In contrast, p38 MAPK pathway was important for the control of IL-6 and IL-8 release by HBMEC: specific p38 MAPK inhibitors SB 203580 and SB 202190 reduced IL-6 and IL-8 release by about 50% and 64%, respectively. Genistein, a protein tyrosine kinase inhibitor, decreased both invasion of *N. meningitidis* into HBMEC and IL-6/IL-8 release, indicating that protein tyrosine kinases, which link signals from integrins to intracellular signaling pathways are essential for both bacterial internalization and cytokine secretion by HBMEC. Uncovering the molecular mechanism of *N. meningitidis*-endothelial interaction is the first step in understanding the pathophysiologic mechanisms that lead to bacterial B-CSF barrier invasion.

Pilin antigenic variation during gonococcal infection of model polarized epithelia.
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Neisseria gonorrhoeae (Gc) has a remarkable capacity to vary the expression and composition of its surface-exposed structures, which is thought to contribute to successful immune evasion by this pathogen. Changes in pilin, the major subunit of type IV pili, arise from the DNA recombination-based process termed antigenic variation (Av). Pilin Av is a high frequency event, occurring in vitro at approximately 10^{-2} per colony-forming unit.[1] Colonies isolated from experimentally infected human volunteers and from the basolateral medium of cultured epithelial cells have changed pilin sequence from the starting inoculum, indicative of Av having occurred, but it is not known if this is reflective of an increased frequency of Av or selection for nonparental pilin sequences.[2-4] Interestingly, the frequency of Av is stimulated following iron limitation[5], a situation Gc might encounter when inside host cells. Given this background, we have begun investigating the interactions of Gc with polarized T84 cells, a model for the mucosal epithelium, to address the following questions: 1) Does Av occur in Gc inside epithelial cells? 2) During intracellular existence, is there selection for a particular piliation phenotype in Gc? 3) If there is no selection, is the frequency of Av upregulated for Gc inside cells?

Our results demonstrate that Opa-expressing Gc are internalized at a low frequency by T84 cells independent of their piliation status and that intracellular bacterial growth, as assessed by recoverable colony-forming units, is delayed for several hours post-infection. In line with previous reports, Gc begin to exit into the basal medium by 24 hours post-infection, but surprisingly Gc exit at similar levels into the apical medium, demonstrating that egress from polarized T84 cells is non-directional. In contrast to previous observations[4], we observe no selection for or against piliation in these exited Gc; individual epithelial monolayers yield bacterial populations that are predominantly piliated, predominantly nonpiliated, or a mix of the two. Nonparental pilin sequences are detected in both internal and exited Gc (of either piliated or nonpiliated morphology), indicating that Av occurs during epithelial cell contact. Sequence analysis reveals that these changes in pilin are similar to those that arise for Gc cultured in vitro. However, growth inside cells slightly favors non- or underpiliated Gc, which arise through both pilin Av and PilC phase variation. Together, these results provide a complete profile of Gc growth and exit from the T84 model for mature polarized epithelia, and question whether transcytosis is the principal outcome of internalization by these cells. We conclude that if increased pilin Av is found within infected individuals, this process is not specifically influenced by epithelial cell interactions.

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Bacterial Adhesion Under Flow - a real time adhesion assay

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Adhesion of *Neisseria meningitidis* to human cells is a key step in the life cycle of this organism by allowing colonization of the nasopharynx. Bacterial adhesion is also thought to be a prerequisite for crossing cellular barriers such as the nasopharyngeal epithelium and the blood brain barrier, two central events in neisserial infections. Experimentally, bacterial adhesion is usually modeled in the laboratory by incubating bacteria with cells in culture. *In vivo*, however, bacterial adhesion does not occur in static conditions as those generally used but under liquid fluxes. In the airway system, mucus velocity is 0.1 mm/s and punctually reaches higher values upon coughing. In the blood phase of the infection, meningococci adhere to endothelial cells under blood flow (velocity 0 to 300 mm/s).
We subsequently set up an experimental system allowing the study of bacterial adhesion under flow. Adherent endothelial or epithelial cells are placed in a flow chamber and bacteria expressing green fluorescent protein (GFP) are introduced onto the cells under a controlled flow. Digital time-lapse video microscopy is used to monitor fluorescent bacteria. An image analysis software was developed for the purpose of monitoring bacteria movement on the surface of cells. Several parameters such as bacterial attachment, detachment or movement can be measured automatically in real time.
The piliated, serogroup C, 2C4.3 strain was used as a reference strain and different mutants of this strain were tested. We first determined the flux under which bacteria can bind to human cells. Consistently with fluxes found in the nasopharynx or in small capillaries, binding efficiently occurs in fluxes up to 0.5 mm/s; higher fluxes lead to a sharp decrease in the binding ability of the 2C4.3 strain. In capsulated bacteria, type IV pilus was an absolute requirement for adhesion under flow, as non-piliated or *pilC1* mutants did not bind. The flux tolerated before detachment of adhering bacteria was also studied. We found that extreme fluxes resembling those found in large arteries or during coughing are not sufficient to detach wild-type bacteria.
Surprisingly, in the absence of capsule, pilus is no longer necessary for adhesion under flow. In these conditions, non-capsulated and non-piliated bacteria (Caps-/P-) are able to bind to cells even more efficiently than their wild-type counterpart. When higher fluxes are applied to adhering bacteria, however, Caps-/P- bacteria detach unlike the isogenic piliated bacteria. Interestingly, before detaching Caps-/P- bacteria slowly move on the apical surface of the cells presumably by a rolling mechanism. These results demonstrate that neisserial type IV pilus is necessary to maintain efficient attachment in conditions of physical stress. Pili independent adhesion, on the other hand may provide a means to colonize new sites in the epithelium through a rolling mechanism.

Modulation of host cell survival by PorB of *Neisseria gonorrhoeae*

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The obligate human pathogen *N. gonorrhoeae* is the causative agent of the venereal disease gonorrhoea. Gonococci bind to epithelial cells of the urogenital tract via pili and/or engage receptors of the CEACAM-family, heparansulfate proteoglycans or integrins by so-called opacity-associated outer membrane proteins (Opa). Infection with gonococci is accompanied by a massive inflammation and destruction of the tissue. Although in most cases the infection remains locally restricted to the urogenital tract in about 1 % of the cases gonococci invade the blood stream and cause severe bacteraemia.

The neisserial PorB porin has only recently been recognized as factor, which might directly influence the pathogenicity process. We have demonstrated that PorB is required for efficient invasion of epithelial cells via heparansulfate proteogylcans [1]. Van Putten and colleagues showed [2] that the PI.A serotype of PorB mediated invasion under low phosphate conditions in the absence of other invasins like the Opa proteins. Another interesting feature of PorB is its ability to spontaneously translocate as purified protein [3] or from bacterial membranes into mammalian membranes [4]. Translocated porin targets mitochondria by a thus far unknown pathway [4;5].

We have investigated, how different infection conditions affect the survival of host cells. Interestingly, only bacteria interacting via pili or Opa-proteins, but not P.IA, induce apoptosis in epithelial cells. We investigated the signaling pathway activated by P.IA and show that it differs from all pathways engaged by Opa-proteins. Moreover, the narrow host specificity of Neisseria for human cells is not observed for P.IA-mediated invasion since a broad range of cells derived from different species are invaded via this pathway. We currently investigate on the molecular level, why apoptosis induction depends on the interaction with the host.

An exciting feature of PorB expressed in host cells is the efficient translocation to mitochondria, and the induction of mitochondrial permeability transition and mitochondrial swelling in the absence of significant cytochrome c release. Using genetic tools (knock out lines, knock down techniques) we delineated the pathway of transfected PorB to the mitochondria and the damage induced at the mitochondria. Our data suggest that translocation of PorB into mitochondria occurs via the mitochondrial import pathway. However, loss of function analysis in epithelial cells showed that translocation of PorB does not ultimately result in permeability transition but may require the activation of host cell apoptosis effectors. Possible pathways leading to apoptosis induction by PorB will be discussed.

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MODULATION OF HOST CELL APOPTOSIS BY MENINGOCOCCAL SECRETED PROTEINS

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A number of bacterial pathogens have been shown to induce apoptosis in target cells of the host organism: a phenomenon regarded as a component of the pathology of certain bacterial diseases. As well as inducing apoptosis, interaction with certain pathogens or their products may in some cases inhibit cellular apoptosis. We have previously shown that infection of meningioma-derived arachnoid epithelial (meningothelial) cells with *N. meningitidis* results in changes in expression levels of a number of genes with a role in apoptosis. We observed a trend in which pro-apoptotic genes tended to be down-regulated while anti-apoptotic genes were up-regulated. We demonstrated that, at a phenotypic level, cells pre-treated with meningococci were protected from staurosporine-induced apoptosis. Similar results have been observed by others in HUVEC cells infected with gonococci. The outer membrane porin PorB of both *N. meningitidis* and *N. gonorrhoea* has been shown to localise to the outer membrane of host cell mitochondria and to modulate apoptosis. Interestingly, both induction and protection against apoptosis by neisserial PorB has been reported.

Neisseria meningitidis secretes a large number of proteins (meningococcal secreted proteins, MSPs) which are likely to play key roles in the pathogenesis of disease. However, the role of MSPs in meningococcal pathogenesis is poorly understood. Here, we examined the influence of MSPs on gene expression in meningothelial cells and compared these effects with those observed in cells exposed to live meningococci. Meningothelial cells were exposed to either live meningococci (strain MC58) or their endotoxin-depleted MSPs. Total RNA extracted from cell monolayers was used as a template for synthesis of radiolabelled cDNA, which was then used to probe Human Cytokine Expression and Apoptosis Arrays (R&D Systems). Membranes were subjected to autoradiography and spots were analysed using transmission densitometry and NIH ImageJ Software. The ratios for potentially differentially regulated genes were calculated after correction for probe variation using constitutively expressed genes on equivalent arrays. Eleven anti-apoptotic genes and five pro-apoptotic genes were significantly up-regulated in MSP-treated cells including FLIP, IAP-1, XIAP and PARP. Pre-treatement of meningothelial cells with MSPs also afforded protection against chemically induced apoptosis. The observed effects are not thought to be due to contamination of MSPs with PorB as we were unable to detect PorB in MSP preparations. We are currently continuing these studies with 30,000-gene microarrays in order to obtain a more complete picture of the changes in apoptosis-related gene expression in response to MSPs and attempting to determine which components of the MSP preparations are responsible for the observed effects.

This is the first report describing a role for MSPs in modulation of host cell apoptosis and suggests that MSPs may play an important role in meningococcal pathogenesis.

ROLE OF HOST CELL RECEPTORS IN THE INFLAMMATORY RESPONSE OF HUMAN MENINGEAL CELLS ON INTERACTION WITH *NEISSERIA MENINGITIDIS* AND OTHER BACTERIA CAUSING MENINGITIS.

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Introduction: The nature and specificity of the interactions between *Neisseria meningitidis* and cells of the human meninges has been investigated *in vitro* using a model based on the culture of cells from benign tumours (meningiomas) of the meninges. This model identified several of the major meningococcal surface ligands that mediated interactions with meningeal cells and also demonstrated that cells of the leptomeninges are likely to be active participants in the innate host response during meningitis. In the present study, we have extended the use of the meningioma cell model to i) compare and contrast the biological effects induced by *N. meningitidis* with the interactions of several other important bacteria causing pyogenic and neonatal meningitis; and ii) investigate the activation of meningeal cells by LPS and non-LPS components of meningococci and the role of host cell receptors in recognition of bacterial ligands.

Results: There were significant differences between the meningeal pathogens in the dynamics of their interactions. *N.meningitidis* and *H.influenzae* did not invade meningioma cells or induce cell death, but induced a concentration-dependent secretion of inflammatory mediators. *S.pneumoniae* was also unable to invade meningioma cells, low concentrations of bacteria failed to stimulate cytokine secretion, but higher concentrations of pneumococci led to cell death. By contrast, only *E. coli* K1 invaded meningioma cells directly and induced rapid cell death before an inflammatory response could be induced.

These studies demonstrated a complex relationship between expression of meningococcal components and cytokine induction. LPS-deficient bacteria and isolated OM induced significant cytokine secretion by meningeal cells, compared with wild-type (LPS^+) bacteria and OM. By contrast to *E. coli* LPS, recognition of LPS-replete meningococcal OM did not involve TLR4, as determined by RT-PCR on meningeal cells and the use of reporter Chinese Hamster Ovary (CHO) cell lines expressing CD14 and TLR4. In addition, both the LPS-replete and deficient meningococcal OM preparations did not up-regulate expression of TLR2 on meningeal cells. Moreover, fluorescence resonance energy transfer (FRET) and fluorescence recovery after photo-bleaching (FRAP) techniques demonstrated that other known LPS-associated receptor proteins (HSP70, HSP90, CXCR4 and GDF5) did not associate with TLR4 and TLR2 receptors in response to either OM preparation.

Conclusions: Our data demonstrate that the interactions of different bacterial pathogens with human meningeal cells are distinct and that in the case of meningococci, recognition of meningococcal LPS and other OM components occurs independently of TLR and other LPS-associated proteins. Taken together, these data suggest that different intervention strategies may be needed in order to prevent the morbidity and mortality associated with meningitis caused by different bacteria and their components.

Caveolae are involved in *Neisseria*-host cell interaction

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An initial step of *Neisseria gonorrhoeae* (GC) infection, localized adherence, is mediated by type IV pili. Caveolae are plasmalemmal invaginations implicated in signal transduction and vesicular transport. Here we report that infection with GC induces recruitment of caveolae markers to bacterial microcolony. Cholesterol depletion and perturbation of caveolae stability, abolishing caveolin recruitment, and down regulation of caveolin expression prevent the microcolony formation on the surface of host cells and induce bacterial invasion. The latter event depends on Src kinase activation. Thus, caveolae are involved in regulation of initial attachment of GC to the host cell and impede early bacterial entry.

Session V. Bacterial Genetics, Physiology and Metaboblism

Action of a sequence in the *pilE* upstream regions followed by branch migration of Holiday Junctions is essential for gonococcal pilin antigenic variation.

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The lack of immunity, even after numerous gonococcal infections, remains one of the hallmarks of gonorrhea. Antigenic variation of the pilus is one of the reasons immunity does not develop and this is mediated by a high-frequency gene conversion reactions, which utilize the homologous recombination machinery of the bacterium, between *pilS* storage copies and the *pilE* locus.

We developed a semi-quantitative RT-PCR assay to measure the contribution of all the hypervariable regions of the 18 silent pilin copies of strain FA1090 to changes at *pilE*. We found that a non-uniform distribution of silent copies contributes to pilin variation and that the particular spectrum of silent copies used for pilin variation depends on the starting *pilE* sequence. These results show that the starting *pilE* sequence influences the repertoire of variant pilins produced in a strain during infection.

We have also conducted a genetic screen, based on a loss of pilus-dependent colony variation, to isolate genes required for pilin variation. 13 genes were found, that when inactivated by *in vitro* transposition and transformation, significantly reduced the frequency of pilin variation. Several of these genes were previously identified as members of the RecF-like pathway of homologous recombination including *recO, recQ* and *recJ.* The screen revealed *recR* as an additional member of the RecF-like pathway. Two transposons that completely inhibited pilin variation were found upstream of the *pilE* promoter. Further transposon mutagenesis of this region of the chromosome and deletions within this region defined a ~100 bp sequence that is essential for pilin variation. Furthermore, a transposon insertion into either the *ruvA* or *recG* gene each disrupted pilin variation and also produced DNA repair and DNA transformation phenotypes. Both RecG and RuvA are involved in the branch migration of Holiday Junctions (HJs), but are involved in separate pathways of HJ processing. Inactivation of *ruvB* and *ruvC* produced strains with phenotypes similar to the *ruvA* mutant. Double mutants were made between *recG* and *ruvA, ruvB,* or *ruvC,* in a strain where *recA* expression is regulated by IPTG. Induction of RecA in any of the HJ-processing double-mutant strains resulted in a severe growth defect. Bacteria surviving IPTG induction were predominately nonpiliated, which was due to the selection for *pilE* deletions. Introduction of a *recO* mutation, or one of the transposon insertions in the upstream regions of *pilE* that block antigenic variation, prevented the RecA-dependent lethality observed in the HJ-processing double-mutant. This result shows that RecO and the upstream sequence act before the HJ processing machinery. We conclude that branch migration of HJs, by both the RecG and RuvABC pathways, is required for antigenic variation, but when both HJ processing pathways are inactivated, that a recombination intermediate is formed that is problematic for gonococcal growth.

Type IV pilus biogenesis in *Neissseria meningitidis*. PilW is part of a multi-protein complex in the outer-membrane that stabilizes the fibers and modulates their functionality.

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Type IV pili (Tfp) play a critical role in the pathogenic lifestyle of *N. meningitidis* and *N. gonorrhoeae*, notably by facilitating bacterial attachment to human cells. However, our understanding of Tfp biogenesis, during which the fibers are formed in the periplasm, then stabilized and finally emerge onto the cell surface, remains fragmentary. We therefore identified all the genes required for Tfp formation in *N. meningitidis* by screening a genome-wide collection of mutants for those that were unable to form aggregates, another phenotype mediated by these organelles. Fifteen proteins, of which only 7 were previously characterized, were found to be essential for Tfp biogenesis. One novel component, named PilW, was studied in more detail and was found to present features similar to the extensively studied PilC. PilW is an outer-membrane protein necessary for the stabilization of the fibers since Tfp could be restored in a *pilW* mutant by a mutation in the twitching motility gene *pilT*. However, Tfp-linked properties such as adherence to human cells were not restored in a *pilW/T* mutant, which suggests that PilW also participates in the functional maturation of the fibers. Together with the finding that PilW stabilizes the PilQ multimers, which suggests that these two proteins interact, our results extend the current model for Tfp formation by indicating that a multi-protein complex in the outer-membrane, probably centered around PilQ and comprising at least PilC and PilW, is involved in the terminal stage of Tfp biogenesis during which growing fibers are not only stabilized, as previously thought, but also maturated, which is of paramount importance for their functionality.

Evidence for cooperative interactions between gonococcal transferrin binding proteins

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Neisseria gonorrhoeae has the capacity to acquire iron from the human glycoprotein transferrin, via surface expression of a complex receptor comprised of two dissimilar proteins, TbpA and TbpB. TbpA is an integral outer membrane protein that belongs to the family of TonB-dependent transporters. This group also includes the siderophore and vitamin B12 receptors expressed by *E. coli* and other enterics. TbpB is believed to be largely surface exposed outside the outer leaflet of the outer membrane, to which it is tethered by a lipid modification on the amino terminus. Various analyses have suggested that TbpA and TbpB interact with one another in the outer membrane and in doing so effect the surface exposure and binding characteristics of each other. We utilized immunoprecipitation to demonstrate a stable association between TbpA and TbpB, without the addition of exogenous cross-linkers. Gonococcal TonB is also detectable in these immunoprecipitatable complexes. We generated several isogenic mutants of *N. gonorrhoeae* that no longer express components of this TonB-energized receptor complex. We analyzed the rate at which holo-transferrin associates with the wild-type and mutant receptors and also characterized the rates at which transferrin dissociates from these receptors. The results of these analyses indicate that both TbpB and TonB-derived energy contribute to the rates of transferrin association with and dissociation from the gonococcal cell surface. This implies that the mechanism by which TbpB facilitates transferrin-iron acquisition is both by increasing association of ligand with the receptor and by promoting release of ligand from the receptor, presumably after transferrin has been relieved of its iron, although this latter point has not yet been formally tested. In addition to these studies, we have constructed epitope-tagged forms of TbpA, with which we evaluated surface exposure of particular TbpA epitopes. Of the 12 mutants constructed, seven were incapable of transferrin-iron utilization. However, the growth defects of three of these mutants were overcome by the simultaneous expression of a wild-type TbpB, indicating that the wild-type TbpB is capable of compensating for deficiencies imposed by the TbpA insertion mutation. Interestingly, we isolated revertants of two other epitope-insertion mutants after extended growth on plates containing transferrin as a sole iron source. By PCR analysis, we confirmed that the HA insertion was retained in its original position in the revertant. Furthermore, these revertants could only be isolated in a strain that expressed TbpB. We are currently characterizing the genetic basis for this reversion event. Overall, the results indicate that TbpA and TbpB form a complex in which TbpB contributes to efficient association and rapid dissociation of ligand. Moreover, functions eliminated by in vitro mutagenesis of TbpA can be compensated for by the presence of TbpB.

Role of the lactate permease in the pathogenesis of meningococcal infection and as a potential vaccine candidate EXLEY RM[1], SHAW J[2], READ RC[2], GOODWIN L[2], SUN Y-H[1], LI Y[1], SMITH H[3], TANG CM[1]. [1]The Centre for Molecular Microbiology and Infection, Flowers Building, Imperial College London, Armstrong Road, London, SW7 2AZ, U.K. [2]Division of Genomic Medicine, F-floor, University of Sheffield Medical School, *Beech Hill Road, Sheffield, S10 2RX, U.K.* [3]The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

To cause disease, *Neisseria meningitidis* must acquire essential nutrients to multiply in the systemic circulation, while avoiding exclusion by host innate immunity. We have found that the utilisation of carbon sources by *N. meningitidis* determines its ability to withstand complement-mediated lysis. The gene encoding the lactate permease, *lctP*, was identified and disrupted, resulting in a strain with reduced growth rate in cerebrospinal fluid and impaired capacity to colonise human nasopharyngeal tissue compared with the wild-type. The *lctP* mutant was also attenuated during bloodstream infection. However, this was not due to an effect on growth, but resulted from loss of resistance to complement-mediated killing; the link between lactate and complement was demonstrated by the restoration of virulence of the *lctP* mutant in complement deficient ($C3^{-/-}$) animals. The underlying reason for attenuation is the reduction in sialylation of lipopolysaccharide when lactate is unavailable. We show that lactate influences LPS sialylation through a direct link between intermediary metabolism and sialic acid biosynthesis in the bacterium, demonstrating the essential relationship between carbon source utilisation and resistance against innate immunity in *N. meningitidis*.

The ability of recombinant LctP to protect against challenge with live *N. meningitidis* was established. Mice immunised with LctP were significant protected against systemic infection compared with control animals receiving adjuvant alone. Therefore, LctP and other proteins involved in metabolic functions may prove to be successful vaccines for preventing meningococcal infection.

Genomic analysis of the c-type cytochromes of Neisseria gonorrhoeae: mutational and biochemical analysis of cytochrome *c'*, a nitric oxide-binding lipoprotein important for adaptation to micro-aerobic growth and its implications for pathogenicity

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Neisseria gonorrhoeae was previously reported to be a prolific source of *c*-type cytochromes. Six of the seven *c*-type cytochromes predicted from *in silico* analysis of the gonococcal genome are expressed during aerobic growth. The seventh, a cytochrome *c* peroxidase (CCP), was previously shown to be a lipoprotein that is expressed during oxygen-limited growth. Five of the constitutively expressed cytochromes are predicted to be components of the cytochrome bc_1 complex, cytochrome *c* oxidase cbb_3, or periplasmic cytochromes involved in electron transfer reactions typical of a bacterium with a micro-aerobic physiology. The final *c*-type cytochrome, CycP, like CCP, includes a lipobox required for targeting it to the outer membrane. Mutagenesis of the gonococcal *cycP* gene results in an extended lag phase during micro-aerobic growth in the presence, but not in the absence, of nitrite, suggesting that nitric oxide generated from nitrite reduction during adaptation to oxygen-limited growth is toxic to the *cycP* mutant. The *cycP* gene was over-expressed in *Escherichia coli*. The recombinant CycP was targeted to the *E. coli* outer membrane, and binds NO *in vitro*. The absorption spectra of gonococcal cytochrome *c′* were similar to those of other previously characterised cytochrome *c′* proteins. Spectroscopic evidence will be presented that it binds NO, and its NO binding properties are more closely related to those of denitrifying bacteria than to those of photosynthetic bacteria.

The demonstration that two of the seven gonococcal *c*-type cytochromes fulfil specialised functions and are outer membrane lipoproteins suggests that in both Gram-positive and Gram-negative bacteria, the localisation of lipoproteins close to the bacterial surface provides effective protection against external assaults from reactive oxygen and reactive nitrogen species. We further propose that this might be a far more general aspect of microbial physiology rather than a specialised adaptation of pathogens.

A structural analysis of the DNA-binding regulatory proteins of the pathogenic *Neisseria*.

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Introduction. The regulatory systems of the *Neisseria* species display many interesting features and raise important questions. Their limited range of stress responses, the differences in IHF relationships with growth curves, the restricted set of sigma factors, the divergence of regulators from those of other species, a relatively small number of regulators and a high number of phase variable genes, and questions over which systems are intact and which are not, all indicate the need for detailed assessment of these regulatory systems. We are therefore pursing a protein expression and structurally based study of the complete repertoire of DNA binding proteins in both *N. meningitidis* (NME) and *N. gonorrhoeae* (NGO), with a view to defining the structure-function relationships of these proteins, and to provide key reagents for experimental studies of regulation.

Candidate gene selection. The complete genome sequences of all 4 available *Neisseria* were assessed for homologues of DNA binding proteins with potential regulatory functions. Representative orthologues of each regulator in each genome were identified with the genes selected in order of preference from NME strain MC58, NGO strain FA1090, NME strain Z2491, and NME strain FAM18. A total of 68 proteins were selected for cloning into the expression vector systems.

Cloning and expression strategy. Candidate genes were amplified from genomic templates by PCR and inserted in parallel into T7 promoter based expression plasmids (pDEST17, pET15G and pDEST14) using Gateway™ ligation independent cloning. A N-terminal hexahistidine tag was added to each sequence for purification. Constructs were screened for expression in *E. coli* BL21(DE3) strains (Rosetta pLysS and B834) using NiNTA magnetic beads to harvest soluble proteins in a 96-well format. Expression was analysed by SDS-PAGE and showed that 51/62 cloned genes were expressed, of which 39 showed some soluble product. Selected proteins were also cloned and expressed into pNSX4 and used for protein preparation.

Purification and crystallisation strategy

Prioritised on the basis of expression characteristics, proteins (both native and selenomethionine labelled) were purified by a combination of NiNTA affinity chromatography and gel filtration. For crystallisation trials, proteins were set up in 200nl (100nl protein @ >/=10mg/ml + 100nl precipitant) sitting drops in 96 well plates and incubated at both 21 ^{0}C and 4 ^{0}C.

Progress.

To date, 5 of the first 13 proteins that have entered the crystallisation screening process have been crystallised. Diffraction datasets have been obtained from 2 of these (NMB1995 and NMB0736) and the structures solved by molecular replacement. The structures of the proteins will be presented and discussed, together with strategies for further exploitation of these proteins.

Lytic transglycosylases act in the release of proinflammatory molecules in *Neisseria gonorrhoeae*

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Peptidoglycan (PG) fragments are released by *Neisseria gonorrhoeae* and cause the death of ciliated fallopian tube cells in the organ culture model of pelvic inflammatory disease. The most abundant PG fragments released are two 1,6-anhydro PG monomers. The 1,6-anhydro disaccharide tetrapeptide is identical to the tracheal cytotoxin elaborated by *Bordetella pertussis*. The 1,6-anhydro disaccharide tripeptide is identical to the PG monomer shown to activate the inflammatory response during *Shigella flexneri* infections. Despite their importance in the pathogenesis of these diseases, the genes for cytotoxin production have not been characterized in any of the pathogens that produce them.

The anhydro bond on the released PG fragments suggested that the toxins are produced by lytic transglycosylases. Therefore we mutated each of six lytic transglycosylase genes in the gonococcal chromosome. The mutants were characterized for PG fragment release, extent of autolysis, and the cell morphology and growth rate. We have previously shown that a mutation in *ltgA* caused a reduction in PG monomer release by 40%, and that the mutant released more multimeric PG fragments. The mutant was not affected in growth, except for showing increased survival in late stationary phase culture. Mutations in *ltgB* gave only slight differences in these assays. However, mutation of *ltgD* reduced fragment release and shifted the size of released fragments to larger multimers. An *ltgABD* triple mutant showed no release of PG monomers and was not reduced in growth. However, mutation of *ltgC* inhibited cell division and separation, causing the mutants to grow in groups as large as 10 cells. The growth of the *ltgC* strain was severely impaired, and the bacteria were more prone to cell lysis. Mutation of *atlA* or *ltgX*, the lytic transglycosylase genes found in the gonococcal genetic island, did not lower PG fragment release. However, *atlA* mutations resulted in a loss of type IV secretion of DNA. Secreted DNA may be pro-inflammatory since purified gonococcal DNA was found to signal through TLR9.

These results indicate that LtgA and LtgD are primarily responsible for toxic PG fragment production, but are not required for growth. The question remains as to why gonococci release PG fragments, whereas most gram-negative bacteria efficiently recycle them. The identification of LtgA and LtgD as crucial enzymes will allow us to determine other interacting proteins involved in PG fragment production and characterize the control of their production or localization. The *ltgABD* mutant is being used in cell culture infections in order to judge the role of released PG fragments in cytokine induction. The finding that LtgC is required for normal growth and division shows that the mechanisms of gonococcal cell division differ from those of *E. coli* and suggests that this lytic transglycosylase may be a target for antimicrobial therapy.

Session VI. Epidemiology

Serogroup W-135 Meningococcal Disease in Burkina Faso, 2002 and 2003.

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Background: *Neisseria meningitidis* (Nm) causes large epidemics in African "meningitis belt" countries. Historically, these epidemics have been caused by serogroup NmA and occasionally by serogroup NmC. Bivalent (A/C) meningococcal polysaccharide vaccine is used for epidemic control through district level reactive mass vaccination campaigns, once the WHO-defined epidemic threshold (10 cases/100,000 population) has been surpassed. In 2000, the first NmW-135 epidemic occurred in Saudi Arabia among Hajjis. In 2001, NmW-135 cases occurred in Burkina Faso and Niger prompting enhanced surveillance and laboratory confirmation of meningitis epidemics during 2002.

Methods: We conducted prospective surveillance and laboratory confirmation of suspected meningitis cases in Burkina Faso during the 2002 and 2003 epidemic seasons. Based on the WHO case definition, a suspected case was defined as a person with sudden onset of fever with neck stiffness, headache, purpura or a bulging fontanel; a confirmed case had Nm detected by latex agglutination or isolated from cerebrospinal fluid (CSF). CSF specimens were collected from suspected meningitis cases, inoculated on trans-isolate (T-I) media and transported to national reference laboratories within 2-5 days for culture and serogrouping.

Results: From 1/1-5/19/2002, 12,790 suspected meningitis cases and 1,469 deaths (case fatality ratio [CFR] 11.5%) were reported in Burkina Faso. The overall attack rate was 108/100,000 population and 30 (56%) districts surpassed the epidemic threshold. A pathogen was cultured from 205 of 599 CSF specimens collected. Of these, 179 (87%) specimens were laboratory confirmed. Among these, 167 (81%) were Nm W-135 and only 12 (6%) were NmA. Response to the 2002 epidemic was hindered by lack of serogroup W-135-containing meningococcal vaccine. This epidemic triggered, however, rapid production and licensure of a new trivalent (A/C/W-135) meningococcal polysaccharide vaccine for evaluation and use in Africa.

From 1/1-6/1/2003, 7673 cases and 1146 deaths (CFR 14.9%) were reported in Burkina Faso and 17 (32%) districts surpassed the epidemic threshold. The overall attack rate was 61/100,000 population. A pathogen was cultured from 511 of 1098 CSF specimens collected. Of these, 368 (33.5%) specimens were laboratory confirmed. Among these, 264 (71.7%) were NmA and 104 (28.3%) were NmW-135. In contrast to 2002, W-135-containing vaccine was available in 2003, and decisions about mass vaccination with bivalent (A/C) or trivalent (A/C/W-135) vaccines were guided by serogroup distribution and district attack rates. Eleven epidemic districts received trivalent vaccine.

Conclusion: In 2002, Burkina Faso experienced the largest meningococcal disease epidemic caused by serogroup W-135 ever documented. Data from 2003 demonstrate the continuous role of NmA as a cause of epidemics and reveal the co-existence of NmA and W-135 during epidemics. The emergence of NmW-135 underscores the need to continue prospective epidemiological surveillance and serogroup confirmation of meningitis cases to monitor disease trends and guide vaccine selection for epidemic response.

Epidemic group A meningococcal disease in the Sudan despite high levels of naturally-acquired serum group A antibodies

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We recently described a meningococcal polysaccharide immunogenicity study conducted in Khartoum during the height of the 1999 group A epidemic (Ismail, et al, Pediatric Infectious Disease Journal, in press). Despite mass immunization, 3377 cases were reported in Khartoum over a five-month period. Serum anticapsular antibody concentrations of unimmunized Sudanese bled at the peak of the epidemic were indistinguishable from those of unimmunized subjects bled a month later when the epidemic had begun to wane with onset of the rainy season (geometric means of 31.4 and 27.3 micrograms/ml, respectively, for adults, and 13.2 micrograms /ml and 14.1 micrograms /ml for the 3- to 17-year olds). The data underscored difficulties of raising immunity of a population by vaccination during an epidemic before widespread exposure to the organism has occurred. Little is known about why group A strains are epidemic in some populations but rarely cause disease in others. Here we describe studies of naturally-acquired serum group A antibodies in a new group of 47 unimmunized Sudanese adults bled during the 1999 epidemic, 57 adults from Uganda, which borders Sudan but is outside the African "meningitis belt," and 132 adults from North America where group A exposure rarely occurs. Serum anticapsular antibody concentrations were prevalent in all three populations but were, on average, 6-fold higher in Sudanese (geometric mean of 31.5 μg/ml) than in North Americans or Ugandans (geometric means of 5.4 micrograms /ml and 5.3 micrograms/ml, respectively, $P<0.001$). 66% of Sudanese had serum bactericidal titers before vaccination that correlated with protection (• 1:4 with human complement), as compared with 27% and 23%, respectively, of North Americans and Ugandans ($P<0.001$). Based on inhibition studies, the majority of the bactericidal antibodies in the Sudanese sera were directed at the group A polysaccharide, whereas only 17% and 6% of bactericidal sera from North America or Uganda, respectively, were inhibited by group A polysaccharide ($p<0.0005$). Approximately 50% of the non-bactericidal Sudanese sera had high IgA anticapsular antibody concentrations, which were rarely detected in sera from Uganda or North America. Thus, naturally-acquired serum group A anticapsular antibodies are prevalent in all three populations. However, cross-reacting antibodies in Ugandan and North American sera are usually not bactericidal while antibodies in Sudanese sera, which likely reflect exposure to the epidemic group A strain, are bactericidal, although occasionally bactericidal activity may be blocked by high concentrations of IgA antibody. Meningococcal epidemics in Sub-Sahara result from unique environmental factors that favor spread of a virulent epidemic strain, which can occur despite a high prevalence of naturally-acquired serum anticapsular antibodies. Routine meningococcal immunization, therefore, will prevent epidemics only if vaccination reaches a large portion of the population and not only prevents disease in individuals but also decreases transmission of the organism in the population.

Antigenic shift associated with increased serogroup Y meningococcal infection in Maryland: A population-based, molecular epidemiologic study.

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Background. The incidence of invasive serogroup Y infection increased in Maryland during the 1990s, with a doubling of both incidence and the proportion of all meningococcal cases that were serogroup Y. Nucleotide sequence-based methods enabled the changes in genotype and antigenic properties of these organisms to be monitored.

Methods. Multi-locus sequence typing and antigen gene sequence typing was performed on all available serogroup Y isolates from Maryland residents with invasive disease from 1992-2001. The antigens targeted were the deduced PorA (P1) variable regions (VRs) 1 and 2 and the FetA (F) protein VR.

Results. There were 106 serogroup Y isolates, which contained 16 sequence types (STs). From 1992-2001 100 of these isolates (94%) belonged to the ST-23 complex; the remaining 6 (6%) comprised a group of 4 related STs. When the ST-23 complex isolates were classified according to OMP profile, 59% were P1.5-1, 2-2: F.5-8, which predominated during the early 1990s; 37% were P1.5-2,10-1: F.4-1; and 4% consisted of other OMP profiles. The incidence of infection caused by P1.5-2,10-1: F4-1 isolates increased 14-fold during 1992-2001 (p value for trend <0.001). However, the increase in serogroup Y infection was initially caused by P1.5-1,2-2: F5-8 isolates but was later sustained by the increase in P1.5-2,10-1: F4-1 isolates.

Conclusions. During a period of increasing serogroup Y infection, the vast majority of disease was caused by ST-23 complex isolates. In the early 1990s, the predominant clone had a different antigenic profile than the clone that predominated during later years. Less population immunity to P1.5-2,10-1: F.4-1 likely allowed it to emerge in the face of a decline in the incidence of the clone that initially predominated. The diversity we observed is consistent with strong immune selection acting on 2 genes located on different parts of the chromosome and the non-overlapping antigenic structure was most likely imposed by herd immunity. These data suggest that increases in meningococcal incidence during endemic periods could be caused in part by antigenic shift.

Distributed web-accessible databases developed for meningococcal typing and epidemiology

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Meningococcal typing is increasingly being performed using nucleotide sequence-based methods on both housekeeping and antigen genes. Sequence data offer the most fundamental level of discrimination while being unambiguous in assignment and easily portable by electronic means. We have developed a number of web-accessible, inter-connected, databases that can aid the public health communities with the rapid assignment of sequence types and antigen variants.

The Neisseria MLST website and databases, located at http://pubmlst.org/neisseria/, have definitions for over 3500 sequence types, many of which have been assigned to clonal complexes. The databases have a distributed structure, with allelic profiles and allele sequences separated from isolate data. This distributed structure enables any number of isolate databases to be set up with the advantage that they can be customised to an individual project or laboratory with specific access restrictions. The isolate databases can all query the profiles database for allelic profile definitions, so there is no duplication of data, preventing integrity problems such as multiple assignments of the same profile or sequence. Along with the 'PubMLST' general isolate database that anyone can submit data to, a database containing information on the highly-characterised 107 isolates used in the original MLST validation is available, as well as others that contain datasets described in particular papers. The distributed structure allows databases to be geographically separated, with queries performed between them over the Internet. The profiles and 'PubMLST' isolate databases are easily copied and we have set up mirror sites to provide a robust service that will remain online even if the primary site becomes temporarily unavailable. The database software can retrieve information from PubMed enabling searches for isolate sets from a particular paper or by an individual author.

The MLST isolate databases can also be linked easily to those for antigens, which are currently available for *porB* and the PorA and FetA variable regions (http://neisseria.org/nm/typing/). By including fields for these antigens within the MLST database, antigen sequences can be retrieved automatically for individual isolates. The antigen databases themselves provide tools for the easy assignment of antigen variants, incorporating BLAST searches so that either nucleotide or peptide sequences can be queried and an assignment made or the nearest variant determined. The integration of such multiple data sources over the Internet provides powerful tools for epidemiology and vaccine development.

Diversity in pathogenicity can cause outbreaks of meningococcal disease

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Neisseria meningitidis, the meningococcus, is a major cause of bacterial meningitis and septicaemia world-wide. Infection in most cases leads to asymptomatic carriage and only rarely to disease. Meningococcal disease often occurs in outbreaks, which are both sporadic and highly unpredictable. The occurrence of disease outbreaks in a host population in which the aetiological agent is widely carried is not well understood. A potential explanation lies in the fact that genetically distinct meningococci are diverse with respect to disease-causing potential. We formulated a stochastic mathematical model to investigate whether diversity of the bacterial population is related to outbreaks of meningococcal disease. In the model strains that occasionally cause the disease appear repeatedly in a population dominated by a non-pathogenic strain. When the pathogenicity, i.e. the disease-causing potential of the pathogenic lineage, was low the model shows distinct outbreaks and the size distribution of the outbreaks follows a power law. The ratio of the variance to the mean number of cases is high in such cases. Analysis of notification data of meningococcal disease showed that the ratio of the variance to the mean was significantly higher for meningococcal diseases than for other bacterial invasive diseases. This lends support to the hypothesis that outbreaks of meningococcal disease are caused by diversity in the pathogenicity.

Session VII. Host Response

Defining targets for C3 and C4 on pathogenic Neisseria

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Activation of the classical complement pathway is essential for mediating serum killing of pathogenic Neisseriae. Previously, we identified lipooligosaccharide (LOS) as an acceptor for the complement component C4b. Presently, we have identified a second acceptor for C4b, having a molecular mass of ~18-22kDa, that in addition is phase-variable. One such major outer membrane phase-variable protein in Neisseria is opacity-associated protein, called Opa. A single neisserial strain can possess 3-4 (in Neisseria meningitidis) or 11 (in N. gonorrhoeae) unlinked chromosomal alleles that encode distinct Opa variants. The C4b-protein complex, formed by incubating normal human serum (NHS) with Opa expressing Neisseriae, was evidenced by co-localization with anti-Opa and anti-C4 antibodies in western blots. The linkage between C4b and Opa was amide in nature, by virtue of its resistance to nucleophilic attack by 1M Methylamine, pH 11. We insertionally inactivated the expressed opa gene in an unencapsulated meningococcal strain. Although the total amount of C4b bound to both the wild-type and mutant strains (Opa+ and Opa-) was similar, the resultant Opa- mutant was more resistant to direct complement-mediated killing by NHS (45% killing in 10% NHS compared to 85% killing of the wild-type, Opa+ strain).

N. gonorrhoeae can express one or more Opa proteins out of the 11 opa alleles (OpaA through OpaK). We examined the ability of different Opa proteins to bind C4b, by using a series of Opa variants derived from the gonococcal strain FA1090, which expressed a particular Opa predominantly, or no Opa at all. All Opa variants bound C4b via an amide bond. In addition to Opa and LOS, we also detected C4b binding to the porin (Por) molecule on gonococci.

Because C3b, akin to C4b, possesses an internal thioester bond, we sought to also examine the linkages formed between C3b and neisserial surface targets. In contrast to the amide linkages formed by C4b, C3b bound the same targets on meningococci and gonococci via ester-linkage predominantly.

These findings may serve to explain why Opa- phenotypes are observed in meningococci recovered from the bloodstream and gonococci recovered from the female genital tract at the time of menses, where the organisms encounter a high level of complement. Possessing a phase-variable target for C4b could provide a means for these bacteria to modulate complement activation during different stages of disease.

The role of meningococcal secreted proteins in host-cell interaction

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Neisseria meningitidis secretes a large number of proteins (meningococcal secreted proteins, MSPs) which are likely to play key roles in the pathogenesis of disease. However, MSPs have been poorly characterised and their relative contribution to host-pathogen interactions remains unknown. Here, we examined the differential gene expression of meningioma-derived arachnoid (meningothelial) cells in response to purified MSPs compared to live meningococci.

The host cells were exposed to either live meningococci (strain MC58) or their endotoxin-depleted MSPs. Total RNA extracted from cell monolayers was used as a template for synthesis of radiolabelled cDNA, which was then used to probe Human Cytokine Expression and Apoptosis Arrays (R&D Systems). Membranes were subjected to autoradiography and spots were analysed using transmission densitometry and NIH ImageJ Software. The ratios for potentially differentially regulated genes were calculated after correction for probe variation using constitutively expressed genes on equivalent arrays

A striking finding was the 2876-fold up-regulation in transcription of cyclooxygenase-2 (COX-2) within 8h of exposure to MSPs but not after exposure to live meningococci for the same time. By 24h, however, live meningococci induced greater up-regulation of COX-2 transcription than MSPs. COX-2 is an important multi-functional protein involved in the synthesis of prostaglandins and pro-angiogenic factors. It is believed to be an important mediator of the response to bacterial sepsis, including stimulation of endothelial cell migration and prevention of apoptosis. COX-2-deficient mice have increased susceptibility to bacterial peritonitis and endotoxaemia.

Transcription and translation of COX-2 in the human meningothelial cells were confirmed by real time RT-PCR and immunoblotting, respectively. Although the MSP preparations used were LOS-depleted, the possibility that the observed effects were due to residual LOS on COX-2 expression could not be ruled out. Immunoblots of meningothelial cells exposed to MSPs prepared from strain H44/76 and its LOS-deficient derivative H44/76[pLAK33] revealed equivalent expression of COX-2 confirming that the MSP-induced COX-2 up-regulation was unrelated to LOS.

These findings indicate that MSPs may play important roles in the host-pathogen interaction and that COX-2 is involved in the pathogenesis of meningococcal disease. Up-regulation of COX-2 expression was recently reported in urethral epithelial cells following exposure to *N. gonorrhoeae*. However, this is the first report of COX-2 expression in response to meningococci or its secreted proteins.

Meningococcal porin PorB activity is abrogated in TLR2 ko B cells.

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Purified neisserial porin PorB or porin from intact bacteria co-localizes with mitochondria of different cell types, including murine splenic B cells. This interaction has been shown to lead to protection of mitochondria from staurosporine-induced membrane depolarization. Accordingly with what previously published by our group, by interacting with mitochondrial porin VDAC, neisserial porin PorB helps maintaining the integrity of the mitochondrial membrane, preventing mitochondrial depolarization induced by staurosporine and thus preventing release of cytochrome c from the inner membrane space and the following activation of the apoptosome complex. The interaction of PorB with mitochondria from TLR2 ko B cells has been investigated and it does not seem to require the presence of TLR2 on the B cell surface, since we could detect porin association with mitochondria isolated from either wt or TLR2 ko B cells. Interestingly, PorB failed to protect TLR2 ko B cells from staurosporine-induced apoptosis as compared with B cells from wt mice.

One of the most important characteristics of neisserial porins is their ability to up-regulate the expression of the co-stimulatory factor CD86 on the B cell surface, thus increasing the cross-talk between B cells and T cells in vivo, and inducing a strong potentiation of the immune response. Our group has also shown that this characteristic effect of PorB is abrogated when the porin is incubated with B cells from TLR2 ko mice. We have then investigated if the lack of anti-apoptotic activity of PorB and its inability to up-regulate CD86 in TLR2 ko B cells might be a consequence of a blockage in the B cell activation pathway induced by PorB.

Neisseria-host cell interactions in CD46 transgenic mice.

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An important interaction occurs between pili of *Neisseria* and CD46, a human cell surface protein involved in regulation of complement activation**.** In order to develop an experimental infection model that mimics the human host, we used transgenic mice that express CD46 with human-like tissue specificity, and showed that CD46 transgenic mice are susceptible to meningococcal disease.
CD46 transgenic mice challenged intraperitoneally (IP) with *N. meningitidis* developed bacteremia and lethal disease in a dose-dependent manner. Nontransgenic mice survived challenge, although they had similar bacterial blood counts compared with CD46 transgenic mice. This strongly supports that CD46 is a major factor in development of lethal disease. Immune responses such as macrophage levels, neutrophil levels, and cytokine production, were analyzed in IP fluid, blood and brain tissue at different time points post-challenge. Macrophages and neutrophil numbers in blood were similar in transgenic and nontransgenic mice, however, neutrophil levels in IP fluid were higher in CD46 mice at 1 h post-challenge, but showed similar levels compared with nontransgenic mice at later time points. TNF, IL-6 and IL-10 levels in serum were significantly higher in CD46 transgenic mice compared with non-transgenic mice. Inoculation with LOS deficient meningococci or the medium alone did not induce cytokines. Thus, challenge of CD46 transgenic mice with meningococci trigger inflammatory responses more efficient compared with nontransgenic mice.
Crossing of the blood-brain barrier by bacteria occurred in CD46 mice, but not in non-transgenic mice, indicating an important role of CD46 in meningococcal meningitis. These data argue that spread from blood to CSF is facilitated by the presence of CD46. Intranasal infection of CD46 mice required piliated bacteria for development of disease, supporting that CD46 facilitates pilus-dependent interactions at the epithelial mucosa. Taken together, these data demonstrate a crucial role of CD46 in meningococcal disease and reveal a novel experimental system for rapid consideration of vaccine candidates as well as to study *Neisseria* pathogenesis.

Neisserial Porin Induces Dendritic Cell Functional Activity, which is MyD88-dependent

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Neisseria meningitidis PorA (class 1 protein) and PorB (class 2 or 3 proteins) and *Neisseria gonorrhoeae* PIA (protein IA) and PIB (protein IB) are the major outer membrane proteins of the pathogenic Neisseria. It has been shown that Neisserial porins act as B cell mitogens and immune adjuvants. The mechanism of the immunopotentiating ability of porin is mediated predominately by its up-regulation of the T cell co-stimulatory ligand CD86 (B7-2) on the surface of B cells. Because of Neisserial porin's ability to activate B cells and potentiate immune responses, we hypothesized that porin also employs the potent immune stimulatory function of dendritic cells (DC). In this work, we examined the ability of purified *N. meningitidis* PorB to induce maturation of murine splenic and bone marrow-derived DC. We have previously shown that incubation of murine DC with meningococcal PorB induces DC maturation as demonstrated by increased expression of CD86 and class I and II MHC molecules. In addition, PorB treatment enhanced the allostimulatory activity of DC, as evidenced by their increased activity in the mixed lymphocyte reaction (MLR), as compared with medium treated DC. To further characterize the functional activity of PorB-matured DC, we examined the ability of PorB to enhance presentation of the model protein antigen chicken egg ovalbumin (Ova) to DO11.10 Ova specific, $CD4^+$ transgenic T cells. DC co-treated for 24 hours with PorB and Ova protein induced activation of naive, DO11.10 T cells greater than that induced by DC treated with Ova protein alone, as evidenced by T cell proliferation. Hence, PorB-matured DC are capable of inducing activation of T cells in an antigen-specific manner. In addition, we have demonstrated the ability of PorB to induce IL-6 production by DC. This is significant as IL-6 is induced during infection with *Neisseria meningitidis* and may be involved in the inflammatory process observed with this disease. Our group has previously demonstrated the requirement of both MyD88 and Toll-like receptor 2 (TLR2) for PorB-induced B cell activation. In this work, we implicate Toll-like receptor signaling in PorB-induced DC maturation. *N. meningitidis* PorB did not induce maturation of MyD88 knock out DC. This work is significant for understanding the mechanism of Neisserial porin's immune stimulatory activity.

Meningococcal Lipopoly(oligo)saccharide Is a Potent Activator of Both The TLR4/MyD88-Dependent and –Independent Signaling Pathways

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Background: Meningococcal lipopoly(oligo)saccharide (LOS) is a major inflammatory mediator of fulminant meningococcal sepsis. Meningococcal LOS induces macrophage activation in a CD14/TLR4-MD-2 dependent manner. However, quantitation of meningococcal LOS activation of the MyD88-dependent and -independent signaling pathways in comparison to other endotoxins has not been determined. **Methods**: Human macrophage-like cell lines (THP-1, U937 and MM6) or murine macrophage RAW 264 cells were stimulated with equal molar amounts of highly purified endotoxins (protein, DNA, peptidoglycan and phospholipid free preparations, standardized based on lipid A content). Harvested supernatants (that contained released cytokines) from previously stimulated cells were also used to stimulate RAW264.7 or 23ScCr (TLR4-deficient) macrophages (i.e. indirect induction). **Results**: Meningococcal LOS at patho-physiologicaly relevant, picomolar concentrations was a very potent inducer of TNF alpha, IL-1 beta, MCP-1, MIP3 alpha, nitric oxide, IP-10 and IFN beta . *E. coli55:B5* and *Vibrio cholerae* LPS at the same molar concentration induced TNF alpha but did not induce significant amounts of nitric oxide, IFN beta or IP-10. In contrast, *Salmonella* (*minnesota* and *typhimurium*) LPS in time-course and dose-response experiments induced the release of significant amounts of nitric oxide, IFNβ and IP-10 but very little TNF alpha and MIP-3 alpha. No response was seen in TLR4-deficient macrophages (C3H/HeJ or 23ScCr) which indicated that all endotoxins tested utilized the TLR4-dependent pathway. Exogenous IFN beta induced nitric oxide in a dose-dependent manner; anti-IFN beta polyclonal antibody neutralized the effect of induced IFN beta and significantly reduced nitric oxide release; and when the type 1 IFN receptor was blocked with IFN alpha/beta receptor 1 antibody, a significant reduction in nitric oxide release was observed. Blocking the MyD88-dependent pathway by DNMyD88 resulted in significant reduction of TNF alpha release but did not influence nitric oxide release. The data suggest that meningococcal LOS and *Salmonella* LPSs in contrast to *E. coli* and *V. cholerae* LPS differentially induced the MyD88-independent pathway. Meningococcal LOS is a potent activator of the human macrophages CD14/TLR4-MD-2 receptor complex via both the MyD88-dependent and -independent signaling pathways.

CEACAM1-specific Opa proteins suppress dendritic cell maturation in response to *Neisseria gonorrhoeae*: Implications for gonorrhea and HIV

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While gonorrhea is typified by an intense inflammatory response, the specific immune response to *N. gonorrhoeae* is weak and re-infection is common. Previous work established that gonococcal Opa protein binding to human CEACAM1 suppressed the activation and proliferation of $CD4^+$ T lymphocytes in response to various stimuli. This effect appears dependent upon the immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic domain of CEACAM1, which recruits cellular phosphatases that counter the kinase-dependent activating signals. While $CD4^+$ T cells direct the development of immunity, their activation *in vivo* requires antigen presentation by professional phagocytes. Immature dendritic cells that reside in peripheral tissues are induced to mature upon exposure to pathogen-associated molecular patterns, such as bacterial endotoxin binding to human TLR-4. This maturation process results in a reduction in the dendritic cell's phagocytic function, and a concomitant increase in efficacy of antigen presentation in a manner that stimulates lymphocytes. We observed that dendritic cells increase CEACAM1 expression upon exposure to *N. gonorrhoeae*, suggesting that neisserial expression of CEACAM-specific Opa proteins could impact bacterial binding and/or the dendritic cell response to infection. Flow cytometric analyses indicated a consistent change in the expression of most markers of dendritic cell maturation, regardless of whether *N. gonorrhoeae* expressed pilus, the heparan sulfate proteoglycan receptor-specific Opa_{50} protein, or the CEACAM receptor-specific Opa_{52} protein. However, an exception was apparent: CD83 is not expressed in response to the Opa_{52}-expressing strain. The functional significance of neisserial binding to CEACAM1 was apparent by a highly significant (~50%) reduction in the dendritic cells' ability to stimulate an allogeneic T cell response following exposure to Opa_{52}-expressing gonococci. Significantly, a similar effect was evident when response to a defined epitope was assessed, as dendritic cells exposed to Opa_{52}-expressing bacteria were defective in their ability to stimulate lymphocytes in response to an HIV-derived epitope when cells were isolated from an HIV-1-infected patient. These inhibitory effects do not result from simple adhesin-dependent differences in bacterial association with the dendritic cells, as no significant difference in gonococcal binding was observed between Opa_{50}- and Opa_{52}-expressing strains. Furthermore, the effects cannot be explained by Opa_{52}-dependent interactions with CEACAM1 on T lymphocytes, as the gonococci were removed prior to the addition of lymphocytes. The Opa-dependent suppression of T cell activation, both directly by binding to CEACAM1 expressed by T lymphocytes and indirectly via the CEACAM1-dependent reduction in CD83 expression by the antigen presenting cell, reflects a powerful capacity to block the adaptive immune response at multiple levels, and undoubtedly contributes to the evolutionary success of this remarkable pathogen.

Lipopolysaccharide-mediated targeting of *Neisseria meningitidis* to dendritic cells: binding of *lgtB* LPS to DC-SIGN

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Dendritic cells (DC) play a crucial role in the initiation of an immune response, both during natural infection and in response to vaccination. Immune responses to bacteria are initiated by DC, which internalize and process bacterial antigens for presentation to T cells. We have shown that *Neisseria meningitidis* lipopolysaccharide (LPS) plays a major role during interactions with human DC, as inactivated meningococci of a completely LPS-deficient mutant are taken up very poorly as compared to the wildtype strain. Moreover, LPS is required for both internalization of bacteria as well as full activation of the DC. To determine which part of LPS is involved, a set of stepwise truncated *N. meningitidis* oligosaccharide mutants as well as lipid A mutants expressing altered fatty acyl patterns were tested. Remarkably, greatly enhanced binding and uptake by DCs was found for the *lgtB* oligosaccharide mutant lacking only the terminal galactose residue of the lacto-*N*-neotetraose part. Increased binding was observed for both live and dead bacteria. In contrast, all the other oligosaccharide mutants showed similar or reduced binding and internalization as compared to the wildtype. In the case of the lipid A mutants, somewhat reduced binding and internalization by DCs was seen for all mutants. The rate of internalization was found to correlate with subsequent DC activation. Opa proteins could be shown not to play a role in DC interaction.

The differences in DC association prompted us to investigate whether this process is mediated via specific DC receptors. Therefore, we analyzed binding of the wildtype and the oligosaccharide mutants to C-type lectin receptors that are abundantly expressed on immature DCs and can serve as antigen receptors for sugar-containing antigens. First, binding of a panel of C-type-lectin-Fc chimeras to wildtype and oligosaccharide mutant whole cells was studied in a soluble adhesion assay. Strong binding of the *lgtB* mutant to DC-SIGN-Fc was found, whereas no binding was seen for the wildtype and the other oligosaccharide mutants. Binding of the *lgtB* mutant to DC-SIGN was subsequently demonstrated in HEK293T cells transiently transfected with DC-SIGN. Finally, the specificity of *lgtB* LPS for DC-SIGN was unequivocally demonstrated by studying DC binding and internalization of the *lgtB* mutant in the presence or absence of the anti-DC-SIGN blocking antibody AZN-D2.

Taken together, our data demonstrate that the LPS-mediated association of *N. meningitidis* with DCs seems to critically depend on the interaction of both the oligosaccharide and lipid A part with multiple receptors. In the case of the *lgtB* mutant, the C-type lectin receptor DC-SIGN was identified to mediate uptake and internalization. This finding has major implications for future vaccine development against *N. meningitidis* since the use of *lgtB* LPS might facilitate direct targeting of antigens to DCs thereby increasing vaccine efficacy**.**

Session VIII. Surface Structures

THE THREE-DIMENSIONAL STRUCTURE OF THE SECRETIN PILQ AND ITS INTERACTION WITH TYPE IV PILI

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PilQ is an integral outer membrane protein and an essential component of the type IV pilus (Tfp) biogenesis machinery. We have determined the three-dimensional (3-D) structure of the PilQ complex by cryo-electron microscopy to 12 Å resolution, a considerable improvement from the resolution of 25 Å obtained previously (Collins *et al.*; 2003; J. Bacteriol. **183**, 3825-32). The higher resolution provides much more detail of the PilQ complex structure: the dominant feature is a large central cavity, formed by four 'arm' features which spiral upwards from a squared ring base and meet to form a prominent 'cap' region. The cavity, running through the centre of the complex, is effectively sealed at both the top and bottom. Symmetry analysis of the complex found a strong C4 rotational symmetry with a weaker C12 rotational symmetry, consistent with PilQ possessing true C4 symmetry but with C12 quasi-symmetry. This unusual structure suggested that the PilQ complex may not function solely as a passive portal within the outer membrane. Further evidence for this hypothesis came from Far-Western analysis and sucrose density gradient centrifugation, which provided evidence for a specific interaction between PilQ and purified pili. Transmission electron microscopy of preparations of purified pili, to which the PilQ complex had been added, showed that PilQ was uniquely located at one end of the pilus fibre. Determination of the 3-D structure of the PilQ-pilus fibre complex at 25 Å resolution showed that the C4 rotational symmetry was still present. Comparison with the structure of the PilQ complex alone revealed that the PilQ oligomer undergoes a major conformational change on association with the pilus fibre. The 'arm' features identified in the unliganded structure appear to move outward, the chamber is filled and the 'cap' feature dissociates in order to allow the pilus fibre to emerge from the top of the structure. Further evidence for specific structural changes in the PilQ complex mediated by pili was obtained from the cryo-electron microscopy 3-D structure of an intermediate form of PilQ at 16 Å resolution, determined with data from PilQ particles which had been exposed to limiting amounts of pili. The chamber in this form is largely void, the 'arm' features have moved outwards and the 'cap' appears to have descended about 15 Å to close off the top of the chamber. Taken together, these results show that the PilQ secretin exerts a specific interaction with pili and also that this interaction causes specific structural changes which may allow the secretin to support the mature assembled pilus fibre. The latter observation suggests that the PilQ complex does not function merely as a passive portal within the outer membrane but could play a pivotal role in anchoring the base of the pilus fibre.

The meningococcal secretin PilQ: Actions and interactions

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Secretins (TC 1.B.22) are a large family of bacterial proteins associated with translocation of single proteins and macromolecules across the outer membrane. A subset of this family, termed PilQ proteins (TC 1.B.22.2.1), are required for type IV pilus biogenesis in *Neisseria meningitidis*, the causative agent of meningococcal disease. Meningococcal PilQ is particularly interesting because it can induce bactericidal antibodies[1], making it relevant as a meningococcal vaccine candidate. PilQ is found as a highly stable complex and the lipoprotein PilP is thought to be important for complex stabilisation. Meningococcal PilQ is unique among secretins because of its abundance in the outer membrane and its N-terminally located polymorphic region containing repetitive elements. We have previously purified the native PilQ complex from meningococcal outer membranes and shown that it is a ring-shaped structure of approximately 900 kD, organised out of 12 identical subunits[2]. Our data indicate that the PilQ complex is the pore through which its substrate, the moving pilus fibre (polymerised PilE), is directed to the bacterial surface.

New genetic techniques have allowed us to construct defined mutants to characterise the functional domains within PilQ. In particular, PilQ complex multimerisation and orientation in the outer membrane, as well as surface exposure, are being assessed. The application of Far-Western analysis and ultracentrifugation demonstrated that PilQ and the pilus fibre directly interact. We have also identified the PilQ domains that are involved in the PilQ-pilus interaction. Furthermore, the DNA binding behaviour of full and partial recombinant as well as complex PilQ was evaluated in DNA band-shift and plasmon resonance analyses. The PilQ-mediated binding of DNA was not enhanced by the presence of DNA uptake sequences (DUS). Surprisingly, the binding of single stranded DNA was much more evident than the binding of double-stranded DNA. This work is critical to understanding how the PilQ complex functions; our aim is to detail the dynamics of PilQ complex interaction with other components such as outer membrane proteins and nucleic acid during pilus biogenesis, pilus retraction and DNA uptake in transformation.

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A unique capsular epitope recognized by bactericidal, non-autoreactive anti-N-propionyl group B polysaccharide mAbs

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Neisseria meningitidis group B polysaccharide (MBPS), alpha (2→8) N-acetyl neuraminic acid, is chemically identical to human polysialic acid and is, therefore, poorly immunogenic and elicits autoantibodies. However, polysaccharide-protein conjugate vaccines prepared from MBPS in which the N-acetyl groups of MBPS have been replaced with propionyl groups (N-Pr MBPS) are reported to elicit serum bactericidal antibodies in mice and non-human primates, and a subset of these antibodies have minimal autoreactivity. We also described a panel of murine bactericidal and protective anti-N-PR MBPS mAbs that reacted with encapsulated group B strains, but not with capsular-deficient mutants, and these MAbs exhibited little or no reactivity with human polysialic acid antigens (Granoff et al*, J. Immunol*. 1998). Together, the data suggest that antibodies raised to the N-Pr MBPS conjugate vaccine recognize a polysialic acid epitope unique to group B organisms. To better understand the epitope, we cloned and sequenced the variable region genes of five anti-N-Pr MBPS mAbs and compared them to those of an autoreactive anti-MBPS mAb (735) whose x-ray structure has been determined. The genes encoding the antibody V regions are derived from a restricted set of germline V, J, and D genes. We constructed 3-dimensional structural models of the combining sites by computational modeling using the peptide sequences encoded by the V genes. Models of non-autoreactive anti-N-Pr MBPS mAbs (SEAM 2 and 3) have a deep groove in the combining site that could accommodate a hapten, whereas the structure of the combining site of the autoreactive anti-MBPS mAb 735 is characterized by a shallow wide groove that could accommodate a larger structure. Structural models of minimally autoreative anti-N-Pr MBPS mAbs (SEAM 12, 18, and 35) have combining sites with characteristics that are intermediate between autoreactive and non-autoreactive mAbs. To determine whether anti-N-Pr MBPS mAbs recognized a substructure of the polysaccharide, we measured the ability of intermediates in the synthesis of N-Pr MBPS to inhibit binding to solid phase N-Pr MBPS in an ELISA. Surprisingly, binding of two of the anti-N-Pr-MBPS mAbs (SEAM 3 and 18) was inhibited by de-N-acetylated MBPS while binding of a third MAb (SEAM 12) was inhibited by re-N-acetylated MBPS. In contrast, none of the anti-N-Pr-MBPS mAbs was inhibited by unmodified N-Ac MBPS. We used the anti-N-Pr MBPS mAbs linked to magnetic beads to purify the antigen and MALDI-TOF mass spectrometry to determine the structure. Preliminary results show that the mAbs recognize, at a minimum, a disaccharide containing one or more de-N-acetylated MBPS residues. These results suggest that human polysialic acid and native group B capsule differ in that the bacteria may contain significant amounts of de-N-acetylated polysaccharide. Furthermore, these sites may be useful targets for bactericidal and protective group B anticapsular antibodies that lack auto-reactivity.

Selection for Opa-positive Gonococci Occurs during Experimental Murine Genital Tract Infection in the Absence of Human CEACAM Receptors

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The opacity (Opa) proteins of *Neisseria gonorrhoeae* are a family of phase variable outer membrane proteins that mediate interactions with host cells. Gonococci can express 8-10 antigenically distinct Opa proteins, most of which mediate adherence to, and invasion of epithelial cells by binding to members of the carcinoembryonic antigen cell adhesion molecule family (CEACAM); some Opa proteins bind heparin sulfate proteoglycans (HSPG). The importance of Opa proteins during infection is supported by the recovery of a majority of Opa-positive variants from male volunteers following inoculation with a predominantly Opa-negative population. A similar result occurs during experimental genital tract infection of female mice. It is not known whether these observations are due to selection of a pre-existing population of Opa-positive gonococci or increased *opa* gene phase variation in vivo. Of note is that, with the possible exception of CEACAM1, murine and human CEACAMs are not highly related. Here we explored the kinetics of Opa expression in mice by determining the Opa phenotype of vaginal isolates following inoculation with defined mixtures of Opa variants of strain FA1090. In mice inoculated with a mixture of primarily Opa-negative and OpaI variants (an HSPG-binding Opa protein), three phases of Opa expression were defined: an early phase (days 1-3), in which a high percentage of OpaI variants was recovered from a majority of mice, a mid-phase (days 4-7) characterized by significantly decreased recovery of Opa-positive variants, and a late phase characterized by a resurgence of Opa-positive variants. To investigate the basis for the increased recovery of Opa-positive variants in the early phase, the recovery of selected Opa variants of a chloramphenicol-resistant (Cm^R) derivative FA1090 strain was followed over time. Mice were inoculated with defined mixtures of OpaI,Cm^R and Opa^-,Cm^S gonococci. A predominance of OpaI variants was recovered from 75% of mice within 48 hours post-inoculation, the majority of which were Cm^R. Results from reciprocal experiments using a mixture of OpaI,Cm^S and Opa^-,Cm^R variants were consistent with selection of the OpaI,Cm^S population. To test if Opa proteins that do not bind HSPG are also selected for in vivo, we inoculated mice with a mixture of OpaB,Cm^R and Opa^-,Cm^S gonococci. In all mice, selection of OpaB variants occurred early in infection, the majority of which were Cm^R. We conclude that the isolation of Opa-positive gonococci during the early phase of murine infection is due to selection of a pre-existing Opa-positive population. Enhanced resistance of Opa-positive variants to innate factors of the immune response, adherence to murine CEACAM1 and/or HSPG receptors, or adherence to unidentified receptors may be responsible for these results. Studies to identify selective factors are ongoing, as is an investigation of the basis for the mid and late phases of Opa expression during experimental murine infection.

The outer membrane protein Imp/OstA (NMB0280) is required for lipopolysaccharide transport to the bacterial cell surface

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Lipopolysaccharide (LPS) is an essential component of the outer membrane (OM) of most Gram-negative bacteria. LPS is synthesized in the bacterial inner membrane, a process that is now quite well understood. In contrast, the mechanism of its transport to the outer leaflet of the outer membrane has remained enigmatic. Recently, the Imp/OstA protein was found to be essential in *E. coli* and to function in cell envelope biogenesis (1). We hypothesised therefore that Imp might have a role in LPS transport. An Imp-deficient mutant of *Neisseria meningitidis* strain H44/76 was viable, demonstrating Imp is not essential in *N. meningitidis.* The Neisserial *imp* mutant demonstrated a very similar phenotype as an *lpxA* mutant, which is completely devoid of LPS. Both strains showed reduced growth rates, enhanced colony opacities and an elevated leakage of periplasmic proteins, while outer membrane proteins (OMPs) were normally expressed and assembled. These similarities indicate an effect of Imp on LPS biogenesis. However, in contrast to the *lpxA* mutant, the i*mp* mutant still produced minimal amounts of full-length LPS. KDO measurements indicated that only 6% of normal levels were produced. The surface location of LPS was tested by assessing the accessibility of sialylated LPS to neuraminidase. Sialylated LPS of the *imp* mutant was completely accessible to the enzyme in cell envelope preparations, but only poorly in intact cells, indicating that LPS was mostly absent from the cell surface. Surface localization of LPS was also investigated by measuring modification of LPS by PagL. PagL is an OM-located deacylase capable of modifying LPS within the OM (2). Expression of *Bordetella bronchiseptica*-derived PagL in H44/76 wild-type bacteria resulted in complete modification of LPS during growth. In contrast, in the *imp* mutant strain, PagL expression did not result in any detectable modification of LPS during growth, despite the presence of a functional PagL enzyme, as shown by in vitro assays.
These data clearly show an LPS transport defect in a Neisserial *imp* mutant. Since Imp is an OMP, as shown by its presence in purified *E. coli* and Neisserial OMs, Imp likely represents the transporter that mediates LPS translocation over the OM. The role of Imp in the biogenesis of an essential bacterial component and its high conservation among Gram-negative bacteria, make it an excellent target for the development of novel antibacterial compounds. Furthermore, Imp-deficient strains may be useful vaccine strains, since outer membrane vesicles (OMVs) prepared from these strains will contain only minute amounts of LPS, thereby relieving the need for detergent extraction of the OMVs which might result in undesired removal of other OM components besides LPS.

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lpt6*, a gene required for addition of phosphoethanolamine to inner core lipopolysaccharide of *Neisseria meningitidis

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Phosphoethanolamine (PEtn) is found at several positions within *Neisseria meningitidis* (*Nm*) lipopolysaccharide (LPS) both in the inner core region attached to the beta chain heptose (HepII) and on the lipid A moiety[1]. PEtn is found at the 3-position (PEtn-3) on HepII in immunotypes L1, 3 and 7 [2,3,4] and confers relative resistance to the bacteria in bactericidal killing mediated by a monoclonal antibody (mAb) L3-B5 *in vitro*. The amino acid sequence of the gene *lpt3* required for this addition[5], was used to search against the completed genome sequence of *Nm* serogroup B and identified the gene responsible for the addition of PEtn on lipid A and subsequently named *lptA*[7]. However it has long been recognised that some strains possess PEtn at the 6-position (PEtn-6) on HepII as seen in immunotypes L2, 4 and 6[2,4,7] so a study to identify the third PEtn transferase was undertaken.

To investigate the genetic basis of PEtn-6 addition, we screened a library of transposon induced mutants in a *galE* background of strain 89I (immunotype L4) for reactivity with the mAb L2-16, the reactivity of this mAb is dependent upon expression of the PEtn-6 residue[8]. Eighteen mAb L2-16 non-reactive colonies were identified from approximately 20,000 colonies of the library of transposon insertion mutants. The DNA sequence adjacent to the different transposon insertion sites was determined for each mutant then used to search the available *Nm* genome sequences. Lack of mAb L2-16 reactivity could be caused by functional loss of one of more genes required for the addition of PEtn-6 to HepII, or interruption of any of the genes required for the synthesis of the PEtn-6 containing LPS epitope. Nine of the mutants had transposon insertions in genes known to be related to LPS biosynthesis and electrophoretic profiles of LPS extracted from these mutants were consistent with the expected phenotype that would result from disruption of the corresponding LPS genes.

The remaining transposon insertion sites were found to be clustered in a region of the genome previously designated as Lgt-3[9]. Transposon insertions occurred independently in four mutants of the open reading frame NMA0408 of the *Nm* serogroup A genome sequence. Insertional inactivation of NMA0408 resulted in the loss of mAb L2-16 reactivity and structural analysis of LPS from the mutant showed no PEtn-6 to be present. Alignment of the translated amino acid sequence of NMA0408 and the two previously characterised PEtn transferases, Lpt3 and LptA, showed they were of similar length and contain a number of conserved residues across the length of the proteins. We conclude that we have identified a third PEtn transferase in *Nm* and have named the gene *lpt6* (*lipopolysaccharide phosphoethanolamine transferase at position 6*).

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Lipooligosaccharide (LOS) inner core biosynthesis in *Neisseria meningitidis* : Identification of the O-6 PEA transferase and role of glycine

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The meningococcal LOS inner core [Hep_2-(GLcNAc)-Kdo_2-Lipid A] may be decorated by phosphoethanolamine (PEA) at the O-3 and O-6 positions of Hep II. The O-3 and O-6 PEAs are determinants of LOS immunotype and have recently been shown to form amide linkages with C4b, important in complement-mediated killing of meningococci. A putative *lpt-6*, encoding the O-6 PEA transferase, was identified by *in silico* analysis of the genomes of *N. meningitidis* MC58 and Z2491. A PEA transferase motif generated from an alignment of eukaryotic and prokaryotic PEA transferases was used to search the COG database. One candidate ORF that contained the PEA transferase motif was found in the genome of Z2491 but was absent in strain MC58. The putative *lpt-6* gene was insertionally inactivated in meningococcal strain NMB and the oligosaccharides were analysed by Maldi-TOF mass spectrometry. Strain NMB expresses a lipooligosaccharide (LOS) population consisting of L2 and L4 immunotypes. L2 immunotype structures are characterized by the attachment of O-3 glucose and O-6 PEA groups to HepII, additions which are catalyzed by LgtG and Lpt-6, respectively. L4 immunotype structures lack O-3 glucose but retain O-6 PEA attachments to the inner core. Inactivation of the putative *lpt-6* gene in strain NMB resulted in the complete absence of O-6 PEA on the LOS inner core of the mutant. The ^{1}H NMR and ^{31}P NMR spectrums of the oligosaccharides from the *lpt-6* mutant confirmed the loss of the O-6 PEA group from the LOS inner core.

Interestingly and despite the fact that strain NMB contains an intact and transcribed *lpt-3* gene, no O-3 PEA attachments to the LOS inner core were detected in the parental strain NMB or in a mutant in which *lgtG* was inactivated. However, mutations which result in the loss of the *N*-lactoneotetraose chain such as *lgtK* (formerly *rfaK*) and *pgm*, result in the absence of O-3 linked glucose and the appearance of both O-3 PEA and O-6 PEA groups on the inner core. The ^{1}H NMR and ^{31}P NMR spectrums of the oligosaccharides purified from an *lpt-3/lgtK* double mutant confirmed that the O-3 PEA group was absent whereas the O-6 PEA group was retained. Further, Maldi-TOF mass spectrometry also revealed the presence of glycine on the LOS inner core at the O-7 position of HepII of parental strain NMB but that this attachment was absent in all *lgtK* mutant derivatives. Therefore, from this data we conclude that attachment of glycine to HepII of the inner core of LOS occurs at some point after the addition of the terminal GlcNAc residue. As well, it appears that the absence of glycine on the *lgtK* mutant LOS inner cores enables Lpt-3 to donate O-3 PEA groups to HepII.

POSTER SESSIONS

Poster Session I

Epidemiology

The continuing diversification of *Neisseria meningitidis* W135 as a primary cause of meningococcal disease, after its emergence in 2000.

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The occurrence of a clonal outbreak of serogroup W135 (ET-37 clonal complex) meningococcal disease among Hajj pilgrims 2000 has lead to enhanced surveillance of the evolution of this particular serogroup, formerly considered as rare, in invasive infections. Since the first case of meningococcal disease due to a serogroup W135 strain was detected in France in 1994, all strains were characterized phenotypically. We further used phenotypic and genotypic approaches to type all serogroup W135 strains (n=101) isolated from invasive meningococcal diseases in France in 2001 and 2002. Overall, 55% of these strains had Hajj-related phenotypes (60% and 52% in 2001 and 2002, respectively) though only 45% belonged to the ET-37/ST-11 clonal complex. Moreover, pulsed field gel electrophoresis of the ET-37 clonal complex strains showed that only 32% of W135 strains were indistinguishable from the Hajj-2000 strain. The association of strains of serogroup W135 with arthritis was statistically significant. Moreover, in arthritis, the majority of strains of serogroup W135 belonged to the clonal complex ET-37/ST-11.

Our results suggest a continuous emergence of new genetic lineages of serogroup W135 independently of the 2000 global outbreak.

Different evolutionary histories for the two type I secretion channel-tunnels of *Neisseria meningitidis*

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Introduction

In both published complete genome sequences of *N. meningitidis*, two genes containing outer membrane efflux protein domains are present (*mtrE*/NMB1714 and *natC*/NMB1737, respectively). These so-called channel tunnel proteins form trimeric channels that allow export of a variety of substrates via type I secretion. These substrates include antimicrobial agents and toxins in other Gram-negative bacteria, making the channel-tunnels possible targets for intervention.

Aim

To assess the diversity of genes encoding the channel-tunnel proteins and their products in *N. meningitidis*

Methods

From the database of the Netherlands Reference Laboratory for Bacterial Meningitis (RIVM/AMC, Amsterdam), 23 isolates from five serogroups, representing twelve clonal complexes isolated in a 34 year period in 3 different continents, were selected. Both genes encoding the channel-tunnel proteins were PCR amplified and 1296 bp parts were sequenced.

Results

Twelve different *mtrE*/NMB1714 alleles with 69 polymorphic sites were identified, encoding ten different polypeptides. In marked contrast, only 3 alleles with 4 polymorphic sites encoding 3 different proteins were found for *natC*/NMB1737.

Conclusion

Whereas *mtrE*/NMB1714 has a similar number of polymorphic positions compared to housekeeping genes, *natC*/NMB1737 had remarkably few polymorphic positions (including synonymous positions). It was previously shown for housekeeping genes, that polymorphisms are far more likely to be the result of recombination than mutation. We propose that divergence of *natC*/NMB1737 is limited, due to the absence of this gene in *N. gonorrhoeae* and *N.* lactamica, resulting in a smaller genepool as compared to the genepools for *mtrE*/NMB1714 and housekeeping genes. The introduction of parts of the highly divergent alleles from other Neisseria will result in a high number of polymorphic sites, which can subsequently spread through the meningococcal population, as observed for both *mtrE*/NMB1714 and housekeeping genes. In contrast, in a *N. meningitidis*-specific gene such as *natC*/NMB1737, only polymorphisms which accumulated within the meningococcal population will spread via recombination. Apparently, the number of accumulated polymorphisms is very low in *N. meningitidis*. This implies, that introduction of alleles from other Neisseria plays a major role in generating sequence diversity in neisserial genes, whereas species-specific genes remain more conserved.

Confirmation of *Neisseria gonorrhoeae* infection and transmission partners by *por* VR typing from non-cultured clinical specimens.

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Nucleic acid amplification tests (NAAT) are used increasingly for diagnosis of sexually transmitted diseases. NAAT positive tests may require confirmation, especially in low prevalence populations. In an ongoing study of asymptomatic *Neisseria gonorrhoeae* (GC) infections, individuals enrolled at an urban U.S. Emergency Department were screened by NAAT from urine (Abbott LCR) or self-collected vaginal swab (Roche COBAS). Follow-up (f/u) of positive individuals included repeat NAAT, traditional microbiologic evaluation and treatment. A number of NAAT-positive participants were negative for GC at f/u without intervening treatment. *por* variable region (VR) typing was conducted to evaluate these individuals.

por VR typing was performed using PCR amplified *porB* DNA from clinical specimens and checkerboard hybridization with 40 *por* VR probes. Clinical samples included whole urine, urine pellets, swab eluates, and/or NAAT preps. DNA purification, and nested *porB* PCR were performed on 60 NAAT positive screening visit samples (visit 1), and 89 samples collected from 35 individuals at f/u.

Of the 60 participants with NAAT positive screening samples, 26 had available follow-up data. The screening visit sample from 2 of 16 participants who were subsequently negative at f/u were *porB* PCR positive, only one of which was *por* VR typeable. The screening visit samples from 6 of 8 participants who were GC positive at follow-up were *porB* PCR positive and *por* VR typeable. Two participants had mixed results at f/u with one positive and one negative NAAT; the screening samples from both were negative for *porB*. Among the 34 participants with no f/u to date, 11 (34%) of screening samples were *por* VR typeable.

At f/u, samples from 9 of 12 participants who were positive for GC by NAAT and/or culture were *por* VR typed. In contrast, *porB* was amplified and typed from 2 of 20 participants who were GC negative. F/u samples from three participants who had mixed NAAT results were all negative for *porB* PCR. In a subset of 9 individuals who had more than one test positive for GC at f/u, 8 were *por* VR typeable.

Overall, *por* VR type was determined for 29 participants, and in all but one, multiple samples for the same individual had the same *por* type. Two individuals had partners enrolled, and the *por* VR type of each partner matched the index case, one of whom was culture negative.

These preliminary data suggest that *por* VR typing may serve to confirm positive GC infections identified by screening NAAT and will be useful in examining transmission patterns of asymptomatic, NAAT positive infections. The etiology of positive NAAT at screening in individuals subsequently negative at follow-up remains unclear. The almost complete inability to amplify and type GC *porB* from these samples suggests that they may represent false positive results.

Diversity and dynamics of *Neisseria lactamica* carriage in infants

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Neisseria lactamica, a non-pathogenic organism predominantly found in the upper respiratory tract of infants, is closely related to *Neisseria meningitidis*. Evidence suggests that it is involved in the increased immunity to *N. meningitidis* that occurs in childhood despite low rates of meningococcal carriage and is therefore a potential vaccine candidate. Despite the interest in the exploitation of this bacterium in vaccines, little is known of the genetic character of this bacterium and its precise relationship to the meningococcus. To examine the dynamics of carriage and population diversity of *N. lactamica* in infants, 250 *N. lactamica* isolates collected from 316 infants from two longitudinal studies of bacterial carriage (one of six months duration, the other of two years) in Oxfordshire, England were obtained and genetically characterized by Multi-Locus Sequence Typing (MLST). The MLST system devised for *N. meningitidis*, using the same seven loci, was used but modified to accommodate the genetic differences between the two species.

Virtually all of the *N. lactamica* isolates were obtained from throat swab samples as opposed to concurrent swabs taken transnasally, demonstrating that this is the more efficient route for collection of *N. lactamica*. No meningococci were detected in any of the children included in these studies. Only seven of the 75 infants who had swab samples positive for *N. lactamica* carried more than one detectable strain during the course of the studies. Parents and siblings of some of the infant participants were screened for *N. lactamica* carriage. None of the parents' strains were shared with the infants, but a number of infants shared a strain with a sibling. A maximum carriage rate of around 40% at 48-56 weeks of age and an acquisition rate of 41% per year were calculated. Duration of carriage was generally long (one infant carried an organism with the same genotype between eight and 96 weeks of age) with an average rate of loss of under 1% per week during the 28 weeks following acquisition.

Genetic characterization of *N. lactamica* demonstrated that the organism is highly variable with a population structure and genetic diversity comparable to *N. meningitidis*. A total of 69 genotypes were isolated from 75 infants, and despite a relatively small sample population, collected over a two-three year period, it was possible to resolve many of the genotypes into clonal complexes. This study examined the longitudinal carriage of *N. lactamica* in young children at a genotypic level, advancing our knowledge of the biology of the organism, its diversity and duration of carriage. The observation that many infants carry genetically identical organisms for long periods with little evidence for strain replacement may have implications for the design and development of vaccines based on this organism.

EUMenNet: Genetic characterization of European meningococcal disease isolates

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EUMenNet, which is funded by the European Commission, was established as a pan-European infrastructure for the research and surveillance of European meningococcal disease. Data from 18 EU countries are presented here.
The European Meningococcal MLST Centre (EMMC) provides a centralised high-throughput facility for multi-locus sequence typing (MLST) of meningococcal isolates. The EMMC aims to promote the implementation of MLST across Europe by reducing sequencing costs, increasing speed, and disseminating high-throughput technology. Defining an MLST profile for an isolate involves: submission of samples (boiled cell suspensions that can be sent via mail) to the EMMC, PCR amplification and nucleotide sequence determination using robotics, data assembly, collation and archiving employing custom designed state-of-the-art software that has been developed as part of the EMMC (http://neisseria.org/nm/emgm/emmc/).
A representative sample of 3000 disease isolates was chosen from the participating countries from the years 2000, 2001 and 2002. These isolates are being typed by MLST. To date (May 2004), 654 different Sequence Types (STs) have been found. Despite the diversity of STs found, a small number accounted for most of the isolates, ST-11 (19%), ST-32 (8%) and ST-41 (7%). The isolates were resolved into 20 different clonal complexes, the main ones being ST-41/44 complex (Lineage 3) (22.1%), ST-11 (ET-37) complex (21%), ST-32 (ET-5) complex (18%), ST-8 complex (Cluster A4) (8%) and ST-269 complex (7%). The clonal complexes found throughout the countries were broadly similar, although prevalence of each was slightly different among countries. Differences included the absence of ST-8/Cluster A4 isolates and the higher prevalence of ST-269 complex isolates in the UK in comparison to other countries. Across Europe, there were increases in the prevalence of the clonal complexes ST-41/44 and ST-11 from 2000 to 2001. The ST-41/44 complex increased from 20% to 22% while the ST-11 complex increased from 16% to 21%. There was a decrease in numbers of isolates of the ST-8 complex, from 13% to 6% over the three years.
The outcome of the EUMenNet project and its integrated epidemiological and population genetic studies will be an improved understanding of the spread of hyperinvasive and antibiotic resistant meningococci throughout Europe.

Understanding the population structure and antigenic diversity of *Neisseria meningitidis*

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The high levels of antigenic diversity observed in *Neisseria meningitidis* populations have important implications for the design of vaccines. Although outer membrane proteins, such as PorA and PorB, are immunogenic and potentially candidates for inclusion in a vaccine, the diversity of these loci complicates vaccine design. In addition, high rates of recombination mean that particularly transmissible or virulent clones can switch their antigenic characteristics in response to selection pressures from the immune system. It is important, therefore, to understand the mechanisms which shape the structure of the pathogen population, so that we can understand how it might respond to vaccination and other control policies.

The population structure of *N. meningitidis* is characterised by the presence of distinct strains, defined by the expression of particular antigens, which are widespread geographically and persist for many years. Mathematical models have shown that these strains can be maintained by strong immune selection, despite high rates of recombination (Gupta et al., 1996). More recently the population structure of *N. meningitidis* has been described in terms of strains which are defined by housekeeping genes, using MLST to group them into clonal complexes. Certain clonal complexes, so-called hyperinvasive lineages, have been shown to be particularly virulent. Clonal complexes usually associate preferentially with certain antigens, however these associations can change over time, presumably driven by pressures from the immune system. Czech carriage isolates collected between 1970 and 1993 have been characterised antigenically and using MLST, providing an excellent opportunity to investigate the long-term association between these two traits. The clonal complexes display fluctuating antigenic types over long time scales in the data, suggesting that different selection pressures are acting on the different genes.

We developed an individual-based model on a random-mixing network in which clonal complexes respond to ecological competition, determining transmission, and 'antigenic types' respond to immune selection. These combined selection pressures can result in the changing association between antigens and genotypes seen in the data. This has implications for vaccination policy, since suppression of antigenic types may provide sufficient selection pressure to result in virulent clonal complexes changing their phenotype. In addition, it emphasizes the importance of monitoring the antigenic characteristics of clonal complexes in order to understand how *N. meningitidis* is evolving.

Carriage of *Neisseria meningitidis* in 18-22 years old males in Norway, 2003

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Introduction: After 25 years of an hyperendemic wave of meningococcal disease caused by the electrophoretic type-5 (ET-5) clonal complex/sequence type-32 (ST-32) complex, the incidence of disease in Norway is back at an endemic level (1.1/100 000 in 2002). Carrier studies performed during the period of elevated incidence in Norway have revealed risk factors associated with carriage and the prevalence of the hypervirulent clones in the asymptomatic population. The aims of this study were to determine whether the carriage rate has changed in recent years and to estimate the present carriage of hypervirulent meningococcal clones in the Norwegian population.

Materials and Methods: Nasopharyngeal swabs were collected from 164 healthy military recruits (18-22 years old) from the whole country directly upon arrival in a training camp. Cultivation was performed on selective media and one meningococcal colony per throat sample was further investigated. The isolates were serogrouped, serotyped and serosubtyped using a dot-blot method with monoclonal antibodies. The STs of the isolates were identified using multilocus sequence typing as described (http://neisseria.mlst.net).

Results: Forty-eight (29%) recruits were carriers upon arrival in the camp. For comparison, the carriage rate among recruits who had been together in that same camp for a year was 53%. The dominant serotype among the 48 strains was 4,7 (31%), but the serosubtypes of the strains were very heterogeneous. Three serosubtypes were represented by 6 strains or more, each: P1.14,22; P1.19,15; P1.16 with or without P1.7; and P1.5 with or without P1.2. A total of 40 STs were identified among the 48 isolates, with ST-60 and ST-26 represented by 3 isolates each. The ST-269 complex and the ST-41/44 complex (lineage 3) predominated with 6 and 5 isolates, respectively. Only two isolates represented clones of the hypervirulent lineage ST-32/ET-5 complex, but these two variants have not been found associated with disease.

Conclusions: The overall meningococcal carriage rate appears somewhat lower than it was during the Norwegian epidemic, when a rate of over 40% in the 20-24 years old males was found. The prevalence of the hypervirulent clones has changed, with ST-32 and ST-11 no longer detected in the asymptomatic population.

Duration of carriage and measurement of multiple carriage of *Neisseria meningitidis*

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Introduction: The high degree of genetic diversity of meningococcal populations has been attributed to its capacity to readily generate new genotypes through recombination. Horizontal genetic exchange between meningococcal strains must then occur during the carriage stage in the throat of healthy individuals colonized by multiple clones. Although numerous carrier studies have been performed, usually a single meningococcal colony from each sample is analysed and it is unknown how frequent carriage of multiple strains is. In an attempt to address that question, multiple colonies were isolated and characterized from consecutive throat samples of healthy carriers in the Czech Republic, Greece and Norway.
Materials and Methods: Nasopharyngeal swabs were obtained monthly for a period of 5-6 months from 206 secondary school children (15-19 years old) from the Czech Republic, 105 university students (18-24 years old) in Greece, and 126 military recruits (18-22 years old) from Norway. Cultivation was performed on selective media and up to 20 meningococcal colonies per nasopharyngeal sample were picked for storage and further investigation. The genetic homogeneity of the colonies in each sample was screened by randomly amplified polymorphic DNA (RAPD) or by a variable number of tandem repeat (VNTR) method (Yazdankhah et al., manuscript submitted).
Results: The numbers of carriers identified during the study period were 33 (16%) in the Czech Republic, 23 (22%) in Greece and 77 (61%) in Norway. Of these, 4 (12%), 9 (39%) and 21 (27%) individuals, respectively, had all samples taken and were stable carrier in the whole period. More than 4000 colonies were further analysed by the RAPD or VNTR methods. Two carriers in the Czech Republic and four carriers from Norway were found to harbour more than one genotype within a single nasopharyngeal sample.
Conclusions: The study demonstrated that more than one-fourth of the carriers identified were persistent carriers. The large majority of the samples contained only a single meningococcal strain. However, a few cases of multiple carriage were detected, indicating that colonisation by multiple clones may result in the generation of novel genotypes that can be selected within the human host.

Genotypic Comparison of Invasive Serogroup Y Meningococci from the United States and South Africa

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Over the last decade, several countries including the U.S., Israel, Sweden, and more recently South Africa, have reported an increase in the incidence of invasive meningococcal disease caused by *Neisseria meningitidis* serogroup Y (NMSY). In a previous study, serologic and genotypic comparison of isolates collected during 1992-1998 from the U.S. and Israel indicated that the high incidence of NMSY in these two countries was not attributed to a single clone of NMSY. In an expanded prospective study, we have included isolates from South Africa and compared them to isolates from the U.S. from the same time period. We analyzed 150 and 144 NMSY strains collected in South Africa and the U.S., respectively, through the national active laboratory-based surveillance programs of each country during the period 1999-2002. Strains were characterized using 16S rRNA gene sequencing and PorA VR (Variable Region) typing.

South Africa: 16S rRNA gene sequencing of the 150 isolates revealed 21 different 16S types, 15 (10% of isolates) of which were novel types. 16S types 21 and 19 were the predominant types, identified in 71% and 14% of the isolates, respectively. PorA typing revealed 9 PorA types. Two of these, PorA type P1.5-1,2-2 (75%) and P1.5-2,10-1 (14%) occurred most frequently. Seventy percent of isolates were 16S type 21, PorA type P1.5-1,2-2, and 12% were 16S type 19, PorA type P1.5-2,10-1. *United States*: 16S typing of the 144 strains from the U.S. revealed 18 different 16S types, 10 (8% of isolates) of which were novel types. In contrast to South Africa, 16S type 19 (85%) was predominant. 16S type 21, the most prevalent type in South Africa, was not seen among the U.S. NMSY isolates. Sixteen PorA types were observed in the U.S., with PorA types P1.5-2,10-1 (58%) and P1.5-1,2-2 (27%) being predominant. Fifty-two percent of isolates were 16S type 19, PorA type P1.5-2,10-1.

In addition to 16S type 19, only 2 other 16S types (types 3 and 62) were common to both countries, yet these represented <1% of the isolates from either country. Both countries had 3 PorA types in common in addition to the 2 predominant types, however, these contributed <6% collectively to the total from each country.

These results indicate that while both South Africa and the U.S. have fairly diverse sets of NMSY isolates by 16S typing and PorA typing, the majority of isolates collected during this time period from the individual countries was a single clone, 16S type 21, PorA type P1.5-1,2-2 in South Africa and 16S type 19, PorA type P1.5-2,10-1 in the U.S. We, therefore, provide further evidence that there is not a global clone singularly responsible for the high incidence of NMSY disease in the countries we have investigated to date.

Multi-Locus Sequence Analysis (MLSA) of meningococci in Scotland before, during and after the introduction of meningococcal serogroup C conjugate vaccines.

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The Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) started using multi-locus sequence analysis (MLSA) as a routine method for the characterization of isolates of *Neisseria meningitidis* in 1999. MLSA involves the sequencing and subsequent analysis of seven or more housekeeping and antigen genes. Meningococcal serogroup C conjugate (MenC) vaccines were introduced in the UK towards the end of 1999. The SMPRL introduced MLSA prior to the introduction of MenC vaccines because those strains within the electrophoretic type 37 (ET-37) complex, occurring during case clusters of disease, are often indistinguishable by standard methods. All invasive meningococci received from regional hospital laboratories throughout Scotland that were isolated from blood, cerebro-spinal-fluid (CSF), and eye were characterised by MLSA during 1999, 2000, 2001, 2002 and part 2003. Nucleotide sequencing was performed on seven housekeeping genes and one outer membrane protein gene, *porA*. Data was analysed using databases and software available through the MLST website (www.mlst.net) and the *porA* website (neisseria.org/nm/typing/porA/).

Over 400 strains were analysed from invasive disease using semi-automated robotics and automated DNA sequencers. Serogroup C disease declined by 30% after the introduction of the MenC vaccines although the decline was slow due to the length of the vaccination campaign. At the same time the overall number of cases fell from 349 in year 1999, to 343 in year 2000, to 271 in year 2001 and 194 in year 2002. Interestingly, serogroup B disease increased by 30% between 1999 and 2001 and in 2002 over 66% of all meningococcal isolates causing disease were serogroup B. The incidence of ET-37 strains fell in line with the decrease in serogroup C disease; ET-37 strains of other serogroups have not yet increased.

The decrease in serogroup C disease has led to an increase in serogroup B disease. However, this does not appear to be due to direct capsule switch. It is shown that MLSA is extremely important for the surveillance of meningococcal disease over a period of years and, in this study, has been effective not only in monitoring the impact of the MenC vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease.

Simple and rapid molecular serogrouping of *Neisseria meningitidis* by multiplex PCR-based reverse line blot assay.

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Neisseria meningitidis is a leading cause of bacterial meningitis and septicaemia in children and young adults. Rapid and reliable identification of *N. meningitidis* is crucial for judicious and expedient response to cases of meningococcal disease, including public health intervention by vaccination.

Here we describe the application of a multiplex PCR-based reverse line blot assay for the molecular serogrouping of *N. meningitidis*. The assay utilizes a single tube multiplex PCR to amplify the *N. meningitidis* specific capsular polysaccharide transport protein gene ctrA and capsular polysaccharide serogroup specific loci. Following PCR amplification, the amplicons are chemically denatured to form single strands that are then added to an automated hybridisation instrument, containing nylon membrane with immobilized oligonucleotide probes. The assay has the ability to confirm *N. meningitidis* using the *ctr*A gene, in addition to the eight most clinically significant meningococcal serogroups A, B, C, 29E, W135, X, Y, and Z.

A collection of 100 meningococcal isolates expressing a variety of serogroups including some non-serogroupable isolates, identified by established serological methods for meningococcal serogrouping were extracted by boiling and were subject to analysis by the multiplex PCR-based reverse line blot assay. Ninety-five isolates were serogroupable by the conventional methods and were all (100%) correctly genogrouped by the multiplex PCR-based reverse line blot assay, these included 11 serogroup A, 40 serogroup B, 31 serogroup C, 8 serogroup W135, 3 serogroup X, and 2 serogroup Y. Five isolates were reported as non-groupable by the conventional methods; however, by the multiplex PCR-based reverse line blot assay two further isolates were determined to be serogroup B and one serogroup Y whilst two isolates remained non-groupable.

Serogrouping of meningococcal isolates is critical for public health management of meningococcal infection and represents the minimum requirement for strain characterisation. The conventional methods involve the detection of capsular polysaccharide expression and the availability of standardised antisera to capsular meningococcal polysaccharides. With the availability of nucleic acid sequence data identifying capsular polysaccharide specific gene targets there is a growing number of PCR-based assays for genogrouping meningococci as more robust and accurate methods for serogroup identification. We describe here a multiplex PCR-based reverse line blot speciation and genogrouping assay for the clinically relevant meningococcal serogroups which uses basic molecular techniques which are available in the majority of clinical microbiology laboratories and certainly all major reference centres.

Phenotypic characterization of *Neisseria meningitidis* isolates collected from UK 15-18 year-olds at the time of introduction of serogroup C polysaccharide conjugate vaccine and in the two years post vaccination.

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The introduction of meningococcal serogroup C conjugate (MCC) vaccination for the under 18 year-old population of the UK in November 1999 prompted efforts to collect representative *Neisseria meningitidis* isolates to determine the effect on the meningococcal population structure. All Neisseria species collected were characterised using conventional serological methods at either the HPA MRU (England and Wales) or SMPRL (Scotland).
Total numbers of swabs collected acceptable to the study criteria were 13,919, 16,321 and 17,652 in 1999, 2000 and 2001. respectively yielding 2,469 (17.7%), 3,125 (19.1%) and 3,450 (19.5%) presumptive Neisseria species (isolates). The phenotyping of 2,319, 2,941 and 3,318 *N. meningitidis* isolates from 1999, 2000 and 2001 respectively form the basis of this presentation.

The percentage carriage of the *N.meningitidis* serogroups identified from 1999, 2000 and 2001 were: serogroup C (0.4%, 0.1% and 0.1%), B (4.1%, 4.2% and 4.6%), W135 (1.1%, 1.4% and 1.4%), Y (1.0%, 1.1% and 1.1%), 29E (0.8%, 0.7% and 0.5%), X (0.2%, 0.2% and 0.2%), Z (0.01%, 0.06% and 0.1%), Z/29E (0.01%, 0.04% and 0.03%) and Non-serogroupable (9.0%, 10.3% and 10.7%).

Sixty-two, 24 and 16 serogroup C meningococci were isolated in 1999, 2000 and 2001. The predominant serogroup C phenotypes identified in 1999 and 2000 were C:2a:P1.5/NT/NT or C:2a:P1.5/P1.2/NT but it was C:NT:NT/P1.15/NT (3 isolates) in 2001.

Serogroup B meningococci exhibited a great diversity of phenotypes in all years. The predominant serogroup B phenotype was B:1:NT/P1.14/NT (13.8%, 21.4% and 22.0% of serogroup B isolates in 1999, 2000 and 2001 respectively). Phenotype B:4:NT/P1.4/NT accounted for 8.3%, 8.2% and 8.0% of serogroup B meningococci in 1999, 2000 and 2001. B:1 meningococci were the predominant phenotype by 2001; rising from 6.6% of all meningococci in 1999 to 8.1% in 2000 then to 8.5% in 2001. Serotype 1 also increased for all serogroups of meningococci from 12.8% in 1999 to 14.7% in 2000 then to 14.9% in 2001. B:4 carriage declined over the three years from 7.8% to 6.0% in both 2000 and 2001.
Serogroup B sero-subtype P1.14 was detected in 6.1%, 8.0% and 8.3% of all meningococci in 1999, 2000 and 2001. Serogroup B sero-subtype P1.4 was detected in 2.8%, 2.5% and 2.9% of all meningococci in 1999, 2000 and 2001. There was a reduction in B:2a meningococci from 3 isolates in 1999 to 1 isolate in both 2000 and 2001.

A significant reduction in serogroup C meningococci was observed. No phenotypic evidence of "capsule switching" from serogroup C to B was observed. Changes in serogroup B phenotypes were observed but the significance with regard to age matched cases over the same time period remains to elucidated.

Influence of age and carriage status on salivary IgA antibody to *Neisseria meningitidis*

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Introduction: IgA is the most abundant antibody of the upper respiratory tract, and is thought to have an important role in control and protection from microbial infection at the mucosal surface. In view of the association between acquisition of natural immunity against invasive meningococcal disease and commensal colonisation by *N. meningitidis* (Nm), we have sought to define the relationship between salivary anti-meningococcal IgA, age and Nm carriage.

Materials and Methods: IgA levels to Nm OMV (Isogenic derivatives of H44/76, TR4 (P1.5,2), TR10 (PI.7,4), and a PorA deficient strain) across different age groups (3 months, 3 years, 7 years and 18+years) were measured by standard ELISA. In a separate carriage study of 258 young adult volunteers attending two Colleges, carriage was assessed by throat swabbing and salivary IgA levels were measured against lysates of the carriage strains using the same ELISA protocol.

Results and Discussion: We show acquisition of PorA-specific and non-PorA specific anti-meningococcal salivary IgA antibody with age, suggesting acquisition of immunity to a range of Nm outer membrane proteins (OMPs). Amongst these populations, we demonstrate that the prevalence of serosubtype-specific IgA reflects those subserotypes commonly circulating within the UK. In our study of meningococcal carriers, we show higher anti-meningococcal IgA levels in the saliva of current carriers than in non-carriers ($p < 0.009$). This antibody was cross-reactive against other serogroup B strains circulating in the College communities. In line with previous studies showing a relationship between Nm carriage and smoking, our data shows higher IgA levels amongst smokers, irrespective of current carriage status ($p < 0.0017$).

Conclusion: Anti-meningococcal IgA PorA specific and non-specific antibody increases with both age and current meningococcal carriage. The relationship between higher IgA levels and smoking may reflect increased intensity or length of carriage, past or present, or represent a direct effect on mucosal immunity. This data supports the assumption that IgA has an important role in mucosal defence from *N. meningitidis*, the precise mechanism that underlies this process remains to elucidated.

Identification of a new clonal complex (ST-213 Complex) of hyperinvasive meningococci among disease and carriage isolates

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Multilocus Sequence Typing (MLST) is a tool that unambiguously characterizes bacterial isolates. Internal fragments of seven housekeeping genes of 450-501 base-pairs of length are sequenced on both strands, and each fragment is assigned a unique allele number. The combination of the alleles at each of the seven loci defines the sequence type (ST). Genetically related sequence types are classified into clonal complexes each of which contains a central genotype and all the STs that share alleles at 4 or more loci. Although there is no formal way to define the central genotype, the following criteria are used: prevalence, the central genotype is generally the most frequently isolated in the population; persistence over time; geographical distribution, it is widespread over a large geographical area, such as a continent; and population genetic techniques such as split decomposition or the BURST (Based Upon Related Sequence Types) algorithm, the central ST occupies a central position. Currently an international committee agrees on the designation of new clonal complexes.

ST-213 (B:1:P1.14) was first described in a disease isolate in the UK in 1998, and has now been isolated from a variety of sources. Further analyses have revealed that this is the central genotype of a new clonal complex currently comprising 85 genetic variants which appear to be spreading rapidly and causing disease all over Europe, and especially in the UK. Sporadic cases caused by members of the ST-213 Complex had been detected since 1998 in the UK, but the prevalence has risen from 3.1% in 2001 to 8.2 % in 2002, whereas it remained steady during the same period in the rest of Europe with 1.3% of cases in 2001 and 1.2% in 2002.

Among the UK carried population of meningococci a large increase of ST-213 Complex has been detected; it represented 5.7% of the population in 1999 prior to the introduction of the MCC vaccine, and it increased to 9.7% in 2001 and to 12% in 2002. As almost all the STs belonging to this complex have only been detected recently, this could indicate that new variants are being rapidly generated from the central genotype and occupying their own ecological niche within the population. Tracking the evolution of this clonal complex may provide invaluable information on the population dynamics of *Neisseria meningitidis*

A closer look at invasive *Neisseria meningitidis* isolates from Sweden.
A negative print of our immunity?

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One of the biggest challenges today is to develop a working vaccine covering *Neisseria meningitidis* (Mc) group B since there is no available broad vaccine for this serogroup, which is the major cause of invasive meningococcal disease in many counties. Different approaches are being tried based on a number of antigens and formulations.

In order to get a better understanding of the complex problems concerning Mc serogroup B we have gathered our data of all known invasive Mc isolates in Sweden during 2001 to 2003 (n=139). The clean impression is that an extensive heterogeneity is found between the isolates. Serogroup B dominated (n=81, 56% resistant to sulphonamide), seconded by serogroup C (n=37, 65% resistant) and the other serogroups (n=21, 33% resistant).
About 45% of all isolates were not serotypable by ELISA.
Genosubtyping of the serogroup B isolates showed 15 different variants of variable region 1 (VR1), and 10 variants among non-B isolates. VR2 showed 25 different variants among serogroup B (18 variants among non-B isolates) and VR3 showed 8 different variants among serogroup B (6 variants among non-B isolates).
Multilocus sequence typing (MLST) results for the isolates will be presented and comprise more than 27 different sequence types (ST) belonging to more than 8 different clonal complexes.

All together the great heterogeneity without any major clustering of cases or isolates form an important clue concerning the general immunity to the mucosal inhabitant *N. meningitidis*.

Epidemiology of invasive meningococcal disease following introduction of serogroup C conjugate vaccination in England

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Serogroup C meningococcal disease rates rose throughout the latter part of the 1990s in the United Kingdom. After phase 1 and 2 studies of a number of candidate vaccines, meningococcal C conjugate (MCC) vaccination was introduced to the UK infant schedule in November 1999. Concurrently and throughout 2000, an immunisation program targeting all under-18 year olds was also undertaken through general practices and the school nursing services.

The impact of this major public health initiative has been monitored by enhanced epidemiological and laboratory surveillance which began two years prior to commencement of the program and remains in place. The aim of this has been to determine age specific vaccine efficacy and to characterise any change in disease causing strains.

Serogroup C disease incidence overall has fallen by 90% in the 4 years since MCC introduction. This disease reduction has occurred predominantly in those immunised but significant falls have also been seen in unvaccinated individuals indicating a herd immunity effect across a broad age spectrum. Carriage studies have shown significant falls in serogroup C isolates recovered.

Serogroup B infections showed rises in 2000 and 2001 but then fell by 15% in 2002, remaining at this level in 2003. Incidence in the first part of 2004 has been the lowest since the early 1990s. There has been no evidence of 'capsule switching' however there has been some change in the clonal composition of invading serogroup B strains with ST269 complex isolates becoming more common and ST213 emerging as a cause of about 6% serogroup B cases in 2003.

Continuing close surveillance of invasive meningococcal disease and the causative strains is necessary to identify future vaccine requirements and to assess possible needs for revaccination or boosting with MCC.

Epidemiological troubles on invasive meningococcal disease in Moscow

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Introduction: It was shown, that epidemiological situation with invasive meningococcal disease (IMD) in Moscow noticeably changed during 2002-2003 years. The present study describes: the overall incidence of IMD and purulent bacterial meningitis (PBM); the main etiological agents of PBM (including IMD) in Moscow; the age distribution of IMD; the serogroup distribution of meningococci; overall cases fatality rates of IMD and PBM.

Materials and methods: Samples of cerebrospinal fluid (CSF) and blood were taken for laboratory examination from patients, who were clinically diagnosed as having a invasive meningococcal disease or some other forms purulent bacterial meningitis. These patients were admitted from the city of Moscow to the 2nd Infection Clinic in the period 2001-2003. In total 1521 cases of IMD and PBM were registered in Moscow during 2001-2003. In 971 (63,8%) cases etiology of IMD and PBM was conformation (bacteriological and serological methods) by isolation of pathogen from CSF and/or blood samples.

Results: On the basis of the reserved data in 2001, 2002 and 2003 the parameters of disease PBM increase and has made 5,28; 5,27 and 7,0 (respectively) per 100000 population including IMD - 2,21; 2,36; 3,64 (respectively). Out of all patients (n=971, during 2001-2003) with laboratory conformation diagnosis, 65,6% (n=637) were due to N.meningitidis; 21,3% (n=207) to Str.pneumoniae; 3,8% (n=37) to H.influenzae type b and 6,1% (n=59) to other microorganisms (staphylococci, streptococci and other). A total of 287 (45,1%) laboratory conformation cases of IMD were due to serogroup A, 189 (29,7%) were due to serogroup B and 148 (23,2%) were due to serogroup C. Particular attention should paid to the increase of meningococci of serogroup A during I-VI months 2003 in comparison with I-VI months 2002 (101cases - 57,7% and 37 cases - 35,6% respectively). The age group at high risk for IMD was adults since 25 year (32,4%). Comparison age distribution of patients with IMD during two periods (I-VI 2002 and I-VI 2003) shown that among adults percent of cases increase from 47,7% in the period I-VI 2002 up to 52,1% in the period I-VI 2003. The mortality rate of PBM (2001-2003) was 17,1% and of IMD - 10,3%.

Conclusions: Such a state of epidemiological parameters should be assessed as a reflection of epidemic situation of invasive meningococcal disease in Moscow, however absence of centers of infection of IMD (all cases were incomplete), low parameters circulation of meningococci of serogroup A among carriers, belonging of strains of A meningococci only to VI and X subgroups (MLST method) specifies features of current of epidemiological process. During IX-XII 2003 hundreds of thousand of Muscovites were immunized with A polysaccharide vaccine. The incidence rate and the proportion of serogroup A meningococci decreased and have remained stable through 2004.

Use of single nucleotide polymorhism (SNP) analysis to determine relatedness of *N.meningitidis* serogroup B strains from South Australia pre and post introduction of the conjugate serogroup C vaccine

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In South Australia there are usually 30-40 cases of invasive meningococcal disease every year most of which are regarded as being sporadic rather than outbreaks although there are occasionally epidemiological links between cases. The conjugate serotype C vaccine was introduced in 2002 and since that time a noticeable and as expected, substantial, decrease in serogroup C disease has been observed. Continuing surveillance and characterisation of strains including the predominant serogroup B isolates remains a high priority. This study looked at the relatedness of 11 isolates of serogroup B strains prior to and 11 after the introduction of the conjugate serogroup C vaccine using molecular characterisation including single nucleotide polymorphism (SNP) analysis, MLST and porin sequencing. For the SNP analysis, a computer program that can identify highly informative sets of SNPs in entire MLST databases has been constructed. The SNPs either define a particular user-specified sequence type (ST) or provide a high value for Simpson's index of diversity (D). This system is unique in terms of the assembly of SNP sets on the basis of maximising the Simpson's Index of Diversity (D) with respect to the *Neisseria meningitidis* MLST database. It is not always possible to determine exactly the MLST of a strain by using only 7 SNPs but they do provide a rapid way of determining whether or not two isolates are likely to be the same - and doing that is a way that can be related precisely to the MLST database. SNP analysis identified a range of profiles (and corresponding STs) for individual isolates and also identified 3 groups of isolates. One group equated to the ST-41/44/complex/Lineage 3 (as did a number of individual isolates) but the other 2 could not be classified as currently described clonal complexes. Overall, SNP analysis characterised 12 isolates as belonging to clonal complexes (as defined in the Neisseria MLST database). For the remaining isolates, the BURST analysis of the STs defined by the SNPs, performed by the Neisseria MLST curator, shows that no currently identifiable clonal complex was available. The reasons for this may include; a) These isolates belong to a new ST that SNP analysis cannot resolve or b) These isolates belong to a new clonal complex specific to our geographic region and therefore don't fall into the currently established clonal complexes.
A sample of CSF from which no isolate was recovered but for which meningococcal DNA was detected by real time PCR also yielded directly a sequence type using the SNP analysis.
This technique is useful in rapidly determining the relatedness of isolates of *Neisseria meningitidis* in sporadic and outbreak situations as well as providing sequence type data directly from clinical specimens at a reduced cost.

A nationwide study on invasive meningococcal isolates in Iceland between 1977 and 2003 using multilocus sequence typing

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Neisseria meningitidis is an important cause of meningitis and bacteraemia worldwide. In Iceland, a relatively isolated community in the Mid-Atlantic, invasive meningococcal infections are hyperendemic. Meningococci have frequently caused epidemics in the country. It is of interest to study whether these infections are caused by strains unique to a well-defined Icelandic population , by using sequence-based molecular typing. A nationwide registry of all cases of meningococcal disease has been kept in Iceland since 1975. In this study our laboratories performed multilocus sequence typing (MLST) on all viable strains from invasive infections which were collected during the period 1977-2003. This did not include imported cases. Nucleotide sequences of seven housekeeping genes (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) were determined. Alleles and sequence types were assigned using the MLST database (http://neisseria.org/nm/typing/mlst/).

Currently 236 strains from a total of 356 have been studied; 3.4% of serogroup A, 62.6% of serogroup B, 32.7% of serogroup C, and 1.3% of other serogroups. The most common strain is that of sequence type (ST) 32 (causing 57/236 or 24.2% of all infections), followed by ST 10 (causing 37/236 or 15.7% of all cases) and then a novel ST 3492 (causing 24/236 or 10.2% of all cases). In total, 37 different ST's have been described with nine of these (ST's 1, 10, 11, 32, 60, 206, 1314, 1323, 3492) accounting for 179/236 cases (75.8%). To date, nine new ST's have been described accounting for 14.8% of all invasive infections and two previously undescribed gene sequences (one for *aroE* and one for *gdh*) have been discovered. Between the years of 1977-1979, ST 10 was the predominant strain, however appears to have disappeared almost completely after 1983. This has been replaced by ST 32 which having appeared in 1979 seems to have remained endemic in the region through the entire period. The newly described ST 3492, which was almost exclusively serogroup C, appeared in 1983 and has remained in the community. This study illustrates a substantial heterogeneity of meningococcal isolates, even in a relatively isolated population. Of interest are the number of previously undescribed ST's and the fact that 41.1% of the isolates are of ST's described previously only in Scandanavia.

Increasing prevalence of meningococcal disease caused by *Neisseria meningitidis* serogroup C in Romania

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Introduction: serogroup C is the second most common serogroup of invasive meningococcal isolates in Europe. The increase of the prevalence of serogroup C meningococci was recorded in Romania during 2001-2004.The incidence of meningococcal disease remains low in Romania: $1.15/10^5$ (257 cases) in 2000 and $1.09/10^5$ (236 cases) in 2003.All cases occuring over the last years have been sporadic and mainly due to serogroup B strains, but a significant increase in the proportion of serogroup C strains was observed.

Material and Methods: the study was performed on all *N.meningitidis* strains received by the National Reference Center for Meningococci between Jan.2003 to May 2004.The strains were identified to the species level according to classical criteria. MICs to antimicrobial agents were determined by agar dilution method according to NCCLS 2003. The data about the notification cases were obtained from the Medical Statistics Center.

Results: a total of 43 isolates from cerebrospinal fluid (88.3%), blood (9.3%) and nasopharynx (2.3%) were sent to the reference laboratory in 2003-2004. Over 50% of the cases were among children less than 5 years of age. Overall, there was a higher percentage of cases in males (52.3%) than in females (47.7%). Most cases occurred in the winter months. A very interesting finding was the evolution of the serogroups. As in previous years, serogroup B was predominant 48.8 %, although there was a strong increase in the proportion of serogroup C strains, 44.1% compared to an average very low (1%) in the previous decade (1990-2000).Serogroup A was absent (the moments of maximum incidence coincided with the two big epidemics of meningococcal disease in Romania- 96% in 1970 and 84.5% in 1987). There was only one case due to serogroup W 135. The frequence of nongroupable strains was 4.6%.
Antibiotic resistance: 31 strains of *N.meningitidis* were tested by the agar dilution method to the following antibiotics: penicillin (Pc), ciprofloxacin (Cip), cefotaxime (Ctx), rifampin (Rd), chloramphenicol (Cm), and sulfamethoxazole (Sm).All strains were fully susceptible to Ctx, Cip, Rd and Cm, against 80.6% of strains which were resistant to Sm with MIC >10 mg/l (32.2% serogroup B and 48.3% serogroup C). Regarding Pc, 6.5% of the strains were relatively resistant ($0.125 \leq$ MIC$<$ 1 mg/l).

Conclusions: the incidence of meningococcal disease in Romania remained at the same low level in 2003 ($1.09/10^5$) as in the previous years. Since 2001, an increasing number of invasive disease due to serogroup C meningococci is observed: 33.3% in 2001, 50% in 2002, 47% in 2003, and 42.3% in the first months of 2004.

A simple and inexpensive Modified Trans-Isolate medium for growth and transport of CSF in outbreaks of meningococcal disease

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Bacterial meningitis is an important cause of morbidity and mortality worldwide and particularly in sub-Saharan Africa. Due to the unpredictability of meningococcal disease epidemics in sub-Saharan Africa, the need for a quick and affordable method of culturing cerebrospinal fluid (CSF) specimens from patients with suspected bacterial meningitis obtained during adverse conditions in the field remains high. In addition, rapid and reliable serogroup identification of *Neisseria meningitidis* cultures is essential for making informed decisions regarding vaccination campaigns. Unfortunately, the standard Trans-Isolate (T-I) medium currently used to grow *N. meningitidis* and other bacterial meningitis agents directly from CSF is an expensive product (approximately $1.00 each, plus $1.00 for shipping) and is only produced in three laboratories worldwide. A Modified Trans-Isolate (MT-I) medium has been developed at CDC as a simplified growth and transport medium for meningococci. It is more affordable than the standard T-I and can be produced locally (approximately $0.50 each, needs no shipping), providing a cheaper and more rapid response to the outbreak. The modified medium has been evaluated at CDC using four reference strains of meningococcal serogroups A and W135 from the CDC stock collection. The reference strains were diluted from a suspension equivalent to a McFarland 0.5 turbidity (1.5×10^8 organisms) to dilutions of 1.5×10^3, 1.5×10^2, and 1.5×10^1 organisms/mL, which were used to inoculate the T-I and MT-I. The bottles were inoculated in triplicate and incubated in four different conditions (37°C with CO_2, 37°C without CO_2, 25°C without CO_2, and 42°C without CO_2). A loopful of the inoculated broth (10ul) was removed from each bottle at 24, 48, and 72 hours and streaked onto a Blood Agar Plate (BAP). Growth or survival of *N. meningitidis* in each medium was recorded by observing the presence or absence of colonies in each of the four zones where the culture was streaked (1+ to 4+). Results show that after 24 hours incubation at 37°C, with or without CO_2, growth of *N. meningitidis* was identical for both T-I and MT-I; growth was observed in all four quadrants (4+) of the BAP for dilutions of 10^3 and 10^2 organisms/mL and in three quadrants (3+) at 10^1 organisms/mL. After 48 to 72 hours incubation at 37°C, with or without CO_2, growth in T-I and MT-I was identical (4+) for dilutions of 10^3 and 10^2 organisms/mL with minimal variation at 10^1 organisms/mL, likely due to sampling variation. The comparative ability of T-I and MT-I to support bacterial recovery from CSF is currently being evaluated at Institut National de Recherche en Santé Publique in Bamako, Mali. MT-I may prove to be the ideal medium for immediate use during response to meningitis outbreaks.

Recent increase in slide agglutination discrepancies in serogroup identification of *Neisseria meningitidis* among U.S. public health laboratories

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Reliable serogroup identification of *Neisseria meningitidis* remains an important responsibility of the U.S. public health laboratories. From 1997-2002, the Active Bacterial Core surveillance (ABCs) laboratory network collected 1,298 *N. meningitidis* isolates that ABCs sites and Centers for Disease Control and Prevention (CDC) tested by slide agglutination serogrouping (SASG). Among these isolates, discrepant SASG results were reported for 58 (4%). With the introduction of the new conjugate vaccine in early 2005, and with that the need to accurately monitor meningococcal serogroups to track vaccine efficacy, we conducted a prospective study to track SASG proficiency at both ABCs and non-ABCs state health laboratories, and at CDC, to determine if there had been a change in the rate of SASG discrepancies. Isolates (n=545) collected from January 2003 through April 2004, were tested by SASG at the state health laboratories and at CDC. Real-time serogroup-specific PCR (SGS-PCR) was performed at CDC to resolve discrepancies. There were 102 (19%) discrepant SASG results between all of the state laboratories and CDC. Discrepant results between ABCs laboratories and CDC were reported for 33 of 281 isolates (12%). Discrepant SASG results between state laboratories outside the ABCs network and CDC were reported for 69 of 264 isolates (26%). For 87 (85%) of the 102 total discrepancies, the isolates were incorrectly reported as non-groupable (NG). Of the 102 total discrepancies, 16 (16%) isolates misidentified as NG were attributable to ABCs laboratories, 53 (52%) to non-ABCs state laboratories, and 18 (18%) to the CDC lab. However, all 87 isolates judged NG actually expressed a specific capsule, identified by SASG at either a state laboratory or at CDC, and the serogroup was subsequently confirmed by SGS-PCR. Serogroups B and Y were the majority (92%) of isolates misidentified as NG. These data show that SASG discrepant results among laboratories is a widespread problem; furthermore, the number of discrepancies has increased recently. Also, state laboratories belonging to the ABCs network are more likely to correctly identify meningococcal serogroups than are laboratories outside the network. The factors contributing to these discrepancies are likely a combination of poor quality antisera, lack of standardized criteria for interpretation of SASG results, human subjectivity, and a need for training. Introduction of SGS-PCR into those state laboratories equipped to run real-time PCR would also allow the correct identification of serogroup specific genes.

Worldwide epidemiology of meningococcal disease: examining a global risk

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Background: To determine the worldwide burden of invasive meningococcal disease (IMD) and serogroup distribution of *Neisseria meningitidis* we conducted a systematic analysis of meningococcal epidemiological literature and available data from national meningococcal surveillance networks.

Results: In industrialized countries, IMD is primarily endemo-sporadic with occasional outbreaks, especially in some communities (e.g., barracks, dormitories): annual incidence ranges from 1–3 cases per 100,000 persons. Rates in the US and Canada were 0.8 and 1.13/100,000 in 2001, respectively. In the US, serogroups Y, B, and C are equally present whereas in Canada, serogroup C predominates followed by B, Y, and W135. Incidence rates and serogroup distribution vary significantly between different US states and Canadian provinces. Similarly, wide variations occur between European countries, especially since the introduction of routine meningococcal conjugate serogroup C immunization programs in some countries. Surveillance conducted in 20 European countries showed an overall IMD incidence of 1.82 /100,000 in 2002. Serogroup B disease predominated, followed by C, W135, and Y. In Russia, IMD incidence was 2.4/100,000 in 2002; serogroup A disease predominated, followed by B and C. In 2001, in South America, IMD incidence was 1.25/100,000 in Argentina, 1.52 in Brazil, and 2.40 in Chile. In these regions, serogroups B and C predominate. In Southeast Asian countries and Taiwan in 2001-2002, IMD incidence varied from 0.06 to 1.20/100,000. Serogroup B disease predominated followed by W135, Y, and A. In 2002 the IMD incidence in Australia was 3.0 with serogroups B and C predominant. In New Zealand incidence averaged 13.9/100,000 from 1996-2000 due to a serogroup B epidemic.
In many developing countries, IMD is endemo-epidemic. The highest burden of IMD occurs in the "Meningitis Belt" of sub-Saharan Africa. Until 1999, most cases were due to serogroup A, but recently, W135 has emerged as a potential agent for large-scale epidemics. During the 2002-2003 season, epidemics were reported in five sub-Saharan countries (Benin, Burkina Faso, Ghana, Niger, Nigeria), primarily due to serogroup A, followed by W135. In 2000 and 2001, serogroup W135 was responsible for worldwide outbreaks linked to the Hajj pilgrimage.
In industrialized countries, the highest IMD incidence occurs in children younger than 1 year, with a second peak of sporadic disease in adolescents. In Africa IMD is rare in children up to 3 months of age, highest at about 1 year, and infrequent in persons aged 30 years and older. However, during epidemics, incidence in older children and young adults can increase.

Conclusion: *N. meningitidis*, in both endemic and epidemic forms, is the cause of substantial morbidity and mortality worldwide, with a complex and variable epidemiology. The challenge of changing serogroup distributions, both geographically and over time, enhances the need for broad and global vaccine coverage.

Clonal distribution of Czech invasive *Neisseria meningitidis* isolates

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Clonal feature of all invasive *Neisseria meningitidis* isolates, submitted to the Laboratory and being acquired from approximately 70% of cases of meningococcal disease in the Czech Republic since 1990, has been assessed continually in order to monitor the regional spread of hypervirulent lineages, responsible in general for the majority of cases of the disease and for its most severe forms. Multilocus enzyme electrophoresis typing together with the whole-cell ELISA phenotyping was used for routine screening of isolates, multilocus sequence typing was adopted for characterization of isolates representative of electrophoretic types.

Within the period followed, gradual endemic shift in clonal distribution of meningococcal population has been detected. Due to the predominance of the ET-15, C:2a:P1.5,2 strain of the ST-11/ET-37 complex, representation of serogroup C prevailed since 1993 till 1999 over the country (overal incidence of the disease of 2.2/100,000 reached in 1995). In parallel to the decline of the ST-11/ET-37 complex in the late 1990s, endemic occurence of serogroup B lineages, the ST-32/ET-5 complex and the ST-18 complex of serotype 22-related strains, increased for a temporary period. At present, a repeated increase of occurence of ST-18 and ST-145 subcomplexes of the ST-18 complex was observed.

Strains of the ST-269, ST-23/cluster A3 and ST-213 complexes, related to B:15,23:P1.5, Y:2c:P1.5,2 and B:1:P1.14 phenotypes, respectively, and showing hypervirulent nature being obtained almost exclusively from invasive forms of infection, have been isolated in a smaller extent since the end of the 1990s. Among ST-11 isolates, a clonal variant of the ET-15 strain, differing in the "ME" locus and showing exclusively the C:NT:P1.5,2 phenotype, has been found since 2001. Isolation of hypervirulent ST-41/44 complex/lineage III and ST-8 complex/cluster A4 strains, spread in some of southern and western European states, and of the serogroup B variant of the ET-15 strain was rare over the period. In general, representation of hypervirulent strains among the whole of isolates studied has reached 79% over the past decade.

In opposite to ST-11/ET-37 complex isolates, expressing usually the characteristic phenotype, isolates of some of the hypervirulent serogroup B complexes, for which a comprehensive vaccine is not available, showed a higher extent of phenotype degeneration and phenotyping was thus not fully reasonable for assessment of their clonal relevance.

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Detection of strain specific Single Nucleotide Polymorphisms (SNPs) for real-time non-culture strain characterisation of *Neisseria meningitidis*

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Approximately 50% of cases of meningococcal infection in England and Wales are now confirmed by PCR only, for which no isolates are available for outbreak investigations. Epidemiological surveillance for meningococal infection requires accurate strain characterisation for both culture and non-culture confirmed cases. Rapid strain identification for non-culture confirmed cases is critical for the public health management of meningococcal infection.

Multilocus Sequence Typing (MLST) is now the established method for strain characterisation of *Neisseria meningitidis* and is based upon sequencing a 450-500bp internal region of seven different housekeeping genes. MLST has been adapted for non-culture confirmed cases however, it requires three to five days before a result is available and additionally there is a significant associated cost.

A detailed analysis of the Neisseria MLST databases (i) PubMLST (http:// mlst.net) and (ii) UK Health Protection Agency, Meningococal Reference Unit (MRU) clinical isolate database has determined alleles for MLST loci which are predictive of the four major clonal complexes of *Neisseria meningitidis* found in England and Wales. Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) have been calculated for these alleles in determining clonal complex identity. Sequence alignments of alleles using Bionumerics™ and Sequencher™ computer software has identified a minimal number of single nucleotide polymorphisms (SNPs) which will identify the previously determined key alleles.

Allelic discrimination assays using the Taqman™ real-time PCR system have been designed which detect the ST-11/ET-37 clonal complex. Clinical sensitivity of the assays has been assessed on clinical specimens from culture proven cases where the isolate had been MLST typed. All results were concordant.

These novel assays have been used to screen for ST-11/ET-37 clonal complex cases of meningococcal infection amongst the PCR confirmed cases during the period April 2003 – March 2004 thus providing enhanced surveillance post introduction of the meningococcal C conjugate vaccine. The assays provide cost effective rapid preliminary non-culture strain identification of meningococci for real-time epidemiology and improved outbreak management.

Meningococci from epidemics in northern and southern Ethiopia 2002-03 characterized by phenotypic and genetic methods

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Ethiopia is part of the Meningitis Belt and is affected by meningitis caused by *Neisseria meningitidis* in both endemic and epidemic forms. Recent large outbreaks and epidemics were reported in 1981, 1988-89, 2001 and 2002. The objective of this project was to characterize recent meningococcal isolates in Ethiopia and compare them with those collected during the epidemic of 1988-89 using the same methods.

Patients from the Gondar province and the SNNPR during 2002 and 2003 were included in the study. Cerebrospinal fluid (CSF) specimens were collected from patients with clinical meningitis and were inoculated in Trans-Isolate transport medium if turbid. Meningococci were isolated using standard methods. CSFs from culture-negative patients were subjected to *porA* and capsule-specific PCR. Meningococci were studied by dot-blotting, multilocus sequence typing and characterization of outer membrane extracts by SDS-PAGE. DNA from a selection of new and old strains was subjected to PCR for *nadA* and *tbpB* genes and lipooligosaccharide synthesis genes *lgtA-Z*.

A total of 95 patients from 2002–03 were included. Of these, 71 (74.7%) were confirmed positive for *N. meningitidis* either by culture (n=40) or by PCR (n=31). All 40 strains were A:4/21:P1.9 and belonged to the sequence type ST-7. The 31 PCR-positive samples showed meningococcal DNA from a P1.20,9 strain, and 21 of them were also positive for the serogroup A capsule gene. Protein band patterns in outer membrane extracts were very homogeneous, although some variation was observed in PorA and NadA expression and the repertoire of Opa proteins and lipooligosaccharide types. The 21 strains from 1988–89 were phenotypically very similar to the recent isolates, but belonged to the ST-5. The *nadA* gene was present in all 14 strains selected for PCR. Results from studies of *tbpB* and *lgtA-Z* genes will be presented.

The meningitis epidemics in northern and southern Ethiopia in 2002-03 were caused by serogroup A *N. meningitidis* strains of ST-7, which were antigenically very homogeneous. Epidemiological and microbiological surveillance is essential to ensure that changes are detected in time for preventive measures to be implemented.

Predictors of Immunity After a Major Serogroup W-135 Meningococcal Disease Epidemic, Burkina Faso, 2002

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Background: The African "meningitis belt" regularly experiences epidemics caused by *Neisseria meningitidis* serogroup A. During 2002, Burkina Faso documented the first large serogroup W-135 meningococcal disease epidemic. Exposure to meningococci through oropharyngeal carriage leads to short-term immunity, but carriage and immunity had not been previously studied in a population experiencing a serogroup W-135 epidemic. **Methods:** We conducted a cross-sectional meningococcal carriage and seroprevalence survey with a cluster sampling design in an epidemic district and a non-epidemic district of Burkina Faso. At the end of the epidemic (5/17-5/26/2002), we obtained and cultured oropharyngeal swabs, and collected sera from eligible district residents aged 5 – 25 years. We measured serogroup W-135 serum bactericidal activity (SBA, a functional correlate of protection), opsonophagocytic activity (OPA), and serogroup W-135 IgG and IgM anti-capsular antibodies by enzyme-linked immunosorbent assay. We identified predictors of elevated W-135 SBA titer (equal or above an estimated protective threshold of 1:8) using multivariable logistic regression. **Results:** From 899 eligible participants with oropharyngeal culture results, we obtained 304 sera. The meningococcal serogroup W-135 carriage rate was 25.2% in the epidemic district and 3.4% in the non-epidemic district (Odds Ratio [OR] 9.5, $p<0.0001$). Compared to non-epidemic district residents, epidemic district residents had higher geometric mean concentrations of serogroup W-135 IgG (0.88 vs. 0.22 ug/ml, $p<0.0001$) and IgM (0.83 vs 0.52 ug/ml, $p=0.0004$), higher W-135 SBA titers (68.8 vs. 9.7, $p<0.0001$) and higher W-135 OPA titers (66.4 vs. 26.9, $p=0.0004$). W-135 SBA titers $\geq$1:8 were observed in 60.4% and 34% of epidemic and non-epidemic district residents, respectively; 53.2% of epidemic and 21.3% of non-epidemic district residents had titers $\geq$1:64. In a multivariable model, current meningococcal W-135 carriage (OR 12.7, $p<0.0001$), age greater than 16 years (OR 4.4, $p=0.0001$), and residence in the epidemic district (OR 2.1, $p=0.0435$), were significant predictors of W-135 SBA titer $\geq$1:8. **Conclusions:** Natural exposure to meningococcal serogroup W-135 during the 2002 epidemic produced population immunity. Residents of a district undergoing a meningococcal serogroup W-135 disease epidemic had significantly higher serogroup W-135 carriage rates and immune responses compared to residents of a non-epidemic district. Both direct (carriage) and indirect (district residence, age) markers of meningococcal exposure predicted individual immunity against serogroup W-135. When this immunity wanes, the population may become susceptible to future epidemics.

The Usefulness of PFGE in Subtyping *Neisseria meningitidis* Serogroup B Isolates of the Hypervirulent ET-5 Complex

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Meningococcal disease is an important health problem in the U.S. and world wide. In the U.S., the overall incidence of meningococcal disease has decreased from 1.3/100,000 population in 1997 to 0.6/100,000 population in 2002. However, the percentage of disease due to serogroup B has increased from 29% in 1997 to 36% in 2002. A licensed vaccine to prevent serogroup B disease is currently unavailable in the U.S.; furthermore, *Neisseria meningitidis* serogroup B (NMSB) isolates identified by multilocus enzyme electrophoresis (MEE) typing as being a part of the ET-5 complex may potentially cause epidemics. 16S rRNA typing also differentiates isolates associated with the MEE ET-5 complex. With the increase of serogroup B disease in the U.S., molecular typing of these isolates is extremely important to assess if the isolates belong to the ET-5 complex. While few U.S. laboratories are equipped to perform MEE or 16S typing, many are equipped to perform pulsed-field gel electrophoresis (PFGE) typing as part of PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance. This study was conducted to evaluate the usefulness of PFGE in differentiating NMSB isolates of the ET-5 complex from NMSB non ET-5 complex isolates.

We selected 35 NMSB isolates representing 28 different 16S types, and 11 additional NMSB isolates that were either sporatic, surveillance, or outbreak associated isolates of 16S type 4, the predominant U.S. type. Of the 46 total NMSB analyzed, 18 isolates were of the ET-5 complex. After restriction digestion of NMSB DNA with *NheI* followed by PFGE analysis, 41 different PFGE patterns were identified. Thirty –eight of the patterns were unique and found in only 1 isolate, while 3 patterns were representative of more than 1 isolate. Cluster analysis of the PFGE patterns generated 6 distinct groups. All patterns representing the 18 isolates of 16S types: 4, 83, 85, and 98 associated with the ET-5 complex grouped together in a distinct cluster that was < 60% similar to the other 5 pattern clusters. These preliminary data suggest that clustering of PFGE patterns associated with the ET-5 complex could be useful for laboratories that are not equipped for MEE typing or 16S typing.

INVASIVE MENINGOCOCCAL DISEASE ASSOCIATED WITH VERY HIGH MORTALITY IN THE NORTH-WEST OF POLAND

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Introduction. Invasive meningococcal disease (IMD) may be presented clinically as meningitis and/or as a state ranged from mild transitory meningococcemia to a rapidly evolving circulatory collapse with multiple organ dysfunctions. Especially this late state, known as fulminant meningococcal septicemia is connected with very high mortality rate up to 50%. Last year in the North-West of Poland, bordering Germany several fatal cases of fulminant meningococcal septicemia were diagnosed. Thus the aim of the study was to establish epidemiological links and/or the relatedness between meningococcal isolates responsible for fatal infections in this part of Poland.

Methods. The isolates collected in West-Pomeranian area from April 2003 to April 2004, isolated from patients with IMD and their close contacts were identified and serotyped by standard methods. Macrorestriction fragment length polymorphism analysis of genomic DNA was performed by pulsed field gel electrophoresis (PFGE), using the *Spe*I restriction enzyme for DNA digestion. In the case of negative culture clinical material was collected *in vivo* or *post mortem* from patients with suspected IMD.

Results. There were 22 cases (1.29/100,000) of confirmed or suspected IMD during the studied period in the region, with mortality of 41%. However among the first 11 cases, 8 were fatal, with mortality of 72.7%. All fatal cases developed very rapidly, progressed to the death in several hours and were diagnosed as fulminant meningococcal septicemia connected with Waterhouse-Friderichsen syndrome. The patients were 3 weeks, 6, 7 and 18 months, 2, 3 and 16 (two boys) years old. In two fatal cases diagnose was not confirmed microbiologically, in one Gram-negative diplococci were observed, in one serogroup W135, in one C, and in four serogroup B meningococci were confirmed by culture or PCR. Serotyping as well as PFGE revealed that there were different meningococcal strains responsible for all invasive infections in this region. Only 4 cases of meningitis with good outcome were caused by isolates of the same meningococcal strain C:2b:P1.2,P1.5. These isolates, frequent also in close contact carriers were found in this area as well as in central Poland in 2002.

Conclusions. High mortality due to IMD in West-Pomeranian area was not associated with the dissemination of one new epidemic clone and was not linked with the increase of the prevalence of serogroup C meningococci observed in Poland from 2002. However, this situation accelerated changes in registration system, in which till recently only meningococcal meningitis was notified. From last year there is a compulsory notification of all IMD: meningitis and septicemia.

Characterisation of serogroup Y meningococci in Scotland from 1978 onwards using multi-locus sequence typing

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Neisseria meningitidis is an important cause of meningitis and bacteraemia worldwide. Our laboratory holds a unique collection of invasive meningococcal isolates dating back to 1964. Multi-locus sequence typing (MLST) and *porA* typing methods were used for the retrospective characterisation of invasive serogroupY meningococci. Nucleotide sequences for seven housekeeping genes (*abcZ*, *adk*, *aroE, fumC, gdh*, *pdhC*, *pgm*) and an antigenically variable gene (*porA*) were determined. Alleles and sequence types were assigned using the MLST database (http://neisseria.org/nm/typing/mlst/) and ST's were assigned to lineages using the BURST program. Three variable regions were examined within *porA* VR1, VR2 and VR3 and variant names were assigned using the *porA* variable region databases (http://neisseria.org/nm/typing/pora/ and http://www.show.scot.nhs.uk/smprl/).

The incidence of non-invasive serogroup Y meningococcal disease in Scotland remained level between 1978 and 2004. Only 41 isolates were from invasive disease. These isolates were not associated with any one particular ST or a particular clonal complex. However the most common complex was ST-23 complex/Cluster A3. There were also cases of ST-766 and ST-167, neither of which are assigned to a complex although they are similar and only differ by the *gdh* allele. Invasive serogroup Y meningococcal disease was associated mostly with the young or old. Although serogroup Y meningococcal disease was uncommon and a rare cause of invasive disease in Scotland between 1978 and 2004 it is essential that microbiologists are aware of its potential for increasing in incidence due to the introduction of the MenC vaccine, and its increased incidence in the USA.

Associations between meningococcal phenotype, clonal complex and death from meningococcal disease in Europe, 1999-2002

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The risk of death from meningococcal disease has been previously shown to be associated with the age of the case, clinical presentation and the serogroup and serotype of the infecting organism. We investigated the association between disease outcome and strain characteristics in Europe using cases reported to the EU-IBIS project between 1999 and 2002.

Three datasets, containing different levels of strain characterisation were derived from the EU-IBIS database. The first dataset included all laboratory confirmed cases from those countries reporting outcome. The second was a subset of the first, incorporating all culture confirmed cases, where serogroup, serotype and serosubtype were reported. The third dataset was derived by matching EU-IBIS reports to the results of multilocus sequence typing from the EU-MenNet project (2000-2002 only). Associations between meningococcal phenotype and clonal complex and death were investigated using multivariable logistic regression models controlled for age, country and year.

[*]The overall case fatality ratio (CFR) in the EU-IBIS serogroup dataset (N= 27,048) was 7.3%, with a range between 4.5 and 15% across countries. The odds of death were significantly higher for serogroup C (odds ratio 1.9, 95% CI 1.7 - 2.1) and W135 (odds ratio 1.5, 95% CI 1.2 – 1.9) compared to serogroup B. The odds of death were also significantly higher for those aged >=15 compared to those aged <15 (odds ratio 2.1, 95% CI. Within the culture confirmed dataset (N=14,218), the odds of death were significantly higher (odds ratio >2) for C:P2.2a and W135:P2.2a compared to the baseline strain (B:P3.4). There were no significant associations between clonal complex and outcome in the smaller MLST dataset (N=1,291).

The quality of reporting of cases and outcomes may vary between countries, and even though the models were controlled for country this may not fully account for possible biases. Nevertheless, EU-IBIS is a valuable resource, providing a large amount of data, which can be used to monitor the emergence and spread of hypervirulent strains across Europe.

[*] Provisional results

Transmission dynamic models for predicting the direct and indirect impact of meningococcal serogroup C conjugate (MenC) vaccination

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The meningococcal serogroup C conjugate (MenC) vaccine programme in the UK has been shown to generate high levels of herd immunity in addition to providing direct protection to vaccinated individuals. A realistic age-structured dynamic model was developed to predict both the effects of direct vaccine protection and the indirect effects of reduced carriage of serogroup C meningococci on future disease incidence. The model was parameterised and fitted to epidemiological data from England and Wales although several of these parameter estimates, including the duration of vaccine protection, currently remain uncertain. The equilibrium model (before vaccination) was used to estimate the basic reproduction number of serogroup C infection (carriage and disease), which was 1.36 in the base case. The dynamic model was used to investigate the effects of a range of vaccine strategies, including alternatives to the strategy adopted in the UK. Catch-up vaccination generated substantial herd immunity and controlled disease much more rapidly than routine infant vaccination or routine infant and teenage vaccine strategies. The results were sensitive to changes in the assumptions regarding the method of vaccine action, particularly the duration of protection and efficacy of vaccination against carriage acquisition. However, the ranking of different strategies did not change when these key assumptions are altered. This model can be used to help design future vaccine strategies, both in the UK and elsewhere.

Effectiveness of meningococcal serogroup C conjugate vaccine four years after the introduction of mass immunisation in England.

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The UK introduced the meningococcal serogroup C conjugate (MCC) vaccine into the routine immunisation schedule at 2, 3 and 4 months in November 1999. In addition, the vaccine was offered to all children under the age of 18 years old in a phased catch-up campaign. The vaccine was well accepted with routine infant immunisation coverage of over 90% and coverage of around 85% in the catch-up campaign. The number of cases of serogroup C disease fell by 80% in the targeted age groups within 2 years of the start of the campaign. Early analyses suggested high vaccine effectiveness in all age groups. In addition to this direct protection the vaccine was shown to reduce serogroup C carriage leading to significant herd immunity.

We reanalysed vaccine effectiveness (the percentage reduction in the attack rate in vaccinated compared to unvaccinated children) using data on laboratory confirmed cases of serogroup C disease occurring in England between January 2000 and March 2004 in the targeted age groups. The data were stratified by age at which vaccine was received. The routinely vaccinated cohort was further stratified by age at disease onset (< 18 months old versus • to 18 months old).

Vaccine effectiveness remains high (• 83%) in all children who had been received MCC vaccine in the catch-up campaign between the ages of 1 and 18 years. The effectiveness of routine infant immunisation overall was 65% (95% CI 4 – 86%), however, clear differences can be seen after stratifying by age at onset of disease. In children aged under 18 months, the vaccine effectiveness is high (93% 95% CI 66 to 99%), but vaccine protection wanes significantly (p=0.001) in children aged 18 months or above (-84%, 95% CI –7562 to 71%). Although a negative effectiveness is reported it should be noted that the confidence intervals are very wide and it is unlikely that the attack rate is truly higher in the vaccinated group.

The waning of vaccine protection after only 18 months in routinely vaccinated infants is concerning, but it is important to note that the number of cases in these cohorts remains low, probably due to the high levels of indirect protection in the population. It is clearly necessary to investigate ways in which the duration and level of direct protection conferred by MCC vaccines administered in infancy can be improved, including the possibility of offering a booster dose in the second year of life.

Potential capsule switching from serogroup Y to serogroup B: genetic and antigenic characterization of 3 such *Neisseria meningitidis* isolates causing invasive meningococcal disease in Nanaimo, British Columbia, Canada.

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Three unusual serogroup B *Neisseria meningitidis* isolates recovered from three invasive meningococcal disease (IMD) cases in Nanaimo, British Columbia, Canada are described in this poster. Two isolates were recovered from two related cases living in the same household in January 2001 and the third isolate was recovered from a separate case living in the same community in January 2002. All three isolates were typed by murine hybridoma monoclonal antibodies as B:2c:P1.5. Partial DNA sequences of their *porB* and *porA* genes confirmed that these isolates contain the serotype 2c epitope in their PorB outer membrane proteins (OMPs) as well as the serosubtype epitope of P1.5 in their PorA OMPs. The two isolates from 2001 have identical pulsed-field gel electrophoresis pattern while the third isolate from 2002 gave a DNA fingerprint with only 1 band difference from the pattern given by the 2001 isolates. Multi-locus sequence typing confirmed that all three isolates are related because of an identical sequence type (ST) 573.

Many serogroup Y isolates recovered from Canadian IMD cases have been typed as serotype 2c with the serosubtype antigen of P1.5. Testing of other serogroups of *N. meningitidis* isolates, including over 100 serogroup B organisms from Canadian IMD cases in 2001 and 2002, did not reveal any non-group Y meningococci with the serotype 2c antigen in their PorB OMPs other than the three serogroup B isolates from Nanaimo, British Columbia. Although none of the serogroup Y Canadian isolates was identified as ST-573, some are showing the presence of MLST house keeping gene alleles similar to those found in ST-573. Also no other serogroup B meningococci other than these three B:2c:P1.5 isolates were found to have a MLST profile identical or similar to ST-573. This suggests these B:2c:P1.5 isolates may in fact be serogroup Y organisms and we postulate that they arise from potential capsule switching from serogroup Y to serogroup B.

Molecular characterisation of *Neisseria gonorrhoeae* – identification of one ciprofloxacin-resistant strain circulating in the Swedish society

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In Sweden, the incidence of gonorrhoea has increased almost annually since 1997, mainly due to a rise of domestic cases. In addition, the resistance of *Neisseria gonorrhoeae* (GC) to ciprofloxacin has increased rapidly since mid-1990s. In the first months of the year 2003, an epidemiological core group comprising domestic cases of young heterosexuals of both sexes was identified in a county in the middle of Sweden (Gävleborg county). All GC isolates from these cases (n=19) were designated as serovar IB-10 and showed a high level of resistance to ciprofloxacin.

Aims: To phenotypically and genotypically characterise *Neisseria gonorrhoeae* (GC) serovar IB-10 isolates cultured in Sweden during 2002 and 2003, in order to investigate the homogeneity/heterogeneity of the strains as well as the possibility of a circulation of one ciprofloxacin-resistant strain in the Swedish society.

Materials & methods: GC serovar IB-10 isolates (n=36), cultured in Sweden during 2002 and 2003, were included. Phenotypic characterisation was performed by antibiotic susceptibility testing (Etest) and serovar determination with the Genetic Systems and the Pharmacia (Ph) panels of monoclonal antibodies. Genetic characterisation was performed by means of sequencing of a unique region of the *gyrA* gene by Pyrosequencing technology (as a genetic indicator of resistance to ciprofloxacin and for species verification), sequencing of the entire *porB* gene, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) that analyses internal segments of the *porB* gene and of the *tbpB* gene, and pulsed-field gel electrophoresis (PFGE) with the restriction enzymes *Spe*I and *Bgl*II. Phylogenetic trees were constructed with TREECON v1.3b by using Neighbor-joining method.

Results: All GC serovar IB-10 isolates from the cases of the domestic core group in Gävleborg county (n=19) showed similar MIC values in the antibiograms (differences within ± 1 $\log_2$), were designated as Ph serovar Bropst, had identical *porB1b* gene sequence, and indistinguishable or closely related PFGE fingerprints. Isolates comprising indistinguishable or closely related phenotypic and genotypic characteristics were also identified in other counties of Sweden. The phylogenetic analysis of the *porB1b* gene sequences suggested that the minor differences (one to two nucleotides) of some isolates represented the ongoing evolution of the *porB1b* sequence of the same strain. Further results will be included at the presentation.

Discussion: The thorough molecular characterisation in combination with phenotypic and epidemiological information identified a circulation of one GC serovar IB-10 ciprofloxacin-resistant strain in the Swedish society during 2002 and 2003. The transmission of the strain caused a domestic core group consisting of young heterosexuals in the county of Gävleborg in Sweden and the strain was also identified in several other Swedish counties. Minor diversities between some isolates of the strain were identified, however, this probably only reflecting the ongoing evolution.

Genotypic Characterisation of Invasive Meningococci Isolated in England and Wales Preceding Introduction of the Meningococcal Conjugate Serogroup C Vaccine.

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It has been known for some time that the antigenic and genetic structure of meningococcal populations carried asymptomatically differ from those observed among disease-associated meningococcal isolates. This has led to the concept of meningococci with an increased propensity to cause invasive disease, the so-called hyper-invasive lineages. Descriptions of these meningococci have, to date, relied on observational data concerning the frequency of particular antigenic or genetic types in carriage and disease isolates. This has been hampered to the high diversity of meningococci, especially those recovered from asymptomatic carriage. The advent of height-throughput nucleotide sequecne analysis permits the collection of definitive genetic data from a variety of genetic loci for large numbers of isolates. This permits the application of population genetic techniques to compare populations, providing a means of defining the hyper invasive meningococcal more rigorously and investigating their evolution.

As a first step in such an analysis it is important to identify and characterise a coherent collection of disease-associated isolates. In November 1999 a conjugate vaccine against serogroup C meningococci (MCC vaccine) was introduced into the UK national immunization programme. All individuals under the age of 18 years were offered the vaccine. Implementation of the vaccine may have a direct affect on the population of meningococci causing disease. An indirect effect on disease may also occur as carried meningococci in the human population is likely to change. In order to study the effect of the immunization programme on meningoccal disease, it is necessary to determine base-line and therefore the disease-associated isolates obtained for one year prior to the vaccine introduction are important collection to examine. During 1999 there were 2783 reported cases of meningococcal disease in England and Wales and, for over 1700 of these, a culture was referred to the Meningococcal Reference Unit (HPA, Manchester). Here we present the results of the characterization of these meningococci, by MLST, permitting the genetic composition of this collection to be definitively established. As the UK meningococcal carriage study sampled carried meningococci in the same period this analysis will provide a means of comparing invasive and carried meningococcal isolates on a large scale and developing evolutionary models for the emergence pf hyper invasive lineages.

Impact of vaccination against serogroup C meningococci on the epidemiology of meningococcal disease in the Netherlands

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Introduction Since 1999 the incidence of meningococcal disease increased from 3.6 cases to 4.5 cases per 100,000 inhabitants in 2001. The proportion of serogroup C meningococcal disease rose from 14% in 1999 to 39% in 2001. The increase of meningococcal disease in 2001 was exclusively due to the increase of serogroup C. Nation-wide vaccination of the population in the age group 0-9 year had been accomplished in the period June - November 2002 and one shot vaccination at the age of 14 months has been implemented in the national vaccination program since September 2002.
Materials and Methods Meningococcal isolates were characterised in the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) by serogrouping, serotyping, and sequencing of the variable regions of *porA*, encoding the PorA epitopes. The number of cases of meningococcal disease of the period between January 2001 - April 2002 (pre-vaccination period) was compared with that of the period January 2003 - April 2004 (post-vaccination period).
Results and Discussion In 2003, the incidence of meningococcal disease decreased to 2.0 cases per 100,000 inhabitants, while in 2002 the incidence was 3.8 cases per 100,000 inhabitants. During the pre-vaccination period 593 isolates of serogroup B meningococci were received by the NRLBM. The number of cases of serogroup B meningococcal disease decreased by one third to 400 cases during the post-vaccination period. In contrast, during the pre-vaccination and post-vaccination periods, the number of isolates of serogroup C meningococci received by the NRLBM was reduced by 88% from 417 to 50 cases, respectively.
Among persons with age between 14 month and 18 years the number of cases of serogroup C disease reduced from 266 cases in the pre-vaccination period to only 2 cases (0.8%) during the post-vaccination period. These two patients had not been vaccinated. Remarkably, the number of serogroup C isolates among persons > 18 years was reduced by 68% (from 151 to 48 cases) in the post-vaccination period. This reduction of cases of serogroup C disease among non-vaccinees might be indicative for herd immunity. However, the bimonthly distribution of cases of serogroup C disease showed that the incidence already declined before the introduction of the vaccine independent of the age group.
The NRLBM received no isolates of serogroup C meningococci during the months April-May 2004.
Conclusions In 2003 the incidence of meningococcal disease in the Netherlands showed a decrease of 48%. This decline of meningococcal disease is partly caused by the natural fluctuation in the incidence of serogroup B as well as serogroup C meningococcal disease. Nevertheless, the vaccination against serogroup C meningococcal disease was very effective; after the introduction of the vaccine, cases of serogroup C meningococcal disease were no longer observed among vaccinees by the NRLBM.

Epidemiology of meningococcal disease in Germany, report of the national reference center (NRZM)

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Up to 800 cases of meningococcal disease are reported annually to the Robert-Koch Institute (Berlin). The national reference center for meningococci analyses strains and samples from >530 patients per year, more than 100 of which are referred for culture independent analysis. According to EU-IBIS data, Germany belongs to low-incidence countries in Europe (<1/100.000/a). There is currently no generally recommended C conjugate vaccination campaign. Nevertheless, serogroup C cases increased from 20% before 2002 to 30% of the cases in 2002 and 2003. No increase of the general incidence of meningococcal disease was noted in the same period of time. The reference laboratory focussed on molecular techniques for fine typing, i.e. PorA sequence typing, FetA sequence typing (routinely used since 2004), and MLST. Serotyping is restricted to the serotypes 2a and 2b, because of the good correlation of serotypes 2a and 2b with the ST-11 and ST-8 complexes, respectively. PorB sequence analysis is not applicable for routine use due to the fragment length required for molecular PorB type assignment. FetA sequence typing (Thompson et al. 2003) proved to be extremely helpful for discrimination within the ST-11 complex, especially in cluster analysis. Other molecular tools for fine typing of ST-11 strains in clusters have been developed and will be presented. MLST was performed by the MLST center of the EU-MenNet giving rise to a representative overview of clonal lineage distribution throughout Europe. Approximately 10% of the isolates were tested as intermediate resistant to penicillin. The minority of those isolates exhibited a modified penA gene. Clusters of meningococcal disease in Germany which require public health action occur at low frequency and with a size of less than 10 patients. Most clusters are due to the ET-15 clone (as determined by *fumC* sequencing) with PorA types P1.5,2 and P1.5-1,10-8, and FetA types 3-6 and 4-1.

Geographic differentiation in *Neisseria meningitidis*

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Composition and prevalence of different sequence types (STs) is known to vary with geographic location in populations of carried *Neisseria meningitidis*. Such geographic differentiation may be the result of gene flow restricted by distance, or may be maintained by natural selection, perhaps in response to the genetic make-up of the host population. Therefore geographic differentiation has the potential to inform us about the rate at which types circulate in the population, and investigate what selection pressures shape the pathogen population.

We studied 822 isolates of *N. meningitidis* collected from healthy carriers in Bavaria, Germany in the winter of 1999 - 2000. The isolates were collected from either schools or military barracks. Because STs (defined as the unique combination of alleles at seven loci typed by MLST) tend to emphasize differences between genotypes, we studied genetic diversity at the nucleotide level. We used population genetics techniques to test for evidence of genetic subdivision correlated with geographic distance, age of the host, and institution type (schools versus military).

There was no evidence for genetic structuring on the basis of host age or institution type, indicating that they do not define distinct transmission systems. However, significant genetic differentiation was found between different localities. For schools, whose catchment areas are small, a positive correlation was found between genetic distance and geographic distance for pairs of isolates. For military barracks, no correlation was found, consistent with their large catchment areas which cover the entire region.

Genetic diversity of *Neisseria meningitidis* based on variable number tandem repeat typing (VNTR)

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Introduction: Tandem repeats of mini and microsatellite DNA sequences are a significant source of information for characterization of pathogenic bacteria. The availability of whole genome sequences of *N. meningitidis* has opened the way to find repetitive DNA motifs and their application to epidemiological investigations. We identified novel variable number tandem repeat (VNTR) loci by a bioinformatic approach and analyzed a panel of *N. meningitidis* strains, which had been characterized previously by multilocus sequence typing (MLST) or multilocus enzyme electrophoresis (MLEE).

Materials & Methods: Eighty-two of the 107 *N. meningitidis* isolates used by Maiden *et al*. (1998) in the development of MLST, 47 isolates recovered in different counties in Norway in relation to local outbreaks, and 17 serogroup W135 isolates recovered in several parts of the world were included in the study. The genomic sequences of *N. meningitidis* strains MC58 and Z2491 were analyzed using the Tandem Repeat Finder software and 15 different sequences containing tandem repeats were selected. After testing a set of genetically diverse meningococcal isolates, 4 loci were selected for further analysis of the 146 isolates. PCR products (5 microliters) resulting from amplifications of the 4 VNTR loci for each strain were mixed together and electrophoresed on a 2% agarose gel. Gels were stained by ethidium bromide and photographed under UV illumination. Results were analyzed using the Bionumerics software package.

Results: VNTR-analysis of the meningococcal isolates used in the development of MLST showed low degree of similarity within the ST-8 complex (50-85 %) and the ST-11 complex (40-75 %). The similarity was somewhat higher for isolates of the ST-1 (50-100%), 4 (45-100%), 5 (35-100%), 32 (35-100%) and 41/44 (50-100%) complexes. VNTR-patterns of isolates recovered from local outbreaks in Norway were highly similar and comparable to phenotypic properties and MLST/MLEE data. The serogroup W135 isolates, which all belonged to ST-11 complex harboured different VNTR-patterns. While the Hajj-related isolates from 2000 showed the same VNTR pattern, the W135 isolates from outbreaks in Burkina Faso in 2001 and 2002 showed different and variable VNTR patterns.

Conclusions: VNTR typing is appropriate for short-term epidemiology of meningococcal isolates in relation to outbreaks. The method might be especially valuable to differentiate among ST-11 strains, as shown by the VNTR analyses of serogroup W135 ST-11 meningococcal isolates isolated since the mid 1990s. VNTR-analysis may be used for fine typing of meningococcal isolates, characterized by MLST. Based on PCR primers tagged with different fluorescent dyes, the method can be automated and become a rapid genotyping assay.

Poster Session I

Host Response

Differential host genetic response to secreted proteins of *Neisseria meningitidis.*

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Host-meningococcal interaction is a complex process that involves numerous host cell molecules and signal transduction pathways, depending on the cell type and experimental conditions used. The process also involves numerous meningococcal virulence factors, many of which remain unknown.

We have recently reported that live meningococci induce the expression of a number of inflammatory and apoptosis-related genes. We have also identified and characterized a number of meningococcal secreted proteins (MSPs) which are exported to the extracellular meliu via various secretion mechanisms, including type I and V pathways. However, the relative contribution of MSPs to the host genetic response remains unknown.

We hypothesised that MSPs play essential roles in the pathogenesis of disease and that at least part of the host genetic response is induced by MSPs. To test this hypothesis, we examined differential host gene expression in human meningeal-derived cells, in response to endotoxin-depleted MSPs compared to live bacteria. Using expression arrays, up-regulated expression of several pro-inflammatory and apoptosis-related genes was found to be induced by MSPs. The transcription and translation of representative genes was confirmed by using various methods. Increased interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2) gene transcription was confirmed using real-time PCR. Up-regulated IL-8, IL-6, ICAM-1 and COX-2 protein expression were confirmed by ELISA, flow cytometry or immunoblots. Furthermore, exposure of cells to MSPs or live meningococci induced a modest but significant resistance effect to staurosporine-induced apoptosis.

Secreted meningococcal virulence factors are therefore important in inducing host inflammatory responses and resistance to apoptosis, and they are worthy of extensive investigation.

Neisseria meningitidis–Induced Death of Cerebrovascular Endothelium: mechanisms triggering transcriptional activation of inducible nitric oxide synthase

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The intense host response to meningococcus reflects marked functional and morphological alterations in blood-brain barriers. We showed previously that mouse-derived cerebrovascular endothelium responded to meningococcal lysates with a robust NO response, resulting in the loss of cell viability. To understand how the NO synthase-2 gene in endothelium is activated by meningococcus, we investigated upstream roles for specific protein kinases.

Using known kinase inhibitors, and measuring both mRNA expression and nitrite release, we found MEK2, p38 kinase and PI 3-kinase (but not MEK1 or phospholipase C) to be implicated in the NO synthase-2 response. Recruitment of these kinases by meningococcus did not depend on the prior release of the proinflammatory cytokines TNFα or IL-1β from endothelium. These endothelial cells were found to express Toll-like receptors 2, 4, and 9, and antibodies directed against TLR 2 and 4 (but not TLR 9) blocked the NO synthase-2 response to meningococcus. Both meningococcus-induced translocation of NF-kB, and also endothelial cell death, were blocked by a known inhibitor of p38 kinase. Calpain inhibitor-1 blocked the NO synthase-2 response to meningococcus, which is further evidence of a role for NF-kB.

The pilus and porin of *Neisseria gonorrhoeae* cooperatively induce Ca^{2+} transients in epithelial cells.

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Purified pili and porin from *Neisseria* quickly mobilize calcium (Ca^{2+}) stores in monocytes and epithelial cells, ultimately influencing host cell viability as well as bacterial intracellular survival [Källstrom *et al.* (1998) *J. Biol. Chem.* 273,21777-21782; Müller *et al.* (1999) *EMBO J.* 18, 339-352; Ayala *et al.* (2001) *Cell. Microbiol*. 3, 265-275; Ayala *et al.* (2002) *Infect. Immun.* 70, 5965-5971]. Here, we examined the Ca^{2+} transients induced in human epithelial cells during infection by live, piliated *N. gonorrhoeae*. Porin induced an influx of Ca^{2+} from the extracellular medium less than 60 seconds post-infection. The porin-induced transient is followed by a pilus-induced release of Ca^{2+} from intracellular stores. The timing of these events is similar to that observed using purified proteins. Interestingly, the porin-induced Ca^{2+} flux is required for the pilus-induced transient, indicating that the pilus-induced Ca^{2+} release is, itself, Ca^{2+} dependent. Several lines of evidence indicate that porin is present on pili. Moreover, pilus retraction strongly influences the porin- and pilus-induced Ca^{2+} fluxes. Based on these and other results, we conclude that the pilus and porin cooperate to modulate calcium signaling in epithelial cells, and propose a model to explain how *N. gonorrhoeae* triggers Ca^{2+} transients in the initial stages of pilus-mediated attachment.

Neisserial outer membrane vesicles which recognize CEACAM1 suppress CD4$^+$ T lymphocyte function *in vitro*: Implications for pathogenesis and immunoprophylaxis.

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Outer membrane vesicles (OMVs) derived from the human pathogen *Neisseria meningitidis* are currently employed as a meningococcal vaccine. The colony opacity (Opa) proteins, expressed by this (and other) neisserial species are important virulence determinants, and constitute significant vaccine antigens. We have demonstrated that OMVs derived from *Neisseria sp.* contain functional Opa proteins, some of which recognize carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Interactions between Opa and CEAMCAM1 were consistent with suppressed activation and proliferation of the (leukemic) Jurkat CD4$^+$ T lymphocyte cell line, and further, induced apoptosis in such cells *in vitro*. We propose that the presence of CEACAM1-reactive Opa variants in a vaccine preparation may result in diminished lymphocyte responses, and by corollary, less likelihood of protective immunity. Further, we suggest that neisserial isolates deficient in CEACAM1-binding activity should be used in the future development of OMV based vaccines; and that Opa-containing vesicles may enable therapeutic immunosuppression, (and/or targeted drug delivery) mediated by ligation of CEACAM1, and the subsequent signaling events associated therewith.

Neisseria meningiditis and innate immune evasion? A comparison between mice and men

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Innate immune mechanisms are the first line of defense against invasive bacteria. This is demonstrated by the fact that *Neisseria meningiditis* is efficiently cleared by the murine innate immune system within five days of introduction into the host. In contrast, *Neisseria meningiditis* infection in a human results in passage of the bacteria through the nasopharyngeal epithelium into the bloodstream, culminating in sepsis, meningitis, and inflammation of the meninges and the brain. Upon initial invasion in humans, bacteria encounter the resident immune cells of the nasal lamina propria, but proceed through the tissue and into the bloodstream. To allow this process to occur, we hypothesized that *Neisseria meningiditis* may evade the innate immune system present at this interface. To begin to understand this initial process and the role of innate immune responses in meningococcal disease we examined the recruitment of inflammatory immune cells to the peritoneum in mice in response to both heat killed *Neisseria meningiditis* and Neisserial porin, PorB. Heat killed *Neisseria meningiditis* was injected into wild type C57/Bl6 mice, CD80, CD86, CD80/86, and TLR2 knockout mice, C3H/HeJ LPS non-responsive mice, and cobra venom (C5a depleted) mice to compare cell recruitment in each of these murine strains. After 24 hours, recruitment of PMNs was decreased in CD80 mice; Macrophages, NK cells, and eosinophils were decreased in cobra venom treated mice. Recruitment of both mast cells and CD4+ T cells was decreased in C3H/HeJ mice; interestingly, mast cells, CD4+ T cells, and B1 cells were increased in TLR2 mice. Incubation of PBMCs with live *Neisseria meningiditis*, as measured by upregulation of CD54, was less than observed for incubation with heat killed meningococci. These results suggest that meningococci affect the innate immune response, allowing for subsequent infection and pathology in the host. The differential interactions of the murine innate inflammatory response and the human response are currently under investigation.

Neisseria gonorrhoeae Enhances the Infection of Dendritic Cells by Human Immunodeficiency Virus Type 1 (HIV-1)

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Abstract

Clinical studies indicate that *Neisseria gonorrhoeae* (GC) has the capacity to enhance infection by human immunodeficiency virus 1 (HIV-1). We explored whether GC could enhance HIV infection by activating dendritic cells (DCs). Our results showed that GC had the ability to dramatically enhance HIV replication in human DCs during co-infection. The GC component responsible for HIV infection enhancement might be peptidoglycan, which usually activates Toll-like receptor 2 (TLR2). Involvement of TLR2 was supported by which GC lost its ability to stimulate the expression of HIV in the DCs of TLR2-deficient HIV-1-transgenic mouse. These results provide one potential mechanism that GC infection might increase the viral replication in co-infected patients.

The role of DC-SIGN (CD209), CEACAM1 (CD66a) and Heparan Sulfate in Interaction of Dendritic Cells with *Neisseria gonorrhoeae* and *Escherichia coli*

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Neisseria gonorrhoeae (GC) or *Escherichia coli* K12 (*E. coli*) expressing opacity (Opa) proteins adhere to human host cells and stimulate phagocytosis. These effects are in part due to the binding of certain Opa proteins to CEACAM1 (CD66a) and heparan sulfate (HS) proteoglycan, which promote adherence and phagocytosis of bacteria. While dendritic cells (DCs) possibly express both CEACAM1 and HS proteoglycan, we observed that both Opa^- and Opa^+ GC and *E. coli* avidly bound to DCs. DCs efficiently phagocytosed *E. coli* but not GC. Surprisingly, the interaction of *E. coli,* but not GC, with DCs was partially inhibited by anti-DC-SIGN (DC-specific intercellular adhesion molecule-grabbing nonintegrin, CD209) antibody, but not anti-CEACAM1 antibody and heparin. To prove that DC-SIGN is a receptor of *E. coli*, we showed that HeLa cells expressing human DC-SIGN antigen (HeLa-DC-SIGN) bound and engulfed *E. coli* and these interactions were blocked by anti-DC-SIGN antibody or mannan. Bacterial phagocytosis was confirmed by electron microscopic analysis. We further defined that the *E. coli* ligand for DC-SIGN lies in the core lipopolysaccharide (LPS), and expression of O-antigen at the non-reducing end of the LPS blocks the DC-SIGN-promoted phagocytosis in HeLa-DC-SIGN and DCs. These results demonstrate a role for DC-SIGN in interaction of DCs with *E. coli* and provide a potential mechanism of how O-antigen acts as an anti-phagocytic factor.

DIFFERENTIAL EFFECTS OF *NEISSERIA MENINGITIDIS* AND *LACTAMICA* ON CYTOKINE PRODUCTION.

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Neisseria is a Gram negative diplococcal bacteria which primarily colonizes human mucosal surfaces. It includes many commensal species as well as the pathogenic species *Neisseria meningitidis,* one major cause of bacterial meningitis.
N. meningitidis enters the organism by droplet transfer and mainly results in asymptomatic nasopharyngeal carriage, at times in the presence of commensal bacteria such as *N. lactamica*. However, occasionally, *N. meningitidis* colonizes and invades the nasopharyngeal epithelial cells, from where it can gain access to the circulation, cross the blood-brain barrier and cause meningitis, an inflammation of the meninges in the brain, which can be fatal.

To date it remains unclear why *N. meningitidis* can induce inflammation and *N. lactamica* largely remains a commensal bacteria.
A number of studies have demonstrated that in contact with different cell types, *N. meningitidis* induces inflammatory cytokines (TNFα, IL-8), dependant on the function of toll-like receptor (TLR)-4 (1) and/or TLR-2 (2, 3). Therefore, the outcome or not of disease may result from a differential secretion of cytokines after exposure to *N. meningitidis* and/or *N. lactamica,* in connection to a differential role played by TLRs.

In this study, three strains of *N. lactamica* (8064, 2nd 94 and 23970) were compared with three strains of *N. meningitidis* (H44/76, B16/B6, M101/93) by infecting two different respiratory epithelial cell lines (A549 and D562), which would represent the first line of bacterial infection. Production of the inflammatory cytokines IL-6 and IL-8 using ELISA and intracellular cytokine staining (ICS) techniques, were used to measure the outcome of infection. In addition, the regulation of IL-6 and IL-8 mRNA was measured by Taq Man real-time methodology. To follow up infection, cells were infected for a short time (1, 2 or 3 hours), or for a longer period (18 hours).
Interestingly and unexpectedly, at 10^8 cfu/ml all three strains of *N. lactamica* produced higher levels of IL-6 and IL-8 in both cell lines. A reduction in cytokine secretion with the pathogenic bacteria was not due to an increase in cell death, as this result was observed as early as 3 hours after infection when there was no cell death, suggesting the effect is specific to the presence of the commensal strains. The levels of NFkappaB were also further up regulated in the cells infected with *N. lactamica* strains, suggesting the bacteria are activating the NFkappaB pathway, which may also account for the increased production of the inflammatory cytokines tested. Currently under investigation, is whether a differential expression of TLR-2 or TLR-4 in the cell lines after infection with bacteria, may account for the differences in responses observed.

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3. AC Pridmore *et al* JID 2001 183:89-96

Characterisation of naturally acquired CD4 T cell mediated memory to *Neisseria meningitidis* serogroup B in the mucosal and systemic compartments

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Introduction: Within the nasopharynx immune cells are localised in mucosally associated secondary lymphoid tissues such as the palatine tonsil (PT). Positioned in the upper airways, PT are thought to play a key role in the induction of immunity to locally encountered antigen, such as *Neisseria meningitidis (Nm)*. In support of this, we have previously demonstrated the presence of memory CD4+ T cells responsive to Nm serogroup B (MenB) antigens in the PT that were strongly associated with increasing age. In this study we assess the phenotype and compartmentalisation of CD4 T cell mediated immunity to MenB antigens in the presence and absence of LPS.

Materials and Methods: Putative regulatory CD25+ cells were depleted from tonsillar mononuclear cell (TMNC) preparations using magnetic beads. CD45RO+ memory cells were similarly purified from TMNC and peripheral blood MNC by negative depletion and labelled with carboxyflourescein succinamidyl ester (CFSE) prior to culture. Outer membrane vesicles from MenB, strain H44/76 and outer membrane preparations of a LpxA- mutant, were used as antigens. Samples were removed (days 3-7) and proliferation quantified by tritiated thymidine. Secreted cytokine levels were determined in culture supernatants using cytometric bead array. Intracellular cytokines and Th1/Th2 cell surface markers were examined specifically in the proliferating (CFSE+) memory (CD45RO+) CD4+ T cells by FACS analysis.

Results and Discussion: After 6 days culture 1.6-15.7% of mucosal memory (CD45RO+) CD4+ T-cells were found proliferating in response to MenB antigens. Up to 80% of these cells expressed IL18 receptor (IL18R) and the remainder expressed CRTH2, suggestive of Th1 and Th2 effector phenotypes respectively. Detection of intracellular IFN-gamma in dividing CD4s confirmed the Th1 bias. In contrast, only 0.6-5.4% peripheral CD4+CD45RO+ T cells were responsive to Men-B antigens and the ratio of IL18R and CRTH2 expression suggested these had a balanced Th1 and Th2 effector response, which was confirmed by intracellular staining for both IFN-gamma and IL-13. Increases of up to 1000 picograms per ml of secreted IFN-gamma were detected in both compartments following stimulation, in addition to IL-10 and TNF-alpha in the periphery. Surprisingly, these responses were unaltered by the presence or absence of LPS. CD25+ depletion experiments demonstrated the involvement of regulatory activity that limited the proliferative immune responses against MenB antigens in adult PT and suppressed otherwise detectable responses in children.

Conclusion: We have identified key differences in the naturally acquired anti-MenB memory T-cell phenotype between the mucosa and periphery that may relate to differences in the effector immunity required in these two compartments. How immunisation modulates the complex balance of T helper cell and T regulatory cell activity shown in these experiments is currently under investigation.

The Role of Mannose-Binding Lectin (MBL) in Complement Mediated Killing of *Neisseria meningitidis*

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MBL is a serum collectin that activates complement in an antibody-independent manner when bound to repeating sugar moieties on microorganisms. Genetic deficiency of the MBL pathway is associated with increased risk of numerous infectious diseases including invasive meningococcal disease. We have reported that the primary binding sites of MBL on *N. meningitidis* are not lipooligosaccharide (LOS) but the two major, nonglycosylated, outer membrane (OM) proteins, opacity protein (Opa) and porin PorB. This binding is not lectin-mediated. We now confirm that MBL interacts specifically with these proteins when expressed on the bacterial surface. Purified PorB inhibited the binding of MBL to whole organisms in a dose dependent manner by ELISA. Similarly, an Opa producing clone of *E. coli* bound significantly more MBL than *E. coli* that contained only the vector. Lack of binding to LOS was consistent with the major LOS structures that terminate in galactose or sialylated galactose and are not MBL carbohydrate ligands. However, 20% of strains do make an LOS molecule (L6) that truncates the Lacto-*N*-neotetraose structure at *N*-Acetyl-glucosamine, a carbohydrate that binds MBL. We now report that MBL does bind to this LOS molecule on the L6 prototype meningococcal strain M992. Available evidence indicates that complement activation on meningococcal surfaces is regulated predominantly by factor H (FH) and we confirm that FH binds to purified porB and whole organisms by ELISA. We found that MBL only partially inhibited FH binding to 1 of 3 strains but FH completely inhibited the binding of MBL to the 1 strain tested. In bactericidal assays, MAb 3F8, known to inhibit the complement activation function of MBL, significantly inhibited the killing of meningococci in normal human serum (NHS). The decrease in kill was far more pronounced in post immunization serum from a properdin deficient individual indicating that MBL primarily augments the CP but not the ACP. To study the bactericidal function of MBL in the absence of specific antibody, we absorbed NHS and MBL-deficient serum with a group C strain and tested for residual bactericidal activity for the same strain. The bactericidal activity of the absorbed NHS was only slightly reduced in the absence of antibody, whereas that of the absorbed MBL-deficient serum was completely abolished. We conclude that 1) MBL binds primarily to meningococcal Opa and PorB but can bind to the L6 LOS; 2) MBL primarily supports CP mediated killing of meningococci; and 3) in the absence of specific antibody, MBL is critical for the bactericidal killing of *N. meningitidis*.

Mechanotransduction by *Neisseria gonorrhoeae* Type IV pili activates MAPK signaling and enhances host cell cytoprotection

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The *Neisseria gonorrhoeae* type IV pilus (Tfp) undergoes cycles of extension, substrate tethering, and retraction. We tested the hypothesis that Tfp retraction influences host cell gene expression through exerting tension on the host membrane. We report that during attachment wild-type and retraction-defective bacteria alter the expression of an identical set of epithelial genes. Pilus retraction, *per se*, does not regulate novel gene expression. Rather, retraction enhances the expression level of a subset of infection-regulated genes via MAPK activation. These results can be reproduced by applying artificial force on the epithelial membrane. Importantly, this force-mediated signaling enhances the ability of the cell to withstand staurosporine-induced apoptosis. Our results suggest that pilus retraction stimulates mechano-sensitive signaling pathways that enhance the expression of stress-responsive genes, and lead to the activation of cytoprotective signaling. Taking these and other findings into account, we present a model for the role of pilus retraction in influencing cell survival.

LPS Modulates the Recognition of Meningococci by Human Dendritic Cells.

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N. meningitidis infection depends on the ability of the bacteria to cross the epithelial barrier of their oropharyngeal habitat. Within this barrier, dendritic cells (DCs) are one of the first lines of defence, likely to play a major role in initiating an immune response. We have shown that recognition of *N. meningitidis* by human DCs is efficient for unencapsulated strains. In contrast, expression of the capsule prevents adherence and phagocytosis of the bacteria.
In this study, we examined the role of lipopolysaccharide in *N. meningitidis* – DC interaction. Exogenous and endogenous sialylation of L3,7,9 LPS was found to markedly impair recognition of *N. meningitidis* (3h p.i.). To test, whether the Lacto-N-neotetraose extension of the LPS alpha-chain was also protective for meningococci in the interaction with human DC, we constructed isogenic mutants (lgtA, galE, pgm) of the hypervirulent isolate MC58 in a siaD-negative, unencapsulated parental. In infection experiments, recognition of the mutants expressing a truncated LPS was greatly enhanced compared to wild-type strains. Similar results were obtained with mutants derived from serogroup B hypervirulent strain H44/76, indicating that these features are characteristic for several pathogenic meningococci. Further mutation of the LPS beta- and gamma-chain (lgtG, rfaK) did not enhance recognition of MC58, indicating that the protective effect mainly resides in the alpha chain moiety. In addition, we could show, that expression of serogroup B capsule effectively prevents recognition of strains expressing a truncated LPS isoform.
To test, whether these effects might be of importance in vivo during infection, two independently generated, L8-positive were selected in colony blots. The parental strain was found to switch to an L8-positive phenotype with a frequency of approximately 1:1000. These strains displayed the same level of adherence and phagocytosis as a mutant strain harbouring an inactivated lgtA-allele. Several mutants were constructed to identify relevant bacterial structures mediating the interaction with DC. No effect was found for the major surface antigens opc and opa. Recognition of meningococci by human DC was independent of the mannose-receptor and of DC-SIGN as shown by function-blocking experiments using mannan and a monoclonal anti-DC-SIGN Ab. Interestingly, a significant proportion (50%) of the recognition of meningococcal strains expressing a truncated LPS was mediated via receptors of the scavenger-receptor family and could be inhibited by addition of poly-I and poly-G.
Although the nature of the physical interaction between human DCs and *N. meningitidis* is not clear at the moment, it will be of significant interest to determine bacterial and cellular factors contributing to recognition of *N. meningitidis*. In view of capsule phase variation and a high degree of L8-positive variants especially during colonization, these results might be of relevance for the immune response against *N. meningitidis*.

Macrophage Activation and Cytokine Production Induced by the TLR2 Ligand *Neissseria Meningitidis* Porin, PorB

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Macrophages detect and respond to pathogens through a variety of receptors, including the newly described family of Toll-like receptors (TLR). Activation of macrophages by TLR ligands leads to multiple signal transduction events culminating in the production of various cytokines. Our laboratory has recently described PorB, the major outer membrane protein from *Neisseria meningitidis*, as a ligand for TLR2. We show here that PorB induced activation of peritoneal macrophages from wild type C57BL/6 mice, but not TLR2 or MyD88 deficient mice, leads to mitogen activated protein kinase (MAPK) activation and NF-kappaB nuclear translocation. The signaling events induced by PorB result in an upregulation of B7-2 (CD86), CD40 and CD14 along with the production of the cytokines IL-1beta, IL-6, IL-12p70 and TNF-alpha. This demonstrates a potential role of porins in the induction of the inflammatory response associated with neisserial disease, including gonococcal infection and meningococcal meningitis.

Serum antibody responses in patients infected with serogroup A subgroup III *Neisseria meningitidis*

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Meningitis caused by *N. meningitidis* is affecting Ethiopia in endemic and epidemic forms. While the epidemic in 1988/89 (45,000 cases) was caused by subgroup III meningococci of sequence type 5 (ST-5), epidemics in 2001 (7,000 cases) and 2002 (3,500 cases) were caused by subgroup III meningococci of ST-7. Despite the large number of cases each year, studies of antibody mediated immunity against capsular and sub-capsular antigens in sera from patients with serogroup A meningococcal disease are few; although studies of sera from Finns infected with subgroup III meningococci in the 70s and from Gambians infected with clone IV-1 meningococci in the 80s have been done. This study investigates the antibody responses towards capsular and sub-capsular antigens in convalescent sera from patients infected with serogroup A ST-7 meningococci (A:4/21:P1.20,9) in northern and southern Ethiopia.

Patients were included from the Gondar province and the SNNPR in Ethiopia during 2002 and 2003, if showing clinical signs of meningitis and a turbid CSF. Sera were to be collected from patients in the acute phase (0 to 6 days after onset of disease), early convalescent phase (10 days to 6 weeks) and late convalescent phase (3 to 12 months). Out of the 95 patients included, 71 were confirmed *N. meningitidis* positive either by culture or PCR. Following extensive field tracing of patients confirmed positive with meningococci in distant rural areas, acute and one or two convalescent sera were obtained from 51 of them. Control sera were collected from 80 individuals from the same areas and age groups. The IgG responses towards serogroup A polysaccharide, outer membrane vesicles and lipooligosaccharide (L11) were analyzed by ELISA and compared to levels in control sera. Serum bactericidal activity was used to assess the functional activity in these sera. The analyses are currently ongoing and results will be presented.

Challenge of Human Monocytes with *Neisseria gonorrhoeae*: Effects of Dose on Cytokine Profile

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During natural infections, *Neisseria gonorrhoeae* elicits a powerful inflammatory response involving the infiltration of neutrophils and monocytes to the site of infection. However, the degree of inflammation in an infected individual varies widely. Women infected with gonorrhea frequently experience asymptomatic infections while infected men exhibit a wide range of symptoms. While many different surface components have been identified as being important for the establishment of infection, the role of these components in eliciting an inflammatory response remains unclear. We have hypothesized that variation in the degree of symptoms seen in an infected individual could be influenced simply by the size of inoculum at the time of infection, rather than by variation in the types of surface antigens being expressed by the infecting organisms. In order to test this hypothesis, we challenged primary human monocytes with various amounts of gentamicin-killed, piliated *N. gonorrhoeae*, strain F62, expressing the lacto-*N*-neotetraose LOS structure, and measured resulting cytokine production. When monocytes were incubated with gonococci at an MOI of 100:1 (bacteria to host cell) for 18 hours, significant levels of cytokines were detected (i.e. tumor necrosis factor (TNF), interleukin-10 (IL-10), interleukin-8 (IL-8), and interleukin-1 beta (IL-1 beta). However, at an MOI of 1:10 (bacteria to host cell), IL-8 remained strongly induced while the other cytokines tested were expressed at insignificant levels. To determine whether lower cytokine production in MOI 1:10 challenges was simply due to a slower rate of production, we analyzed cell supernatants from a 24 hour timecourse. There was no observed accumulation of TNF, IL-10, or IL-1 beta. Since IL-8 production by monocytes can be stimulated by very small amounts of TNF and IL-1 beta in culture, we tested whether low levels of either of these cytokines in the MOI 1:10 challenges could be responsible for the observed IL-8 production. We pretreated monocytes with neutralizing antibodies specific for TNF or IL-1 beta and found that production of IL-8 was not ablated, indicating a direct mechanism for IL-8 stimulation during gonococcal infections. These data suggest the direct production of IL-8 in response to the interaction of gonococci with monocytes and demonstrate that a low bacterial inoculum can lead to a dramatic production of neutrophil chemoattractant in the absence of damaging levels of TNF. This scenario could result in immune clearance of infection without involvement of the adaptive immune response.

Activation of $CD4^+$ T cells is induced by Neisserial pili

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Background: Type IV pili of *Neisseria gonorrhoeae* mediate the initial binding of the bacteria to host cells, as well as being involved in cellular signal transduction. The widely expressed transmembrane molecule CD46 participates in the regulation of complement activation and has also been shown to act as a cellular receptor for gonococcal pili and a variety of other pathogens. Recently CD46 was identified as a co-stimulatory receptor for T cells. The aim of this study was to determine whether a change in the activation status of the $CD4^+$ T cells is induced upon pilus binding to cells and to determine whether this effect is mediated via CD46.
Methods: Various strains of *N. gonorrhoeae* with mutations in the major pilus subunit, PilE, and the putative adhesin and minor pilus component, PilC, were used to infect primary $CD4^+$ T cells. The effect of the infection was determined using a variety of assays to study cellular activation, proliferation and cytokine induction following stimulation with bacterial strains and purified pili.
Results: Activation of T cells was enhanced by expression of the PilC pilus component, since all strains, excluding a PilC deletion mutant, were shown to mediate increases in the expression of the early activation marker CD69. Co-stimulation with all gonococcal strains and CD3 promoted T cell proliferation, however, this was enhanced by infection with strains expressing both PilE and PilC, indicating the importance of these pilus components in regulating T cell activity. In the absence of CD3 stimulation all strains of *Neisseria* did not induce proliferation, and at a higher MOI all strains induced much higher but comparable T cell proliferation, probably mediated by other bacterial cell wall components. Additionally, purified pili from the wild-type strain could mediate T cell proliferation in a dose dependent manner, when co-stimulated with CD3, confirming the stimulatory activity of the pili. However, despite this initial activation, the production of IL-10 by T cells following infection with the wild type *Neisseria* strain indicated that the activated cells may be involved in the down-regulation of immune response, possibly via bystander inhibition of other cells.
Conclusion: This data provides further evidence that CD46 is involved in $CD4^+$ T cell activation, since bacterial strains expressing PilC and pili can mediate a change in the activation status of the cells, which has not been noted upon infection with mutant strains. Furthermore, the pilus of *N. gonorrhoeae* has been confirmed as an important bacterial virulence factor that is able to modulate the status of T cells and to induce signals in cells.

OPACITY-ASSOCIATED PROTEIN (OPA) MODULATES SERUM RESISTANCE MEDIATED BY LIPOOLIGOSACCHARIDE SIALYLATION IN *NEISSERIA MENINGITIDIS*

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Neisseria gonorrhoeae and *N. meningitidis* have evolved intricate mechanisms to evade complement-mediated killing. Sialylation of lipooligosaccharide (LOS) uniformly confers high levels of serum resistance in gonococci. However, the contribution of LOS sialylation in serum resistance and pathogenesis of *N. meningitidis* is unclear. We have previously demonstrated that gonococcal LOS sialylation increases factor H (fH) binding. In contrast, LOS sialylation in five diverse strains of *N. meningitidis* (unencapsulated mutants derived from serogroup B strains MC58 and H44/76, and serogroups C, W-135 and Y strains 2120, 171 and Y2220, respectively) did not result in altered in fH binding.

SiaD (unencapsulated, LOS sialylated) mutants were more serum resistant than the corresponding *siaD/lst* (unencapsulated, LOS not sialylated) mutants of all the tested strains, except serogroup B strain MC58, where LOS sialylation did not augment serum resistance. Resistance to serum in sialylated strains was observed only at low serum concentrations (less than 5%). Flow cytometry analysis of complement component binding to the mutants confirmed that the serum resistant mutants bound less C3, C4 and C5b-9; IgG and IgM binding was not altered by LOS sialylation.

In an effort to understand why LOS sialylation did not enhance serum resistance uniformly among all tested strains, we sought to detail the mechanism of complement activation on the meningococcal surface. We focussed on detailing targets for complement component C4 because the classical pathway is essential for initiating complement activation and bactericidal killing on Neisseriae. As we have reported previously, LOS was found to be a target for C4 on all strains. In addition, the MC58 mutants (the only instance where LOS sialylation did not enhance serum resistance) showed an additional C4 target which we identified as opacity-associated protein (Opa). The expressed *opa* gene in strain MC58 (NMB1636) was insertionally inactivated in both *siaD* and *siaD/lst* backgrounds. Deletion of Opa resulted in lower complement activation and increased serum resistance on the *siaD*/Opa- (LOS sialylated) mutant compared to the *siaD/lst*/Opa- mutant.

In conclusion, our data may serve to explain why meningococcal LOS sialylation does not enhance serum resistance in every meningococcal strain. We have identified Opa as an acceptor for complement C4, and shown that the presence of Opa in meningococci can attenuate the complement regulatory effects of LOS sialic acid. It has been observed that most meningococci isolated from the bloodstream and cerebrospinal fluid grow predominantly transparent (Opa-) colonies. Non-expression of Opa may serve to enhance the effects of LOS sialylation and increase serum resistance.

Neisseria gonorrhoeae resists killing and delays PMN apoptosis

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Gonococcal urethritis is a persistent infection despite the large numbers of polymorphonuclear leukocytes (PMN) present in urethral exudates. We developed a system to examine the interactions of *N. gonorrhoeae* with adherent PMN in conditions that model the *in vivo* environment. Transmission electron microscopy (TEM) showed that gonococci are contained within PMN phagosomes and the numbers of intracellular gonococci increased over time. Viability counts demonstrate that *N. gonorrhoeae* strain 1291 resisted PMN killing and the numbers of viable gonococci increased (90.4 ± 35.0% at 1h, 113.9 ± 72.7% at 2h) compared to *E.coli* (2.2 ± 1.7% at 1h, 2.3 ± 1.6% at 2h). *N. gonorrhoeae* strains FA1090, F62, and PID2 resisted killing similar to strain 1291. Chemiluminescence studies were used to characterize the PMN respiratory burst. Responses to gonococci were seen in the presence of both luminol and lucigenin, but not isoluminol suggesting that ROS production is predominantly intracellular and that both NADPH oxidase and myeloperoxidase (MPO) dependent products are generated. Despite the presence of the PMN respiratory burst, intracellular gonococci remain viable. To determine the effect of gonococci on cell viability, PMN were analyzed by TEM for nuclear condensation as an assessment of apoptosis. Both unstimulated (resting) and PMN stimulated with opsonized zymosan particles (OPZ) displayed condensed nuclei when examined at 6-8h. In contrast, PMN challenged with *N. gonorrhoeae* retained normal nuclear morphology at 6-8h. Examination of DNA fragmentation by TUNEL showed that large percentages of resting and OPZ stimulated PMN were TUNEL+. However, almost all *N. gonorrhoeae* challenged PMN were TUNEL-. Annexin V labeling experiments showed Annexin V+ PMN in the resting and OPZ stimulated populations, but lack of staining in *N. gonorrhoeae* challenged PMN. These findings suggest that *N. gonorrhoeae* is able to survive killing and modulate PMN viability to provide an environment for replication.

The nitric oxide reductase of *Neisseria meningitidis* influences cytokine release by human endothelial cells.

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Disease due to *Neisseria meningitidis* is characterised by widespread activation of endothelial cells and phagocytes. Nitric oxide is a key signalling molecule involved in multiple functions of endothelial cells, including expression of cytokines and chemokines and their receptors. The genome of *N. meningitidis* includes the genes *aniA* and *norB,* which encode nitrite reductase and nitric oxide reductase, respectively. We have previously shown that expression of NorB confers resistance to nitrosative stress *in vitro* and intracellular killing of *N.meningitidis* by human macrophages, and furthermore is responsible for depletion of NO from the microenvironment during anaerobic culture.
Here we show that experimental manipulation of cellular [NO] influences cytokine release by the endothelial cell line HDMEC-1. Infection of these cells with *N. meningitidis* wild-type significantly increases the release of TNF-alpha, IL-1beta, and IL-10 but does not affect release of IL-4 and IL-12. Compared with wild-type, infection with an isogenic *norB* mutant results in significantly reduced IL-1beta, IL-4, and IL-10 release by endothelial cells but no effect on TNF-alpha and IL-12 is observed. We conclude that activity of NorB interferes with cytokine release by endothelial cells during co-cultivation with *Neisseria meningitidis*, and that this is likely a consequence of bacterial metabolism of NO.
This work is supported by the Wellcome Trust.

CC and CXC chemokine levels in children with meningococcal sepsis accurately predict mortality and disease severity

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Background: Chemokines are a superfamily of small peptides, involved in leukocyte chemotaxis and the induction of cytokines in a wide range of infectious diseases. Little is known about their role in meningococcal sepsis in children and their relationship with disease severity and outcome.
Methods: Monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1alpha, growth related gene product (GRO)-alpha and interleukin-8 (IL-8) were measured in 58 children with meningococcal sepsis or septic shock on admission and 24 hours thereafter. Nine patients died. Serum chemokine levels of survivors and non-survivors were compared and chemokine levels were correlated with prognostic disease severity scores and various laboratory parameters.
Results: Extremely high levels of all chemokines were measured in the children's acute phase sera. These levels were significantly higher in non-survivors compared to survivors and in patients with septic shock compared to patients with sepsis ($p<0.0001$). Cut-off values for non-survival of 65407 pg/ml, 85427 pg/ml and 460 pg/ml for MCP-1, IL-8 and MIP-1alpha respectively all had 100% sensitivity and 94-98% specificity. Chemokine levels correlated better with disease outcome and severity than tumor necrosis factor (TNF)-alpha and similar to interleukin (IL)-6.
In available samples 24 hours after admission, a dramatic decrease of chemokine levels was seen.
Conclusion: initial phase serum levels of chemokines in patients with meningococcal sepsis can predict mortality and correlate strongly with disease severity. Chemokines may play a key role in the pathophysiology of meningococcal disease and are potentially new targets for therapeutic approaches.

Cleavage of IgA1 antibodies bound to PorA of Neisseria meningitidis requires de novo synthesis of IgA1-protease, eliminates Fc fragments, and reduces binding of Fab fragments

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Neisseria meningitidis secretes a protease that specifically cleaves the hinge region of IgA1, releasing the effector (Fc) domain of IgA1 from the antigen binding (Fab) determinants. Theoretically, the remaining Fab fragments can block pathogen receptors or toxins and still provide protection.
Here, we describe binding of V-gene matched human IgA1 and IgA2 to PorA of strain H44/76. On live meningococci, efficient cleavage of IgA1, but not of IgA2, was observed. Within half an hour, ~40%-80% of the IgA1 Fc-tails were lost from the meningococcal surface. No cleavage of IgA1 was found on an isogenic H44/76 lacking IgA1 protease.
Our data indicate furthermore that PorA-bound IgA1 is masked by the serogroup B polysaccharide capsule, rendering the IgA1 less accessible to degradation by secreted IgA1 protease present in bacterial surroundings. Using protein synthesis inhibitors, we found that *de novo* production of IgA1 protease is mainly responsible for cleavage of PorA-bound IgA1 on encapsulated bacteria. Our data suggest that cleavage of IgA1 by IgA1 protease releases Fab fragments from the bacterium, probably as a result of their reduced avidity compared to whole antibodies.

The accessory protein MD-2 is required for activation of the human TLR4 receptor by meningococcal lipooligosaccharide (LOS).

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Neisseria meningitidis causes severe, rapidly fatal septicemia. Levels of circulating meningococcal endotoxin correlate with severity of shock and death. The human toll-like receptor 4 (TLR4) is a molecular pattern recognition receptor of the innate immune system responsible for initiating the inflammatory cascade of sepsis following interaction with its ligand, endotoxin (LPS). Activation of TLR4 requires association between endotoxin and several adaptor proteins**.** At the cell surface, these include CD14, LPS binding protein (LBP), and the adaptor protein MD-2. MD-2, a 25 kDa protein with a conserved lipid recognition domain, is an essential component for the activation of TLR4 and may determine specific recognition by TLR4. Neither the molecular mechanisms by which endotoxin interacts with MD-2 to activate TLR4, nor the structural requirements of endotoxin for this interaction are understood. Meningococcal lipooligosaccharide (LOS) is a potent activator of the human TLR4 receptor. HEK 293 cells were transfected with expression plasmids for human TLR4 and human MD-2 (Gift Dr. K. Miyake, Tokyo, Japan) alone or in combination. Transfected cells were stimulated with 0.56 pmoles (~ 1 ng)/ mL of highly purified meningococcal endotoxin. IL-8 production was measured by ELISA. To observe the role of soluble MD-2 in activation of TLR4 by meningococcal endotoxin, supernatants from HEK 293 cells transfected with MD-2 alone were added to TLR4 expressing cells, and these were stimulated with LOS. To evaluate dose response, and binding between MD-2 and meningococcal LOS, His-tagged human MD-2 was produced in a *Pichia pastoris* expression system. Secreted MD-2 and MD-2 from cell pellets were purified with Ni-NTA columns and analyzed with SDS-PAGE and Western blotting. Both TLR-4 and MD-2 expression were required for activation of the HEK 293 cells by meningococcal LOS as measured by IL-8 production. TLR-4 transfectants were not responsive without MD-2. Soluble MD-2 from supernatants of transfected cells was able to confer responsiveness upon cells expressing TLR4 alone. The interaction between MD-2 and meningococcal endotoxin is a critical component of the activation of the human TLR4 receptor, and binding to MD-2 may modulate magnitude of response. MD-2 is a specific determinant of TLR4 activation and endotoxin structure may be critically important for binding to MD-2.

Poster Session I

Vaccinology

Opsonophagocytic activity of human sera after vaccination with two different group B meningococcal vaccines in a clinical trial in New Zealand.

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Introduction: Opsonophagocytosis is likely to be an important defense mechanism against meningococcal infections. Effective vaccination against meningococcal disease should induce antibodies with bactericidal and/or opsonophagocytic activity. Two different outer membrane vesicle vaccines against group B meningococcal disease have been developed at the Norwegian Institute of Public Health. One is from strain 44/76, B:15:P1.7,16 (MenBvac), the other is from NZ98/254, B:4:P1.7b,4 (MeNZB). These two vaccines have been tested in a clinical phase I/II trial in New Zealand (V60P1). Groups of 25 volunteers were given three 25 microgram doses of either the MenBvac or the MeNZB vaccine with 6 weeks interval.
Material and methods: Opsonophagocytic activity was measured as respiratory burst (RB) in polymorphonuclear granulocytes with live meningococci as target. The bacteria were grown overnight on Colombia agar and single small colonies were harvested and washed in HBSS/BSA. Serial dilutions of sera from the vaccinee were incubated with bacteria for 30 minutes before human serum (10%) as complement source was added. The incubation was continued for 10 minutes before addition of effector cells (whole blood after ammonium chloride lysis of the red cells) primed with dihydrorhodamine 123. The reaction was stopped after another 10 minutes incubation by keeping the microplates on ice water until the RB was analyzed by flow cytometry. Sera taken at the time of the first dose and six weeks after the third dose were studied. To test for cross- reactivity, sera were analyzed against both strain.
Results: Preliminary results show that both vaccines induce significant RB activities against both the homologous and heterologous strains. The median titer (MT) before vaccination was MT=0 in both vaccine groups against both strains. After three doses of the MeNZB (strain NZ98/254) the RB activity was increased to MT=48 against NZ98/254, whereas against strain 44/46 MT=16 was found. After three doses of the MenBvac (strain 44/76) the RB activity was MT=32 against 44/76 whereas it was MT=16 against NZ98/254.
Conclusion: In this clinical trial we have compared the opsonophagocytic activity in sera from vaccinees given two different group B meningococcal vaccines. Both vaccines induce strong responses, with more than 90% of the vaccinees showing four-fold increase in RB activity against the homologous strain, and about 80% with four-fold increase against the heterologous strain. The high degree of cross-protection observed is encouraging.

Production and real time stability of a meningococcal serogroup C conjugate vaccine with the P64k recombinant protein as carrier (MenC/P64k).

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For all the developed and licensed vaccines only a few proteins have been used as carriers, mainly tetanus toxoide and a non-toxic mutant of the diphtheria toxin polypeptide (CRM197). The characterization of new carrier proteins became a important investigation field since there is an increasing preoccupation concerning the epitopic overload and immune-suppression mediated by carrier proteins due to the repetitive use of them in immunization schedules. The P64k recombinant protein from *N. meningitidis* that has been extensively characterized by physicochemical and immunologic methods is licensed and produced in the Center for Genetic Engineering and Biotechnology. Its low toxicity and high immunogenicity have been demonstrated in a phase I clinical trial**.** A single process was established for the production of a candidate vaccine based on polysaccharide from *N. meningitidis* serogroup C conjugated to the P64k recombinant protein carrier (MenC/P64k). Reductive amination method of a previously meta-periodate oxidized and fragmented polysaccharide was performed for the conjugation of the polysaccharide to the P64k protein carrier. Three consecutives lots were produced under Good Manufactures Production conditions (GMP), characterized and evaluated through 12 months of stability at 4ºC.

The stability of three consecutive lots were studied at 0, 3, 6 and 12 months after production. The final product was presented in lyophilised form and 5µg per doses were formulated in BPS and aluminium phosphate 0.2 mg/mL as adjuvant. For the characterization of the three lots, polysaccharide and protein content. Purity and retention time were performed by gel filtration. Immunogenicity, identity, pirogeny, security, humidity, sterility and chemicals residues from the production process were measured following the FDA recommendations**.**

The stability of three lots were studied by the Quality Control Department of CIGB 1 year period in which the MenC/P64k conjugate vaccine could be conserved lyophilized in storage at 4ºC. The indicative stability properties of three lots were maintained within the established limits.

IMMUNE RESPONSE OF TWENTY HEALTHY MALE VOLUNTEERS TO MENC/P64K: A CUBAN MENINGOCOCCAL SEROGROUP C CONJUGATE VACCINE WITH A NEW RECOMBINANT CARRIER PROTEIN.

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Plain meningococcal polysaccharide vaccines fail to generate memory responses, and induce hyporesponse and a short-lasting protection against the disease, especially in young children. Conjugate vaccines overcome these problems, but for now are available in a limited number of countries. Tetanus toxoide and a non-toxic mutant of the diphtheria toxoide (CRM197) have been widely used as carrier proteins leading to a general preoccupation concerning epitopic overload and hapten-specific carrier-induced suppression that could take place when the same protein carrier is used in subsequent vaccination routines. We studied the immunologic responses to a serogroup C conjugate vaccine using the meningococcal P64k recombinant protein as carrier (MenC/P64k). The MenC/P64k conjugate was obtained by the reductive amination method under Good Manufacturing Practice and had a polysaccharide: protein (w:w) ratio of 1:1 in the final product. Two groups of 10 male healthy volunteers were recruited and immunized with a single dose of 5 µg of the conjugated polysaccharide or 50 µg of the plain polysaccharide in the commercial vaccine Mengivac AC (Pasteur Vaccines, France). No differences in IgG responses against C polysaccharide were detected as well as in bactericidal activity 30 days after vaccination between both groups ($P>0.05$). However, the mean bactericidal activity of the MenC/P64k group was the double of that from the control group. A significant increase in the salivary IgA titers against the polysaccharide was detected only in the MenC/P64k group ($P<0.0001$), as well as in the antibody avidity ($P<0.01$). P64k protein was immunogenic, particularly in 2 volunteers who were previously immunized with it in Phase I safety study 7 years ago, which also showed the higher anti- polysaccharide responses. This finding reveals that the P64k protein induces a memory response. MenC/P64k was immunogenic, even at the low dose of 5 µg, promoting a salivary IgA titer increase and a significant increase in the antibody avidity. All these results allow to continue to a phase II clinical trial with the first Cuban serogroup C conjugate vaccine and the first worldwide using the P64k recombinant protein as carrier.

CONJUGATION OF MENINGOCOCCAL CAPSULAR POLYSACCHARIDE C TO THE P64K RECOMBINANT PROTEIN INDUCES A T-DEPENDENT MEMORY RESPONSE TO THE POLYSACCHARIDE MOIETY: DEMONSTRATION IN A MURINE ADOPTIVE LYMPHOCYTE TRANSFER MODEL.

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Serum antibodies to capsular polysaccharides from *Neisseria meningitidis* confer protection against the disease. However, meningococcal polysaccharide vaccines fail to induce a memory response and they are not immunogenic in children below two years old. Capsular polysaccharide conjugation overcomes this problem, but there is a general concern about the epitopic overload and hapten-specific carrier-induced suppression that could take place when the same protein carrier is used in subsequent vaccination routines. We developed a glycoconjugate composed by meningococcal polysaccharide C (CCPS) chemically bound to the meningococcal recombinant protein P64k by the reductive amination method (CCPS-P64k). The aim of this study was to evaluate the ability of the P64k recombinant protein as carrier protein to induce immunologic memory to the polysaccharide moiety in an adoptive transfer model to glycoconjugate vaccines previously described. We transferred splenocytes from mice immunized with CCPS-P64k, free CCPS or PBS to three groups of naïve mice. After the adoptive transfer, each transferred group was divided in three new groups and immunizations were done with the same antigens and in the same conditions. Adoptive transfer of splenocytes from mice immunized with the CCPS-P64k conjugate vaccine conferred immunologic memory to naive recipient mice. The memory response was characterized by a more rapid kinetics and high levels of CCPS specific IgG antibodies than for the primary response in animals with cells transferred from placebo or CCPS immunized mice. Besides, adoptive transfer of splenocytes from mice immunized with CCPS-P64k conjugate resulted in a more bactericidal and protective response when assayed in a serum bactericidal test and in the infant rat passive protection model than the rest of the evaluated groups. We conclude that chemical conjugation of the CCPS to the P64k protein allowed the memory response generation in mice, changing the immune response to the polysaccharide moiety to a T dependent form.

Identification of Two Immunologically Distinct Domains on the LP2086 Outer Membrane Lipoprotein of *Neisseria meningitidis.* Bentley BE , Ambrose K, Mininni TL and Zlotnick GW. Wyeth Vaccines Research, Pearl River, NY 10965

Neisseria meningitidis strains express an outer membrane lipoprotein, LP2086, capable of eliciting serum bactericidal activity against a broad variety of meningococcal strains despite considerable amino acid sequence diversity. Two subfamilies of LP2086 proteins (A and B) were predicted based on amino acid sequence alignment, derived from over 60 gene sequences. Additionally, the LP2086 protein is predicted to fold into at least two domains, a conserved N-terminal domain and a more diverse C-terminal domain. The N-terminal domain is predicted to consist of the first ~100 amino acids of the mature protein, while the C-terminal domain is contained in the last ~154 amino acids. In the present study, we initiated an investigation to define domain organization using polyclonal and monoclonal antibodies made to recombinant LP2086 A and B subfamily members. N- and C- terminal polypeptides of A and B subfamily members were generated by expressing fragments recombinantly in *E.coli* and cleaving B subfamily members with CNBr. Western immunoblot analysis indicated that polyclonal sera raised against the full length proteins recognized proteins from both subfamilies and N-terminal B subfamily CNBr polypeptides. However, the genetically constructed C-terminal domains reacted only with polyclonal sera produced to a member of the same subfamily. These observations were further substantiated by the reactivity pattern of monoclonal antibodies that specifically recognized the C-terminal domain. Our data support the hypothesis that at least two major domains exist on LP2086 proteins. Additionally, the subfamily determinants of the LP2086 family are located in the C-terminal domain of these proteins.

Antibody persistence and immune memory in 10-month-old infants primed with Tritanrix™-HepB/Hib-MenAC at 6, 10, 14 weeks of age

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Introduction and objectives: The new Tritanrix™-HepB/Hib-MenAC combination showed excellent immunogenicity following 3-dose primary vaccination of infants aged 6, 10, 14 weeks in a previous trial. This trial was conducted to evaluate the immune memory and antibody persistence to Hib, MenA and MenC components of the vaccine in infants aged 10 months in the Philippines.

Methods: Open, randomized, ethically approved study with 10 mcg PSA, PSC (1/5th dose of Mencevax™AC) and 10 mcg PRP administered to subjects primed with Tritanrix™-HepB/Hib-MenAC (N=42) or Tritanrix™-HepB + Hiberix™ (N=36) or Tritanrix™-HepB/Hiberix™ + Meningitec™ (N=39). Serum antibodies were measured prior to and one month after booster (bactericidal assays using rabbit complement for MenA and MenC, ELISA for anti-PSA, PSC and PRP). All vaccines manufactured by GSK Biologicals except Meningitec™ (Wyeth Lederle).

Results:

Persistence: Anti-PRP ≥0.15 mcg/ml persisted in 97.5% of infants in Hib-MenAC group vs 100% in Hiberix™ and Meningitec™ groups. SBA-MenC titers ≥1:8 persisted in 90.5% of subjects in Hib-MenAC group vs 5.7% in Hiberix™ group and 97.4% in Meningitec™ group. All subjects had anti-PSC ≥0.3 mcg/ml in Hib-MenAC and Meningitec™ groups and none in Hiberix™ group. 91.7% of subjects had SBA-MenA ≥1:8 in Hib-MenAC group vs 76.5% in the pooled unprimed groups, suggesting the presence of natural immunity. 92.5% of subjects had anti-PSA ≥0.3 mcg/ml in Hib-MenAC group vs only 6.7% in the pooled unprimed groups.

Immune memory: One month after booster, all subjects had anti-PRP ≥0.15 mcg/ml. Anti-PRP GMC was highest in Hib-MenAC group (66.5 mcg/ml), vs Hiberix™ group (37.1 mcg/ml) and Meningitec™ group (24.4 mcg/ml). 100% of subjects in Hib-MenAC group had SBA-MenC ≥1:8 (GMT 2714) vs 17.6% in (unprimed with MenC) Hiberix™ group (GMT 10), and 100% in Meningitec™ group (GMT 1558). 100% of subjects in Hib-MenAC group had SBA-MenA ≥1:8 (GMT 737), vs 95.4% in the pooled unprimed groups (GMT 525). Of note, 23% of subjects in the unprimed groups had no preexisting MenA bactericidal antibodies: after 10 mcg PSA, 80% had SBA-MenA ≥1:8 (GMT 161). 100% of subjects in Hib-MenAC group had anti-PSA ≥0.3 mcg/ml (GMC 23.6 mcg/ml), vs 70.8% (GMC 0.9 mcg/ml) in the pooled unprimed groups.

Conclusions: Antibody persistence and immune memory to Hib, MenA and MenC in infants primed with Tritanrix™-HepB/Hib-MenAC was excellent: 10mcg PSA, PSC and PRP elicited high levels of corresponding antibodies, demonstrating that priming by the conjugate vaccine was adequate since it is higher than the response in unprimed young children (for MenA and MenC) and similar to Hiberix™ (for Hib). The high level of pre-existing bactericidal antibodies against MenA suggests that anti-PSA IgG-ELISA measurement is a more appropriate method to assess immune memory.

Immunogenicity and safety of 3 doses of Tritanrix™-HepB/Hib-MenAC vaccine administered to infants at 6, 10 and 14 weeks of age

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Introduction: A phase II trial was undertaken with three different formulations of the candidate *Haemophilus influenzae* type b, meningococcal A, C (Hib-MenAC) vaccine administered in combination with Tritanrix™-HepB in infants in the Philippines. The vaccine was produced by GSK Biologicals and is a unique vaccine combination containing the first MenA conjugate vaccine in infants. The new vaccine would greatly benefit countries with high endemicity of MenA and MenC.

Objectives: To evaluate the immunogenicity and safety of Tritanrix™-HepB/Hib-MenAC formulations (A, B or C) as compared to the control groups receiving Tritanrix™-HepB + Hiberix™ or Tritanrix™-HepB/Hiberix™ + Meningitec™.

Methods: Open, randomized, ethics committee-approved study with 5 groups (105 subjects per group) of healthy infants who received a 3-dose primary vaccination at 6, 10 and 14 weeks of age. Serum antibody levels were measured prior to and one month after the primary course. Solicited local and general adverse events were recorded for eight days and unsolicited adverse events for 30 days following each vaccine dose. Serious adverse events were recorded during the entire study period.

Results: Following the primary course, 99%-100% of subjects reached an anti-PRP level of ≥ 0.15 mcg/ml in the 3 Hib-MenAC groups compared to 100% in the Hiberix™ control group. The three Hib-MenAC groups had 99%-100% of subjects with SBA-MenC titers ≥ 1:8 as compared to 100% of subjects with SBA-MenC titers ≥ 1:8 in the Meningitec™ control group. The percentage of subjects with SBA-MenA titers ≥ 1:8 was at least 97.7% in the three Hib-MenAC groups as compared to < 10% in the two control groups not primed with the MenA conjugate. The SBA-MenA GMTs ranged from 316.7 to 418.5 in the 3 Hib-MenAC groups. The percentage of subjects with anti-PSA concentration ≥ 2 mcg/ml ranged between 99-100% in the 3 Hib-MenAC groups (anti-PSA GMCs: 18.10 – 26.51 mcg/ml) as compared to 0-1% in the 2 unprimed control groups (anti-PSA GMCs: 0.15 – 0.17 mcg/ml). The seroprotection levels against diphtheria, tetanus and hepatitis B antigens and the anti-*B. pertussis* toxoid concentration induced by the new combination vaccines were high and similar to the control vaccines, at least for formulation A.

The incidence of clinically relevant solicited/unsolicited adverse events was low and equally distributed among all groups. No serious adverse event related to vaccination was reported.

Conclusions: When administered in the EPI schedule at 6, 10, 14 weeks, the new Tritanrix™-HepB/Hib-MenAC vaccine (formulation A) exhibited excellent immunogenicity and a good safety profile, suggesting that this is a suitable combination vaccine for primary vaccination of infants living in endemic regions for MenA and MenC.

Construction of an isogenic strain panel of *Neisseria meningitidis* mutants to evaluate the contribution of immunogenic components of outer membrane vesicle vaccines.

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Outer membrane vesicle (OMV) vaccines have proved to be efficacious against disease caused by the homologous strain of *Neisseria meningitidis*. Since there is currently no comprehensive vaccine against serogroup B meningococci, vaccines based on combinations, OMVs or other protein antigen components may be developed further to provide cross-protection against heterologous strains. OMVs contain a complex mixture of antigens that display considerable variability in both their expression and antigenic structure. It is therefore important to be able to assess the contribution of key antigenic components to the overall immunogenicity of the vaccine and to facilitate the optimisation of any vaccine formulation. An isogenic strain panel of mutants has been constructed in six isolates of *N. meningitidis* that are representative of commonly isolated virulent genotypes and those that have previously been used in OMV vaccine formulations. Insertion mutagenesis has been used to produce strains of *N. meningitidis* that are deficient in one of five antigens; PorA, PorB, Opc, NspA and FetA. The antigen genes have been cloned into *Escherichia coli* and then disrupted by insertion of a kanamycin-resistance cassette. Constructs were then introduced into *N. meningitidis* by transformation. The panel of mutants can be used in whole-cell or OMV-based ELISAs and serum bactericidal antibody (SBA) assays to assess the impact of antigen deficiency on the reactivity of sera from preclinical and clinical vaccine trials. The panel can be extended relatively easily and also has potential for the evaluation of vaccine formulations containing purified proteins as well as vaccines based on cross-reactive antigens from commensal Neisseria species such as *N. lactamica*.

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

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Outer membrane protein vaccines are being developed against *Neisseria meningitidis* and are now entering clinical trials. These may provide protection against common circulating serotypes and serosubtypes in some countries. However, limited data is available in Europe from genosubtyping meningococci. Our laboratories have been performing genosubtyping for some time which has included analysis of the PorA VR3 region. We have described a number of new VR3 alleles recently and believe that our data indicates the importance of analysing the VR3 region of PorA in addition to VR's 1 and 2. Variation in PorA VR3 is probably indicative of its exsposure to the immune system.

The current numbering system for assigning types and subtypes is proficient but relies on the centralised assignment of new numbers to new types or subtypes. However, new VR3 subtypes are not currently assigned centrally and this may lead to duplication of numbers and therefore confusion. Further development of the meningococcal PorA website (http://www.neisseria.org/nm/typing/pora) is therefore required although a list of known PorA VR3 alleles is available at http://www.show.scot.nhs.uk/smprl. We suggest that a universal nomenclature is required for the PorA VR regions which are being analysed to avoid duplication or misinterpretation. Such data is important for the development of meningococcal outer membrane protein vaccines and epidemiological monitoring.

The association of *Neisseria meningitidis* haemoglobin receptor HmbR with invasive strains and its potential as a vaccine candidate.

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Iron utilisation from host sources is essential for the survival of the meningococcus in man (1). The meningococcus has developed a complex system for iron sequestering involving a number of different proteins and pathways. One mechanism for iron uptake used by meningococcus utilises free haem and haemoglobin via the haemoglobin receptor HmbR (2). Outer-membrane proteins (OMPs) involved in iron uptake have been targeted as *Neisseria meningitidis* vaccine candidates because of their importance for the virulence of the organism (3). Although conjugate vaccines have lessened the incidence of meningococcal group C disease in Europe, this approach does not offer comprehensive protection against meningococcal disease and there remains a need for a vaccine that elicits a broadly protective immune response against all of the virulent serogroups. HmbR has been largely overlooked as a vaccine candidate, even though it is not as antigenically diverse as many other OMP candidates. This is probably because it is phase variable (4) and is not present in all meningococcal isolates (5). This phase variation occurs by slipped-strand mispairing at a poly G tract at position 1164 of the *hmbR* gene. Nevertheless, DNA dot-blot hybridisation, using a *hmbR* probe, against 107 *N.meningitidis* strains, from a global reference set of strains used to evaluate MLST typing, showed that there was a statistically significant ($p=0.001$) association between invasive strains and the presence of the *hmbR* gene. This work was confirmed by nucleotide sequence analysis. From these results, it is postulated that *hmbR* may only be switched on during invasion of the host cells, and therefore avoid immune detection while in a carriage state. Further evidence that HmbR is involved in the virulence of the organism was shown when an *hmbR* mutant was introduced into an infant rat model of meningococcal infection and disease was attenuated (2). Anti-HmbR antisera, produced using refolded recombinant HmbR, will permit an assessment of the potential of this protein as a vaccine candidate.

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Humoral and cellular immune responses in MHC H-2^d and H-2^k haplotype strains of mice induced by vaccination with a conformationally restricted peptide complexed to NeutrAvidin™.

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Oligopeptides that mimic surface antigens have the potential to raise protective antibodies against microbial pathogens. We have recently identified structurally constrained oligopeptides that mimic the epitope of a monoclonal antibody specific to lipooligosaccharide (LOS) of *Neisseria meningitidis*, the causative agent of meningitis and sepsis. To conserve the antigenicity of these peptides they were complexed to NeutrAvidin™ via a biotin linker. Immunisation trials with one of these peptide complexes, C22-Neu, elicited antibodies in mice that cross-reacted with LOS. In this study we demonstrate that this novel antigen is immunogenic in Balb/c and C3H/HeN mice with haplotypes H-2^d and H-2^k, respectively. The adjuvants Freund's and Rehydragel® increased the anti-C22 antibody titres 4-fold in Balb/c mice, but did not increase the titres in C3H mice. Antibodies were detected to constrained and linear forms of the C22 peptide and to NeutrAvidin™. Splenocyte cytokine secretion and antibody isotype profiles indicated that C22-Neu elicited a predominantly Th2 immune response. The structurally constrained C22 peptide primed rapid secretion of cytokines consistent with Th2 responses in vaccinated and unvaccinated mice. NeutrAvidin™ stimulated secretion of cytokines only in vaccinated mice. Antibody titres to NeutrAvidin™ were increased up to 4-fold when complexed to C22. The non-specific priming of cytokine secretion by C22 together with the increased immunogenicity of NeutrAvidin™ when complexed to C22 indicates that this peptide may also have adjuvant-like properties.

Kinetics of Serum Antibody Responses to Meningococcal C Conjugate Vaccine in Adults Previously Immunized with Meningococcal Polysaccharide Vaccine

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Introduction: Polysaccharide-protein conjugate vaccines are generally more immunogenic in infants and young children than unconjugated polysaccharide vaccines but there are conflicting data on whether or not conjugate vaccines are superior in adults. Meningococcal polysaccharide vaccine may also induce group C antibody hyporesponsiveness as measured by impaired antibody responses to a subsequent dose of polysaccharide vaccine. However, only limited data are available on the immunogenicity of group C conjugate vaccines in adults previously immunized with a meningococcal polysaccharide vaccine. The objective of this study was to compare the kinetics of serum group C anticapsular antibody responses of healthy adults immunized for the first time with one dose of a *N meningitidis* group C oligosaccharide-CRM197 conjugate vaccine (MCC, Chiron Vaccines) (n=37) or meningococcal polysaccharide vaccine (MPS, Aventis Pasteur) (n=25). We also determined serum group C antibody responses to a dose of MCC given to adults previously immunized with MPS (MPS/MCC, n=25).

Methods. Serum samples were obtained before, 7 and 28 days after vaccination. IgG anticapsular antibody concentrations were measured by a modified ELISA that incorporated a chaotropic agent in the serum diluting buffer to favor detection of higher avidity antibodies. Bactericidal titers are pending.

Results. Prior to immunization, there were no significant differences in geometric mean serum antibody concentrations (GMC) of adults immunized for the first time with either MCC or MPS (GMC 0.41 U/ml vs. 0.28 U/ml, p=0.26). At 7 days post immunization, serum antibody concentrations in adults immunized with MCC were higher than those of adults immunized with MPS (GMC 1.96 U/ml vs. 0.56 U/ml, p=0.01). At 28 days the respective GMCs were 17 U/ml vs. 6.61 U/ml (p=0.09).

The group previously vaccinated with MPS vaccine and given a dose of MCC (MPS/MCC) had higher baseline serum antibody concentrations (GMC 1.96 U/ml) than previously unimmunized adults given MCC (P<0.001). At 7 days post-immunization, this MPS/MCC group showed increased antibody responses (GMC 3.16 U/ml) compared to their baseline concentrations (p=0.048). However, only 2 of 12 subjects in the MPS/MCC group with < 2 U/ml of antibody in pre-vaccination sera developed • 4-fold increases in antibody concentrations at 7 days post immunization compared to 15 of 28 subjects in the group who only received MCC and had not been previously immunized with MPS (p=0.03). At 28 days, the serum antibody responses of the two groups (MPS/MCC and MCC) were not significantly different (GMC 11 U/ml vs. 17 U/ml, P=0.47)

Conclusions. At 7 and 28 days post immunization, MCC elicited higher group C anticapsular antibody responses in adults than did MPS. MCC also was immunogenic in adults previously immunized with MPS although this group had impaired serum antibody responses at 7 days.

PorA genosubtyping of meningococci in Scotland before, during and after the introduction of meningococcal serogroup C conjugate vaccines

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The Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) started using *porA* sequence analysis as a routine method for the characterization and genosubtyping of isolates of *Neisseria meningitidis* in 1999. Genosubtyping involves the sequencing and subsequent analysis of three variable regions within a class 1 outer membrane porin protein. Meningococcal serogroup C conjugate (MenC) vaccines were introduced in the UK towards the end of 1999. The SMPRL introduced genosubtyping soon after to the introduction of MenC vaccines because those strains within the electrophoretic type 37 (ET-37) complex, occurring during case clusters of disease, are often indistinguishable by standard methods. All invasive meningococci received from regional hospital laboratories throughout Scotland that were isolated from blood, cerebro-spinal-fluid (CSF), and eye were characterised by porA genosubtyping during 1999, 2000, 2001, 2002 and 2003. Nucleotide sequencing was performed on the outer membrane protein gene, *porA*. Data was analysed using databases and software available through the *porA* website (neisseria.org/nm/typing/porA/).

Over 400 strains were analysed and genosubtypes closely associated with serogroup C disease in Scotland (VR1 – variant 5, VR2 – variants 2 and 10 and VR3 variant 36) deceased after the introduction of the MenC vaccines although the significant decline was slow due to the length of the vaccination campaign. Interestingly, genotypes associated with serogroup B disease increased but remained within a defined number of porA variants between 1999 and 2002.

There has been a significant shift in the dominance of specific porA subtypes causing meningococcal disease in Scotland. It is shown that porA genosubtyping is extremely important for the surveillance of meningococcal disease over a period of years and, in this study, has been effective not only in monitoring the impact of the MenC vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease.

From HexaMen to NonaMen: expanding a multivalent PorA-based meningococcal outer membrane vesicle vaccine

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Meningococcal disease in most Western countries is mainly caused by serogroup B for which, in contrast to the successful introduction of Men C conjugate vaccines, still no effective vaccine is available. Outer membrane vesicle (OMV) vaccines are the only experimental vaccines against Men B tested in large Phase III trials. The used wildtype-OMV vaccines gave only protection against isolates expressing the identical outer membrane proteins. For a broad coverage against a variety of meningococcal serosubtypes, a multivalent vaccine was developed by the NVI (former part of the RIVM). HexaMen is based on two strains each expressing three different PorA proteins (P1.7,16; P1.5-1,2-2; P1.19,15-1; P1.5-2,10; P1.12-1,13; P1.7-2,4). HexaMen was safe, well tolerated and immunogenic in infants, toddlers and schoolchildren. Interestingly, the percentage of responders with a fourfold increase in SBA was highest in infants followed by toddlers and was the lowest in schoolchildren. In addition to the SBA response against the vaccine serosubtypes, cross-reactivity of antibodies induced by HexaMen was observed, ranging from 23 to 92 % of vaccine responders for several patient isolate strains, which differ in either single amino acids, a complete VR or the particular combination of VRs compared to the serosubtypes included in the vaccine.
In order to provide an even broader protection, a nonavalent OMV vaccine (NonaMen) has now been developed including the 9 most frequently occurring serosubtypes in the industrialised countries by adding a third trivalent vesicle. NonaMen is produced using three different trivalent *Neisseria meningitidis* strains: HP16215, HP10124 and HP1416. These strains carry two additional chromosomal copies of the *porA* gene, one inserted into an *opa* gene and the other downstream of *rmpM*. Therefore, these strains still express the Class 4 (RmpM) OMP. Each strain produced three different class 1 (PorA) proteins (HP16215: P1.7,16; P1.5-1,2-2; P1.19,15-1 and HP10124: P1.5-2,10; P1.12-1,13, P1.7-2,4 and HP1416: P1.22,14; P1.7-1,1 and P1.18-1,3,6). In addition, all strains lack PorB, express GalE LPS and are capsular polysaccharide negative.
Pre-clinical studies in mice showed after immunization with the third vesicle in mice high SBA titers against P1.22,14 and P1.7-1,1 and lower to P1.18-1,3,6. When mice were immunized with NonaMen (1/10 human dose) high SBA responses were found against P1.7,16; P1.5-1,2-2; P1.5-2,10 and P1.22,14, intermediate responses to P1.7-1,1; P1.18-1,3,6 and P1.12-1,13 and low responses to P1.19,15-1 and P1.7-2,4.
Phase I clinical studies with NonaMen will be performed in the near future.

Antibody responses against homologous and heterologous meningococcal serogroup B strains after a fourth dose of a meningococcal serogroup B OMV vaccine (MenBvac)

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Introduction: More than 20 clinical trials have been performed with the Norwegian meningococcal serogroup B outer membrane vesicle (OMV) vaccine, MenBvac. The vaccine has been shown to induce high levels of bactericidal antibodies after two and three doses and to be efficacious against serogroup B meningococcal disease. In the present study the effect of giving a fourth dose as a booster after a primary schedule of three doses was investigated.
Materials and Methods: 374 teenagers were randomised to receive either MenBvac or placebo in a 2:1 ratio in a double blind clinical trial. A primary schedule of three doses with six weeks intervals was followed by a booster dose after about one year. Blood samples were collected before the first, third and fourth immunization and six weeks and about one year after the third and fourth dose. The immune response was measured as serum bactericidal antibodies against the vaccine strain, 44/76 (B:15:P1.7,16). In addition, sera from a subset of 29 randomly selected MenBvac recipients were tested against a panel of heterologous group B strains. All serum bactericidal assays were performed with external human plasma as complement source.
Results: Six weeks after the fourth dose 78 % of the vaccines showed at least fourfold increase in bactericidal titre compared to prevaccination level against the vaccine strain. The corresponding figure six weeks after the third dose was 75 %. The geometric mean titre (GMT) was significantly higher one year after the fourth dose than one year after the third dose. This shows an increased duration of the immune response after the booster dose. Six weeks after the fourth dose the proportion of subjects with a bactericidal titre $\geq$ 4 against the homologous strain was 93 %. Preliminary data show that against the different heterologous strains this proportion varied between 21 % and 75 %. The level of bactericidal antibodies against the heterologous strains was higher after the booster dose than after the primary immunization, and remained at an elevated level one year after the booster dose.
Conclusion. A fourth dose administered about one year after the primary immunization induces an antibody response of a longer duration than after the primary vaccination, both against homologous and heterologous strains.

The inclusion of colominic acid in test sera enables the use of rabbit complement in the meningococcal serogroup B *Neisseria meningitidis* serum bactericidal antibody (SBA) assay.

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The "gold standard" assay for measuring serological protection against *N. meningitidis* serogroup B is the serum bactericidal antibody (SBA) assay. The preferred complement source is human serum lacking SBA activity and having minimal levels of non-specific bacterial lysis to target strains. To gain such a source many subjects have to be screened and only limited volumes of complement can be taken at each donation. Due to these associated problems of gaining sufficient quality and quantity of human complement the use of a commercially available source is an attractive alternative.

Baby rabbit complement has been used in serogroup A, C, Y and W135 SBA assays but has shown to be associated with elevated SBA titres in the serogroup B SBA assay. This has been connected to the presence of anti-capsular antibody in the test sera. Colominic acid is a capsular homopolymer from *Escherichia coli* K1 that shares the same alpha (2-8)-linked N-acetyl-D-neuraminic acid residue linkage as serogroup B polysaccharide. We have therefore investigated the effect of the addition of colominic acid, as an adsorbent on titres produced by rabbit complement in comparison to human complement.

Using 44/76-SL (B:15:P1.7,16) and NZ 98/254 (B:4:P1.7-2,4) as target strains in the SBA assay, we have compared titres gained using human and rabbit complement to rabbit complement with colominic acid in the bactericidal buffer. Test sera used was that from a phase I trial in New Zealand of two candidate serogroup B outer membrane vesicle (OMV) vaccines and included pre and post vaccination samples.

The addition of colominic acid to the bactericidal buffer decreased rabbit complement titres when compared to titres obtained without colominic acid and resulted in titres more in agreement with those generated by human complement. This leads to the possibility of using commercially available baby rabbit complement with a colominic acid adsorbent in the serogroup B SBA assay.

Effect of sequence variation in meningococcal PorA outer membrane protein on the effectiveness of a hexavalent PorA outer membrane vesicle vaccine in toddlers and school children.

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Though meningococcal conjugate vaccines are effective against serogroup C, there is currently no vaccine solution for serogroup B disease. A hexavalent PorA vaccine in which six PorA outer membrane proteins (OMPs) are embedded in outer membrane vesicles (OMV)(P1.7,16; P1.5-1,2-2; P1.19,15-1; P1.5-2,10; P1.12-1,13 and P1.7-2,4) has been evaluated in phase I and II trials with promising results. However, considerable sequence variation occurs in the variable regions (VRs) encoding these subtypes.
In the U.K. in 2000/1, the serosubtypes 19-1,15-11; 19,15 and 19,15-1 accounted for 13%, 5.7% and 1.25%, respectively, of serogroup B disease. Therefore as 18.7% of U.K. serogroup B, P1.15 disease is due to variants not contained within the current hexavalent vaccine formulation we investigated the cross reactivity of antibody induced by the PorA component P1.19,15-1 against wild type P1.19,15 variants in pre- and post vaccination sera collected from Dutch toddlers and school children.
Pre and post vaccination samples were assayed in the serum bactericidal antibody (SBA) assay using five P1.15 variant target strains (B:4:P1.19,15-1; B:NT:P1.19-1,15-13; B:NT:P1.19-1,15-14; B:NT:P1.19-12,15-11; B:4:P1.19-4,15).
Following vaccination the greatest percentage of samples with titres >4 and with $\geq$ 4 fold rises were against phenotypes B:4:P1.19,15-1 and B:NT:P1.19-1,15-14 with the fewest against B:NT:P1.19-1,15-13 and B:NT:P1.19-12,15-11.
The percentage of subjects with SBA titres of <4, 4, and $\geq$8 varied greatly between the different P1.19,15 variants and this was reflected when $\geq$ 4 fold rises in SBA titre were also examined. This study clearly illustrates the importance of using a large number of meningococcal strains, which represent the true isolate distribution with regards to PorA variants within a single serosubtype. This finding in sera from toddlers and school children may have implications for PorA based vaccines.

Interlaboratory comparison of serum bactericidal titres against 44/76-SL before and after vaccination with the Norwegian MenBvac OMV vaccine

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Following 13 years of a group B meningococcal (MenB) epidemic in New Zealand, a Norwegian manufactured "tailor made" OMV vaccine has been trialled. Before beginning clinical trials, the serum bactericidal antibody (SBA) assay (a correlate of protection against meningococcal disease), was standardised among collaborating laboratories.

This interlaboratory study aimed to compare the SBA assay from each of four different laboratories: HPA Manchester (UK), ESR (New Zealand), NIPH (Norway) and Chiron (USA). The same target strain of MenB (44/76-SL), panel of human sera from lab staff vaccinated with MenBvac and source of human complement were initially used in all four laboratories. Local testing protocols were used and the study highlighted a number of differences in methodologies including media, bactericidal buffer, microtitre plate brand, unknown starting dilutions, rocking of reaction mixture, calculation of titre, and handling of 44/76-SL.

The greatest effect on titres appeared to be due to differences among labs in the maintenance of the MenB test strain, 44/76 SL. Following harmonisation of methodologies all laboratories generated comparable data. Using SBA titres from all samples, and Lab A as the comparator the R^2 for Labs B, C and D were found to be 0.9467, 0.9278 and 0.8334, respectively. From sera obtained from 15 subjects in which all laboratories had assayed both pre and post 3 dose samples, similar rises in SBA (≥ four fold) were seen for the 4 laboratories (12, 11, 9 and 11 subjects). However, the SBA GMTs for the post 3 dose cohort of 15 subjects whom all laboratories tested differed were: Lab A 12.88 (5.75-28.85), Lab B 17.90 (8.16-39.25), Lab C 16.0 (8.37-30.96), and Lab D 32.74 (16.78-63.89).

The serum bactericidal assay has been harmonised between four different laboratories with good agreement on seroconversion rates and fold changes in titres, although absolute SBA titres were still found to vary.

Antibody responses to a meningococcal quadrivalent (A, C, Y and W-135) conjugate vaccine in healthy adults.

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Background
The success of the introduction of the meningococcal serogroup C conjugate vaccines in the U.K. has lead to the development of a quadrivalent meningococcal diphtheria conjugate vaccine incorporating serogroups A, C, Y and W-135 (4μg polysaccharide per serogroup). An open-label, single-site trial in healthy adults was performed to assess the antibody response to each of the portions of the quadrivalent vaccine.

Methods
Sixteen healthy adults received one dose of meningococcal quadrivalent vaccine and blood samples obtained pre- and 1 month post-vaccination. The serological responses to serogroups A, C, Y and W-135 were determined by serum bactericidal antibody (SBA) assay and serogroup-specific IgG, IgG1 and IgG2 ELISA.

Results
The serogroup C SBA geometric mean titre (GMT) pre-vaccination for those 7 subjects with detectable SBA responses was 282 [95% CI: 44-1827] with 9 individuals having SBA titres <4. The SBA GMT increased to 975 [404-2351] post-vaccination. Nine individuals had a pre-vaccination SBA titre of <4 and 8 of these 1 month post-vaccination had a SBA titre >32 with the ninth a non-responder with an SBA of <4. The serogroup C ELISA geometric mean concentration (GMC) increased from 1.4 [0.5-4.1] pre-vaccination to 9.2 [4.0-21.2] post-vaccination. The IgG subclass response showed increases in both IgG1 and IgG2; pre-vaccination GMC of 0.6 [0.1-3.0] and 0.6 [0.2-1.9] and post-vaccination GMC of 1.9 [0.4-9.7] and 6.9 [2.1-22.9], respectively. Results for serogroups A, Y and W-135 are presented in the poster.

Conclusions
These data indicate that vaccination of healthy adults with a meningococcal quadrivalent conjugate vaccine induces protective responses for serogroup C with 94% of individuals having an SBA ≥8. The response to the serogroup C portion is comparable to that previously reported for monovalent serogroup C conjugate vaccination of healthy adults (GMT 1833.6 [1181.7-2845.1]).

A novel conjugation process for production of a highly immunogenic Group A meningococcal conjugate vaccine for use in Africa

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Periodic epidemics of Group A meningococcal meningitis continue to occur in Sub-Saharan Africa despite the availability of a meningococcal A/C polysaccharide (PS) vaccine for over 25 years. An affordable Men A PS-protein conjugate vaccine would be a useful product for preventive immunization of all persons under 30 years living within the "meningitis belt." To meet this need we have produced a group A polysaccharide-protein conjugate vaccine based upon a new paradigm of shared manufacturing with transfer of new technology .

Current production methods use low efficiency conjugation reactions (about 20% yield), which increase costs and limit the number of doses that can be produced. In this study, we developed a new higher-yield conjugation method at the US Center for Biologics Evaluation and Research that employs aldehyde-hydrazide condensation chemistry. The process relies on adding highly reactive groups to both the group A PS and the tetanus toxoid (TT) carrier protein. Aldehyde groups were created on the PS by periodate oxidation. The TT was treated with hydrazine in the presence of EDC to generate hydrazide groups on aspartic and glutamic acid residues. Using this procedure we achieved conjugation reaction yields of over 50%. The resulting aldehyde-hydrazide conjugates, which were prepared from PS made in the Netherlands, and highly purified TT made in India, were used to immunize adult mice. A three dose immunization schedule of 1 mcg given subcutaneously without an adjuvant at two-week intervals induced serum antibody concentrations of over 100,000 antibody units (AU)/ml as measured by ELISA as compared to 100 AU/ml in control mice immunized with unconjugated group A PS vaccine. The anticapsular antibodies elicited by the conjugate vaccine were highly active in bactericidal assays using human complement (titers > 1:6000, as compared to <1:25 for the control mice).

This conjugation process has been successfully transferred for scale-up and production to a vaccine manufacturer in India. Immunogenicity studies are in progress in other animal species, and clinical trials of the new Men A conjugate vaccine will begin in 2005. The new conjugation method and manufacturing paradigm will permit a Men A conjugate vaccine to be made available to African countries at a price of less than $US 0.50 per dose. Thus, the prospect of having an affordable and immunogenic Men A conjugate vaccine for mass immunization in Sub-Saharan Africa has improved considerably.

Map of bactericidal epitopes in *Neisseria meningitidis* GNA 1870

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GNA 1870 is a novel, surface-exposed lipoprotein identified by the genome analysis of *Neisseria meningitidis* MC58 strain, which induces bactericidal antibodies. Three sequence variants of the protein were shown to be sufficient to induce bactericidal antibodies against a panel of strains representative of the diversity of serogroup B meningococcus. Here we studied the antigenic and immunogenic properties of GNA 1870, which for convenience was divided into domains A, B and C. The immune response of mice immunized with each of the three variants was tested using overlapping peptides scanning the entire protein length and using recombinant fragments. We found that while most of the linear epitopes are located in the A domain, the bactericidal antibodies are directed against conformational epitopes located in the BC domain. This was also confirmed by the isolation of a bactericidal murine monoclonal antibody that failed to recognize linear peptides on the A, B and C domains separately, but that recognized a conformational epitope formed only by the combination of the B and C domains. Arginine in position 204 was identified as important for binding of the monoclonal antibody, and secondary structure predictions confirmed that this aminoacid is likely to be located on a surface-exposed loop of the protein. Site-directed mutagenesis of this residue and of additional residues, which are unique for MC58 variant 1 sequence to define the role of each of them in the formation of the functional epitope, is currently in progress.

Immunisation with the meningococcal PilQ complex is protective in a mouse model of meningococcal disease and elicits bactericidal and opsonic antibodies

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Among surface-exposed outer membrane proteins relevant for vaccine development, secretins (TC 1.B.22) should be considered. Secretins comprise a large family of bacterial proteins associated with translocation of single proteins and macromolecules. A subset of this family, termed PilQ proteins (TC 1.B.22.2.1), are required for type IV pilus biogenesis in the pathogenic *Neisseria*. Neisserial PilQ is particularly interesting because it can induce rabbit bactericidal antibodies[1], endorsing its potential as a meningococcal vaccine candidate. Meningococcal PilQ is found as a highly stable complex of approximately 900 kD and is unique among secretins because of its abundance in the outer membrane and its N-terminally located polymorphic region containing repetitive elements. It exhibits sequence conservation in particular domains and is found to be present in all meningococcal strains assessed so far (n>160 strains).

We have purified the native PilQ complex[2] from outer membranes of *N. meningitidis* (NM) and *N. lactamica* (NL) and assessed its immunogenicity and protective activity in a mouse model of bacteraemic meningococcal disease. Mice were immunised with *N. meningitidis* OMVs as a positive control, as these have previously been shown to protect in this challenge model. Purified NM PilQ and NL PilQ were both found to be protective against challenge using the homologous meningococcal strain H44/76. The protective efficacy was similar to that seen with the OMV vaccine positive control. Antibodies elicited by NM and NL PilQ were shown to bind to meningococcal strains representing ET5, ET37 and lineage III clonal lineages in a flow cytometry assay. The NM and NL PilQ sera also showed bactericidal and opsonic activity against strain H44/76, with greater activity observed with the NM PilQ serum.

This work is critical to elucidate and understand the potential role for the PilQ complex as a vaccine component.

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A Pilin Subunit Vaccine Design Strategy for *Neisseria gonorrhoeae.*

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The emergence of resistant strains to each commercially-available antibiotic for *Neisseria gonorrhoeae* has facilitated the need for new treatment and/or prevention options. In the past, type IV pilin-based vaccines for *N. gonorrhoeae* were examined as prevention strategies but were abandoned because they did not protect against challenge with heterologous strains[1]. In *N. gonorrhoeae*, the pilin monomers that make up the type IV pilus filament contain an immunodominant hypervariable region that enables the bacterium to vary pilin epitopes, causing the host to elicit a strain specific immune response. Consequently, the overall goal of this project is to manipulate the structure of pilin from *N. gonorrhoeae* by removing the hypervariable region, in order to create a subunit vaccine candidate, and to assess the ability of this subunit protein to elicit T and B cell responses in mice.

We hypothesize that removal of the immunodominant, hypervariable region of *N. gonorrhoeae* pilin, which forms a beta hairpin and protrudes in pilin monomer and fiber models, could force neighboring conserved epitopes to become dominant. Since the tertiary structure of *N. gonorrhoeae* MS11 pilin closely mimics that of *Pseudomonas aeruginosa's* PAK pilin, an aspartate and glutamine (DQ) linker was taken from PAK pilin and inserted into the subunit protein in place of the hypervariable region. Furthermore, the hydrophobic N-terminal alpha helix was also removed from the subunit protein to facilitate solubility. Since this helix is buried inside the core of the fiber packing and not surface exposed, it would not be an appropriate B cell epitope. The subunit protein was constructed by using gene splicing by overlap extension (SOEing) PCR to amplify the pilE gene while removing the hypervariable region and the N-terminal alpha helix. This protein was cloned as a fusion protein in an expression vector that contains a Tobacco Etch Virus (TEV) protease cleavable N-terminal histidine tagged maltose-binding protein (MBP). The fusion protein was purified with one round of immobilized metal affinity chromatography (IMAC), cleaved with TEV protease, and then the histidine tagged MBP was separated from the subunit protein with a second round of IMAC. X-ray crystallography studies are in progress to verify that the secondary and tertiary structures of the purified subunit protein have not been perturbed by removal of the hypervariable region, and can still provide B cell epitopes. The benefit to using this approach in vaccine development is that it may be applicable to other pathogens with surface antigens that also undergo antigenic variation.

Reference

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Potentials for the use of "tailor-made" outer membrane vesicle (OMV) vaccines against meningococcal disease

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Outer membrane vesicle (OMV) vaccines have proven to be efficacious against serogroup B meningococcal disease in Norway and Cuba. Currently, a public health intervention is ongoing for controlling a serogroup B epidemic in New Zealand. Prerequisites for efficient use of this type of vaccine approach are consistent epidemiology and thorough strain characterization for selection of suitable production strains, representative of the various outbreaks. In addition, the actual vaccine response should be evaluated by a functional immunological assay, as the test for serum bactericidal activity (SBA), to confirm the effect against the circulating strains in the epidemiological situation where a particular OMV vaccine will be used. This type of evaluation is particularly appropriate following a substantial time elapse between isolation of the production strain and the vaccine intervention, or in the case where the vaccine is intended to be used in an altered epidemiological situation (other country, age group etc.). At present, OMV vaccines are still the only available approach for controlling serogroup B epidemics. The recent experience from New Zealand has proven the feasibility of technology transfer, up scaling and manufacturing of an OMV vaccine for a mass immunisation campaign. Some of the challenges in evaluating this type of vaccine are potency tests, surrogate for protection (e.g. SBA) and various regulatory issues connected with the change of manufacturing premises, alteration of production strain etc. It is timely to start the work towards establishing international guidelines for manufacture and evaluation of OMV vaccines.

We propose that the concept of "tailor-made" vaccines, focusing on the sub-capsular antigens, can even be used in sub-Saharan Africa for preventing the reoccurring outbreaks of serogroups A and W135 meningococci. This assumption is based on the epidemiological observation that meningococcal outbreaks in Africa are clonal and strikingly stable regarding their phenotypic characteristics. The sub-capsular antigens in the serogroup A and W135 isolates from Africa are also commonly found in serogroup C and Y strains causing disease in various parts of the world. Thus, by formulating a vaccine including OMVs from representative serogroup A and W135 strains, protection against meningococcal disease caused by group C and Y may be achieved as well. Our pre-clinical data and measurements of functional immunity in mice support this hypothesis. At present, the OMV approach represents a unique opportunity to develop an affordable alternative to a multivalent, A/C/Y/W135 conjugate vaccine in a realistic short period of time.

Epitope Mapping of Protective Monoclonal Antibodies to Meningococcal Vaccine Candidate Genome-derived Neisserial Antigen 1870 (GNA1870).

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GNA1870 is a new meningococcal vaccine candidate that was identified by genome mining. Three variant groups have been described based on antigenic cross-reactivity and amino acid sequence conservation. Variant 1, expressed by ~60% of disease-producing group B strains, is >92% conserved. Our laboratory recently described four murine IgG mAbs prepared against rGNA1870 variant 1 (gene from MC58), and characterized their reactivity against sub-variants of GNA1870 variant 1 strains (Welsch et al., *J. Immunol*, in press). Each MAb appeared to recognize different epitopes. By flow cytometry using live encapsulated bacteria, surface-accessibility of the epitopes was low in most strains. Yet the MAbs were bactericidal and/or activated human C3b deposition on the surface of all sub-variant strains tested. Certain combinations of MAbs were bactericidal against strains resistant to bactericidal activity of the respective individual MAbs. Low doses of the individual MAbs also conferred passive protection against bacteremia in infant rats challenged by strains resistant to bacteriolysis. The basis for differences in MAb binding and functional activity against different sub-variant 1 strains is poorly understood. To investigate this question, we cloned the GNA1870 genes from MC58 and three sub-variant 1 strains into a multicopy plasmid, which was used to express recombinant proteins in *E. coli* and site-directed mutagenesis. By immuno-dot blot, binding of the MAbs to the recombinant GNA1870 proteins paralleled their respective binding with native GNA1870 proteins expressed by the *Nm* strains. We constructed a total of 10 single, double or triple site-specific mutants of MC58 GNA1870 within two segments (region I, residues 165 to 168 and region II, residues 216 to 223). These regions exhibit the largest sequence variability between MC58 and the three sub-variant strains. In dot blots of *E. coli* expressing mutant recombinant proteins, the R223H mutant and three other single amino acid mutations in regions I or II, exhibited decreased or loss of binding with one or more MAbs, but not with the positive control polyclonal anti-GNA1870 antiserum. Binding of the MAbs to the R223H mutant was restored by a second mutation in region I (G166D). In recent experiments, LysC protease peptide fragments of GNA1870 were captured by anti-GNA1870 MAbs linked to magnetic beads, and identified by MALDI-TOF mass spectrometry. Two of the MAbs with the broadest protective activity selected three contiguous peptides between residues 146-194 that contain region I described above. Taken together, the data suggest that at least two of the "protective" epitopes are conformationally-dependent and are located within residues 146 to 194. Further, the epitopes can be altered by reciprocal mutations within regions I and II. GNA1870 is a promising recombinant protein vaccine candidate. Understanding the basis of epitope expression will be helpful for formulating optimally immunogenic vaccines based on this antigen.

Multivalent recombinant PorA liposome vaccines induce serum bactericidal responses against serogroup B meningococci

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Introduction: The PorA outer-membrane protein of *Neisseria meningitidis* has been shown to induce a bactericidal immune response following both natural infection and immunisation with outer-membrane (OM) vesicles. It is therefore a promising target for inclusion in new meningococcal vaccines of defined composition. Variation occurs between PorA proteins of different strains of *N. meningitidis*, and immune responses directed PorA proteins are largely serosubtype specific. Therefore, vaccines based upon PorA proteins must contain multiple PorA serosubtypes to provide protection against a broad range of meningococcal strains. Experimental vaccines containing recombinant PorA (rPorA) proteins of single serosubtypes incorporated into liposomes have been found to induce bactericidal antibodies in animals. In the current study, we have extended this approach to investigate the potential of using liposomes containing purified rPorA proteins of multiple serosubtypes to induce protective immune responses against a broad range of meningococcal strains.

Materials and Methods: Recombinant PorA proteins of serosubtypes P1.7,16, P1.7-2,4, P1.19,15 and P1.5-1,10-4 were expressed in *E.coli* using the high expression vector pQE-30 and purified using 6-histidine tag affinity chromatography. The purified recombinant proteins were incorporated into liposomes individually (monovalent) and also combined and incorporated into the same liposome (tetravalent); in addition, a preparation was made in which equal amounts of the four monovalent liposomes were combined (mixture). These preparations were then used for immunisation studies in mice.

Results: The antisera raised against the four monovalent liposomes induced high titres of antibody against the respective homologous recombinant proteins and OM. They also showed high reactivity to the homologous PorA protein on whole meningococcal cells, as determined by immunofluorescence and importantly, displayed high levels of complement-mediated killing of the homologous meningococcal strain. Antisera raised against both the tetravalent liposomes and liposome mixture preparations showed reactivity to all four homologous PorA proteins and to whole meningococcal cells of the four different serosubtype strains. The multivalent preparations both induced high levels of bactericidal activity against all four homologous meningococcal strains, which was independent of their method of assembly. There were no significant differences in the levels of serum bactericidal activity induced by each of the four PorA serosubtypes, following immunisation either as monovalent or multivalent liposome preparations. A significant finding from this study was that antisera raised against the monovalent liposomes also showed serum bactericidal activity against strains expressing heterologous PorA proteins.

Conclusion: A multivalent liposome preparation containing rPorA proteins of differing serosubtypes not only induces high levels of serum bactericidal activity against each homologous meningococcal strain but also cross-reacting antibodies bactericidal for heterologous strains. Furthermore, there were no differences in the immunogenicities of each PorA serosubtype within the multivalent preparations and such vaccines have the potential to provide protection against a broad range of meningococcal strains.

Identification of Surface Epitopes of Neisserial Outer Membrane Protein 85

JUDD RC

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We identified a Neisserial 85,000 dalton outer membrane protein (Omp85) which has been shown to be required for viability. This protein was highly conserved in Neisseria and widely expressed by Gram-negative bacteria. In *N. meningitidis*. Omp85 was co-transcribed with genes involved in lipid biosynthesis and assembly, appeared to be a component of an auto-transporter complex, and was required for proper incorporation of proteins into the outer membrane. Further, Omp85 appeared to be an important immunogen in meningococcal membrane preparations. Thus, Omp85 may be an important vaccine candidate to prevent meningitis and gonorrhea.

To determine which regions of Omp85 were available to interact with antibody, and may therefore contain epitopes that could induce protective antibodies, we generated a series of overlapping peptide fragments of Omp85. Antisera were generated to each fragment. The binding of the antisera to Omp85 in whole cells was analyzed using immuno-electron microscopy in one meningococcal strain and several gonococcal strains varying in LOS, Por and growth stages.

While all antisera showed some binding to whole cells, two “hyper-reactive” regions, amino acids 111-310 and amino acids 470-630 were identified. Por type did not affect antibody binding but those strains expressing a “deep rough” LOS phenotype showed somewhat greater binding than did strains expressing a “longer” LOS phenotype. Cells in mid-log growth bound more antisera than did lag or late-log stage bacteria. Antisera to the hyper-reactive regions will be used in serum killing assays to determine if these regions represent potential vaccine candidates. The regions can be sub-fractionated to further focus the immune response.

Neisserial Omp85, since it is required for viability and is highly conserved, is a strong vaccine candidate. This study confirmed that antisera to Omp85 bound to whole cells and identified two “hyper-reactive” regions in a meningococcal and various gonococcal strains for further analysis of the vaccine potential of Omp85.

Persistence of serological protection after serogroup C meningococcal glycoconjugate vaccine in toddlers and teenagers.

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Background and aims
Vaccination against serogroup C *Neisseria meningitidis* using a protein-polysaccharide conjugate vaccine (MenC) was introduced into the UK three dose infant primary immunisation schedule in 1999. This was accompanied by a catch-up campaign of a single dose for all children aged 1 to 17 years of age (later extended to 24 years). A number of studies have demonstrated immunological memory following vaccination with MenC but the titre of functional antibody (bactericidal activity of serum) wanes rapidly following the primary series of MenC. Whilst increasing evidence suggests that the serum bactericidal titre correlates with protection against serogroup C *N. meningitidis*, the role of immunological memory is not well characterised. We measured serogroup C meningococcal capsular antibody levels in sera from toddlers and teenagers 3 years after a single dose of MenC to assess persistence of protection based on this serological correlate.

Methods
Serum was available from 94 preschool children who had received a single dose of MenC between the ages of 1.4 and 3.5 years and 50 teenagers who had received MenC 3 years previously. Serogroup C meningococcal serum bactericidal assay (SBA) titre was determined using baby rabbit complement.

Results
Sixty of the 94 (63.2%) toddlers and 4 of the 50 (8%) of the teenagers had an rSBA titre of <1:8, below the putative protective level.

Conclusions
A majority of children who received a single dose of MenC as toddlers in the UK MenC "catch-up" campaign no longer have protective levels of functional antibody in their serum. Conversely, almost all teenagers in this study had antibody titres exceeding the putative protective level. Since antibody titre wanes rapidly after immunisation in early childhood, young children may need a booster dose of MenC to ensure protection through the teenage peak in risk of serogroup C meningococcal disease.

Acknowledgements: Chiron Vaccines sponsored the study in teenagers and the Health Protection Agency supported the analysis of the toddler samples

Serological response to ACYW135 polysaccharide meningococcal vaccine in Saudi children aged under 5 years

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Meningococcal disease remains a serious public health issue in Saudi Arabia where 4 million pilgrims visit each year for religious reasons. In response to a shift in prevalence of disease to those aged under 5 years, a national campaign was conducted in 2003, targeting children from 6 months up to 5 years. Serological responses to the four serogroups included in the polysaccharide vaccine were evaluated.

Six age groups were included in the study; 6 months (n=43), 12 months (n=45), 18 months (n=49), 24 months (n=68), 36 months (n=69), and 48 months (n=92). After taking guardian consent, children were vaccinated with the tetravalent (ACYW135) polysaccharide meningococcal vaccine, (Mencevax ACWY, GSK). Children 24 months or older were given one dose while younger children were given two doses with an interval of 2-3 months. Blood samples were collected before the first dose and one month after the second for children younger than 24 months and before the single dose and one month after for older children. Serogroup specific antibody responses were determined by serum bactericidal antibody (SBA) assays using baby rabbit complement and a tetraplex IgG bead assay.

Pre-vaccination SBA titres increased with age for serogroups A and Y but not for C or W135. Post-vaccination, for the age bands 6, 12, 18, 24, 36 and 48 months the percentage responders (SBA titre • 8) were: serogroup A 19, 29, 42, 80, 87, 94, respectively; serogroup C 10, 9, 11, 42, 42, 48, respectively; serogroup W135 12, 13, 18, 48, 55, 62, respectively; serogroup Y 15, 14, 19, 53, 64, 87, respectively. Serogroup-specific IgG levels increased significantly post-vaccination in all age groups except for those aged 6 and 36 months for serogroup C and 6 month olds for serogroup W135.

To our knowledge, this is the first reported study to evaluate functional antibody responses in young children following tetravalent polysaccharide vaccination. Two-doses of tetravalent polysaccharide vaccine in 6,12, and 18 months old children gives poor protection against serogroups C, Y, and W135. However, for serogroup A, two doses can give 42% responders (SBA titre • 8) at 18 months of age. A single dose of serogroup A polysaccharide from 2 years of age gives good protection and a similar level of protection was observed for serogroup Y at 4 years of age but not in younger age groups. However, for serogroups C and W135 poor response were still evident at 4 years of age.

Safety and immunogenicity of an experimental quadrivalent meningococcal conjugate vaccine (MVC-4) and licensed quadrivalent polysaccharide vaccine (PSV-4) Menomune® in Chilean children.

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Background. An experimental quadrivalent meningococcal conjugate vaccine containing purified PS of *Neisseria meningitidis* serogroups A, C, Y and W-135 covalently bound to diphtheria toxoid was evaluated in a large scale safety trial in 2-10 year old US and Chilean children. A subset of 220 Chilean children was recruited to assess serum bactericidal antibody (SBA) and IgG serogroup specific responses.

Study design and methods: This randomized, modified-double-blind, active-controlled, multi-center trial targeted 3,111 healthy US and Chilean males and females, age 2-10 years. Participants were randomized to receive a single, intramuscular dose of MCV-4 or a single subcutaneous dose of reconstituted Menomune®. Safety endpoints included immediate reactions within 30 minutes; solicited systemic and solicited local events (SSE and SLE) recorded by parents in a diary card during 7 days after vaccination; adverse events (AE), and any severe adverse event (SAE) that occurred over the 6 month study period. Pre and 28 day post vaccination SBA and anti-polysaccharide IgG were measured.

Results (Chile): 548 Chilean children received MCV-4 (mean 5.6 years old) and 488 received PSV-4 (mean 5.7 years old). 11 SAEs occurred and were judged unrelated to vaccination (5 in the MCV-4 and 6 in the PSV-4 group). 36.1% reported at least one SSE in the MCV-4 group while 36.9% were reported in the PSV-4 group. SLE's were slightly higher among PSV-4 recipients (45.1% vs. 34.3%, $p < 0.05$). Mild and moderate pain at the injection site accounted for most of the differences in the SLE between the groups. Pre and post vaccination blood specimens were collected from 119 MCV-4 and 98 PSV-4 vaccinees. In the MCV-4 group, the post vaccination SBA GMT was 2108, 1164, 2615 and 1226 for serogroups A, C, Y and W-135, respectively. These GMT's were not significantly different from those observed among PSV-4 recipients; 1644, 1222, 2019 and 1154 for serogroups A, C, Y and W-135, respectively.

Conclusions: Both PSV-4 and MCV-4 were safe, well tolerated and highly immunogenic in Chilean pre- and school age children. Although the primary responses to both vaccines were comparable, long term protection elicited by MCV-4 is anticipated based on experience with other conjugate vaccines in younger populations.

Development and Evaluation of a Tetraplex Flow Cytometric Assay for Quantitation of Serum Antibodies to *Neisseria meningitidis* Serogroups A, C, Y and W-135

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Introduction

Evaluation of the immune response to meningococcal vaccination entails serogroup-specific serologic measurements. The current method for determining serum IgG concentrations is the ELISA. This sensitive and accurate method is well suited to screening many samples against a single analyte. However assessment of the immune response to meningococcal tetravalent polysaccharide or conjugate vaccines involves measuring an increased number of analytes and testing by ELISA is becoming increasingly difficult. A rapid and simple method for the simultaneous quantification of serogroup-specific IgG antibodies to *Neisseria meningitidis* serogroups A, C, Y and W-135 was developed and evaluated for vaccine evaluation. This assay has also been expanded to include serogroup B for clinical investigations following disease.

Methods

Four microsphere sets were each conjugated with one of the meningococcal capsular polysaccharides A, C, Y or W-135 and the conjugated beads were serologically assessed with the use of anti-meningococcal international reference sera (CDC 1992). Monoplex, biplex and tetraplex assays were compared to determine any interference between bead sets. The tetraplex assay was evaluated by assessing assay specificity, reproducibility and sensitivity. Finally, a comparison of the meningococcal bead assay with the standardised meningococcal ELISA was performed. The quintaplex assay for clinical investigations that includes serogroup B utilizes colominic acid (from the capsular polysaccharide of *E.coli* K1) and was conjugated to the beads and serologically assessed using CDC 1992.

Results

The tetraplex assay was linear over a 24-fold serum dilution range. Studies demonstrated no interference occurring between the bead sets and inhibition studies demonstrated that the assay is specific with < 25% heterologous inhibition. The tetraplex assay was also found to be highly reproducible and sensitive with low intra and inter-assay variation and limits of detection • 650 pg/mL. Comparison of the tetraplex assay and the ELISA showed good correlation between the IgG concentrations obtained from each assay. Assessment of the serogroup B beads demonstrated assay linearity over a six four fold dilution range with both IgG and IgM and comparison of monoplex and quintaplex assays demonstrate no interference occurring.

Discussion

The microsphere based multiplex assay is a specific, sensitive high throughput assay that has the potential to become a viable alternative to the standard ELISA to aid in the evaluation of new multivalent polysaccharide-protein conjugate vaccines.

Serogroup C-specific IgG1:IgG2 ratios in sera collected following disease or polysaccharide or conjugate vaccination.

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Introduction

Meningococcal disease remains a major global health problem with outbreaks and epidemics occurring throughout the world. Little is known about IgG subclass response following vaccination with meningococcal conjugate or polysaccharide, or following disease. Determining values for IgG1 and IgG2 responses following both disease and vaccination with either polysaccharide (MACP) or meningococcal C conjugate (MCC), will provide insight into the immune response elicited by the presence of meningococcal polysaccharide in infants and adults.

Methods

Paired acute and convalescent serogroup C case sera (n = 26), 30 sera from 17–21 year old students who had received MACP vaccine, 48 sera from adults who had received one dose of MCC vaccine and 40 sera from infants who had received one dose of MCC vaccine, were used to determine serogroup C- specific IgG subclass distribution. The method used was previously described by Joseph *et al.,* (1).

Results

Serogroup C case sera were subdivided into age groups: 1-4 yr olds and 6–47 yrs. The GMCs (95% confidence intervals) for acute sera for IgG1 and IgG2 were 0.2 (0.01-3.43) and 0.28 (0.01-6.21) respectively in the 1-4 yr olds and 0.6 (0.18-1.99) and 1.24 (0.26-5.86) respectively for the 6-47 yr olds. Because the levels of IgG antibody are so low within the acute cohorts, it was not practical to calculate a GMC ratio between the subclasses.

Convalescent sera showed a GMC ratio of 1.77 IgG1:IgG2 in 1-4 yrs. The GMC ratio of 6–47 yrs' sera was 0.5 IgG1:IgG2.

The student sera were subdivided into two groups - cohort A received one dose MACP followed by one dose MCC, cohort B received two doses MACP. The GMC ratio for group A was 0.5 IgG1:IgG2, whereas for cohort B, the GMC ratio was 0.34 IgG1:IgG2. For naïve adults who received one dose MCC, the GMC IgG1:IgG2 ratio was 0.39, whereas for infants, who had received one dose MCC, the GMC IgG1:IgG2 ratio was 8.27.

Discussion

This study is the first to demonstrate IgG1 and IgG2 concentrations of antibodies to meningococcal serogroup C in case sera and following vaccination with either MCC or MACP vaccines. Infants receiving MCC vaccine mounted a mainly IgG1 response whilst young children convalescing form serogroup C disease also showed a tendency to a greater IgG1 response. Adults, whether vaccinated with MCC or MACP had higher levels of IgG2.

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B cell ELISPOT to study long-term B cell immunity to PorA in mice

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Introduction: Gradual decline of vaccine-induced serum bactericidal antibody (SBA) titers may falsely be interpreted as limited protective B cell immunity. To study whether frequencies of specific B cells are an alternative (and better) correlate of long-term B cell immunity as opposed to SBA titers in a meningococcal vaccine mouse model, we developed a B cell ELISPOT assay specific for the main vaccine antigen PorA and tested it in different immunization schedules.
Material & Methods: Balb/c mice were primed and boosted with monovalent or hexavalent outer membrane vesicle (OMV) vaccines on day 0 and 28. Different immunization schedules including a long-term experiment were studied. PorA specific ELISA and SBA titers were measured in a standard assay. Spleen and bone marrow (BM) cells were isolated and seeded in PVDF plates coated with αIgG antibodies or PorA, either directly or after *in vitro* culturing for 5 days. After 24 h, plates were developed and PorA specific IgG secreting cells (ISC) were quantitated as permillage of the total ISC.
Results: Using plates coated with either recombinant PorA or OMVs we were able to detect serosubtype specific ISC in BM and spleen from mice with positive ELISA and SBA titers. Frequencies of PorA specific ISC varied between 0.4-36‰ of total ISC. Monovalent priming for a weak immunogenic PorA followed by HexaMen boosting resulted in higher SBA and ELISA titers as well as higher ISC percentages, as compared to the reverse immunization schedule. Preliminary results from a long term experiment (day 105) show that ISC are still present in spleen and BM and that ELISA and SBA titers are still detectable. The group with the highest titers also showed the highest number of specific ISC.
Discussion: PorA specific B cells could be detected after prime-boost in different immune compartments and were more abundant in BM than in spleen. In this setting, trends in frequencies correlated with ELISA and SBA titers, with monovalent priming inducing a better immune response than monovalent boosting. In the long term experiment SBA titers had not yet declined on day 105 and PorA specific ISC were still abundant in both BM and spleen. In more prolonged studies, with declining titers, the B cell ELISPOT assay could be a useful alternative tool to monitor long-term B cell immunity.

Antigenic Variation in the Inner Core Region of Lipooligosaccharides (LOSs) from *Neisseria meningitidis* strains representing the L3 Immunotype.

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Lipooligosaccharide (LOS) is considered as one of the target antigens for a vaccine against meningococci. Even though, twelve different LOS types (Immunotypes) have been documented for meningococci, the development of a commercially viable LOS based vaccine is feasible because of the minimal heterogeneity of the conserved inner core structure of LOS. The previous reports show that the inner core structures are surface exposed and can be targets for bactericidal and opsonic antibodies. Based on the binding studies of LOS specific monoclonal antibody (Mab) MB2-380-91 with LOSs from *N. meningitidis* group B strains *Nm* H44/76 and *Nm* 6275 (both L3 immunotypes), we have shown here that MB2-380-91 recognized *Nm* 6275 LOS but not *Nm* H44/76 LOS in an ELISA and Western Blot analysis. Further studies confirmed that the reactivity of the Mab MB2-380-91 was not dependent on the alpha-chain of LOS molecule but on the phosphorylation of its inner core region. The structures of *Nm* 6275 and *Nm* H44/76 LOSs were determined by glycosyl composition and linkage analysis using ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, ESI-MS and MALDI-TOF mass spectrometry. Structural analysis of *Nm* 6275 LOSs revealed that the inner core region contained two phosphoethanolamine (PEA) moieties attached at *O*-3 and *O*-6 or *O*-3 and *O*-7 of the same L,D-HepII unit. The major fraction (80%) of LOS had PEA substitution at O-3 and O-6 of L,D-HepII, and the minor faction (20%) had PEA substitution at *O*-3 and *O*-7. Structural analysis of *Nm* H44/76 LOS not recognized by MB2-380-91, confirmed the published structure for L3 immunotype [1] with PEA attached at O-3 of L,D-HepII in the inner core. The structure of the alpha-chain was identical in LOSs from both L3 immunotype strains - alpha-Neu$_p$5NAc-(2→3)-beta-D-Gal$_p$-(1→4)-beta-D-Glc$_p$NAc-(1→3)-beta-D-Gal$_p$-(1→4)-beta-D-Glc$_p$(1→4)-L-alpha-D-Hep$_p$-(1→.

These serological and structural data suggest a new serologically distinct antigenic determinant expressed by wild type *N. meningitidis* strain *Nm* 6275. The new epitope is represented by two PEA moieties attached at *O*-3 and *O*-6 or *O*-3 and *O*-7 of the HepII of LOS inner core.

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Standardization and validation of the serum bactericidal assay for measurement of immune responses to serogroup B *Neisseria meningitidis*.

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The serum bactericidal assay (SBA) is used to measure serum antibody responses to meningococcal vaccines to enable licensure. This SBA mimics the host response by measuring immunoglobulin-directed, complement-mediated bacteriolysis. Development of a standardized laboratory protocol and validation of the SBA has been achieved for measurement of functional antibodies to group C disease, but a validated assay has not previously been achieved for measuring antibodies against group B. The trialling of a group B outer membrane vaccine for use in controlling the epidemic of meningococcal disease in New Zealand, necessitated the standardisation and validation of the group B serum bactericidal assay.

An inter-laboratory study involving four laboratories: HPA Manchester (UK), ESR (New Zealand), NIPH (Norway) and Chiron (USA), highlighted a number of differences in methodologies used by each of the participating laboratories. Investigation of the significance of protocol differences led to harmonisation of procedures between the four laboratories. This was followed by another inter-laboratory study in which all four laboratories used the same target vaccine strain and tested the same sera but used their own complement source. Bridging of complement sources to the plasma complement used in the initial inter-laboratory comparison, highlighted issues around use of different complement sources and consistency of results, even when using the same source of human serum complement. Analysis of assay variables showed that reported rates of seroconversion are influenced by the lower limit of quantitation and the base-line titre used to calculate seroconversion. Parameters were determined for the lower limits of quantitation and precision. At the lower end of quantitation, an interpolated titre of 3, or a reciprocal titre of 4, was the lowest limit of precision possible. For interpolated or reciprocal titres greater than or equal to 3 reproducibility testing showed consistent results could be achieved with a standard deviation of ≤ 0.5 $\log_2$ (one-half doubling dilution) and a coefficient of variation of <15%. Reproducibility of titres obtained from assay to assay of $\geq 95\%$ was achievable providing the same complement batch and test conditions were used.

Correlation between PorA protein activity and responses measured in post-vaccination sera derived from trials of a strain-specific vaccine in New Zealand.

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New Zealand has experienced a meningococcal disease epidemic since 1991. Group B meningococci belonging to the clonal complex ST41/44 and expressing the PorA protein P1.7-2,4 have been responsible for 85.6% (2019/2358) of all group B isolates derived from meningococcal disease cases from 1991 through 2003. With few exceptions group B isolates with the P1.7-2,4 PorA belong to three subclones of the ST41/44 complex. In 2001, the New Zealand Ministry of Health contracted Chiron Vaccines in association with the Norwegian Institute of Public Health to produce a tailor-made New Zealand strain outer membrane vesicle (OMV) vaccine targeting the P1.7-2,4 PorA protein for control of the epidemic. To obtain provisional licensure for this vaccine (MeNZB) a series of vaccine trials in different age-groups have been undertaken to measure the immunogenicity of this vaccine and to generate safety data. Immunogenicity measurement for the trials has been based on a validated group B serum bactericidal assay using the vaccine strain NZ98/254 as the target strain. During the trials, serum from immunised subjects receiving MeNZB was tested against two other 'epidemic strain' isolates, NZ94/167 and NZ02/09 and a control strain.
In this separate study, the specificity of the immune response to the P1.7-2,4 PorA protein, the major component of the vaccine, was determined using a range of well characterised meningococci obtained from cases of disease and representing each of the subclones ST41, ST42 and ST44 of the ST41/44 clonal complex, PorA variants of the 'epidemic strain', and strains with different PorAs types belonging to alternative clonal complexes. Sera taken before vaccination and 4 weeks after the third dose from 50 subjects immunised with MeNZB and aged from six months of age through adulthood were analysed. Outer membrane vesicles prepared from each target strain were used as antigen source for immunoblotting. Band intensity was captured using densitometry. Serum bactericidal assays were used to measure the serum bactericidal antibody response. To control for consistency in the serum bactericidal assay the same blended complement source and QC sera were used with the different target strains and sera tested. To determine the specificity of serum antibody reactions sera were absorbed with heat killed cells of target strains. Unabsorbed and absorbed sera were used to demonstrate if antibodies to the P1.7-2,4 PorA protein were present in sera post vaccination compared with the serum from the same individual collected pre-vaccination. Analysis of the association between elevation of serum bactericidal antibody titre and intensity of the specific PorA antigen-antibody reaction in immunoblots was undertaken. During the delivery of vaccine to the wider community, the testing methods developed for this study may be of value for investigating serum antibody responses in any case considered a possible vaccine failure and additionally for monitoring the impact of potential escape mutants.

SELECTION OF PHAGE-DISPLAYED PEPTIDES MIMICKING *NEISSERIA MENINGITIDIS* SEROGROUP A AND C CAPSULAR POLYSACCHARIDES USING HUMAN SERA

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Antibodies against capsular polysaccharides (CPS) from serogroups A and C of *Neisseria meningitidis* correlate with protection against disease. Meningococcal vaccines containing serogroup A and C CPS have been available for more than 20 years, although their routine use is not currently recommended since the vaccines are poorly immunogenic in the age group at highest risk, infants and toddlers, and the serum antibody response is short lived in young children. Meningococcal C polysaccharide-protein conjugated vaccines have been licensed for use in humans and conjugate vaccines against serogroup A meningococcal disease are undergoing clinical studies. They have increased immunogenicity in infants and toddlers versus plain polysaccharide and induce robust immunological memory.
An alternate approach to the development of a T-dependent meningococcal vaccine is through the use of peptides that mimic the CPS. Molecular mimicry of CPS from several pathogens has been reported. Several approaches have been developed for identification of mimotopes of CPS from serogroups A, B and C of *N. meningitidis,* including the screening of peptide libraries displayed on filamentous phages with monoclonal antibodies specific to CPS from serogroups A and B and the development of monoclonal anti-idiotypic antibodies that mimic the serogroup C CPS. Such mimotopes could also be selected from phage libraries using a complex mixture of antibodies, such as whole serum samples. In case of infectious diseases, the screening of peptide phage libraries with patient's sera have the advantage of leading to the recovery of the information left by the pathogen in the immunologic memory of the patient.
In the present work a clinical trial was conducted on healthy adults with the "vaccin méningococcique polyosidique A+C" from Pasteur Vaccins. Based on the levels of IgG, determined by standardized assays, and in the ability to kill *N. meningitidis* group A and C cells in the presence of human complement, two sera were selected to screen a random 15 mer peptide library displayed in the surface of filamentous phage M13.
After panning, individual phage clones were purified and assayed by ELISA using the same sera used for the panning. Positive phages were assayed against a panel of positive sera, i.e. from individuals immunized with the Pasteur vaccine that developed levels of specific antibodies against polysaccharides A and C higher than 2ug/ml of sera. They were also assayed against several negative sera, i.e. sera having levels of specific antibodies against polysaccharides A and C lower than 2ug/ml of sera.
Using this methodology we selected phages displaying peptides unable to bind antibodies from negative sera and specifically reacting with antibodies present in positive sera. Experiments to further characterize these peptides are conducted at present.

Serum bactericidal and opsonophagocytic activity of human chimeric IgG3 antibodies against serosubtype P1.7, P1.16 and P1.4.

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Introduction: The PorA porin on the outer membrane of *Neisseria meningitidis* is an important vaccine antigen. PorA is one of the main targets for bactericidal antibodies induced by two outer membrane vesicle (OMV) group B meningococcal vaccines developed in Norway based on strain 44/76, B:15:P1.7,16 (MenBvac), and strain NZ98/254, B:4:P1.7b,4 (MeNZB). The PorA protein express two immunodominant epitopes located to variable region 1 (serosubtype 7 and 7b) and variable region 2 (serosubtype 4 and 16) on the extracellular loops I and IV, respectively. The relative protective capacity of antibodies to these epitopes is a matter of debate. In order to resolve this problem we constructed chimeric human IgG3 antibodies against all three epitopes and performed functional testing of the antibodies.

Materials & Methods: We constructed chimeric human IgG3 antibodies against the P1.7, P1.16 and P1.4 epitopes by isolating the V_L- and V_H -genes of three mouse mAbs directed against the epitopes. The genes were subcloned into expression vectors followed by transfection to NS0 cells. Chimeric anti-P1.16, anti-P1.4 and anti-P1.7 IgG3 produced by these cells, were tested for bactericidal activity (SBA) by a colony forming unit (CFU) reduction assay and opsonophagocytosis (OP) by flow cytometry using strain 44/76 and NZ98/254 meningococci as target.

Results & Discussion: On molar basis the P1.16 and the P1.4 IgG3 antibodies were equally potent in SBA against the meningococcal strains 44//76 and NZ98/254, respectively. Similarly, the human IgG3 antibodies against the P1.16 and the P1.4 epitopes induced OP equal efficiently on molar basis against strain 44/67 and NZ98/254, respectively. IgG3 antibodies to the P1.4 and P1.16 epitopes were 10 times more efficient in SBA than the corresponding P1.7 antibodies. On the other hand, in an opsonophagocytic assay the IgG3 antibodies were equally efficient against the P1.7, P1.4 and P1.16 epitopes. Thus strains 44/76 and NZ98/254 were equally sensitive for SBA or OP killing when probed with PorA specific human antibodies. Loop I antibodies were 10 times less active than loop IV in SBA, while loop I and IV antibodies were equally active in OP. This observation is important in relation to development of vaccines and antibody preparations for clinical use.

Conclusion. The P1.16 and P1.4 epitopes are better targets for SBA induced by antibodies than the P1.7 epitope, while all three PorA epitopes are equally potent targets for OP activity of antibodies. The strains 44/76 and 98/254 are equally sensitive for OP and SBA when probed with PorA specific antibodies.

MURINE FUNCTIONAL SEROGICAL MEMORY ANTIBODY RESPONSE TO B MENINGOCOCCI AFTER VACCINATION WITH A PROTEIN VACCINE

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The primary goal of vaccination is to prime the immune system so that the host's immune system responds more rapidly and effectively following infection by a pathogen. In this study we investigate the ability of different immunization schedules with the Cuban Men B vaccine to induce immunological memory in a murine model. We analyzed the duration of antibody response (IgG levels and functional antibodies) and the effect of a booster dose on antibody response.

IgG levels were determined by ELISA using OMVs of the vaccine strain (Cu385/83). Bactericidal assays were performed in microtitre plates in the presence of 12.5% guinea pig sera. Opsonic antibodies were measured by a phagocytic killing assay in the presence of 1% human sera as complement source. A neutrophil-to-bacteria ratio of 500:1 was used. Complement sources were negative for bactericidal activity against the target strain (Cu385/83).

We showed that after 2 or 4 doses (primary immunization schedule) of vaccine memory cells were generated and could be activated 7 months after the last dose. The IgG and bactericidal antibody response induced by the primary immunization schedule showed a positive dose-effect. In contrast, a negative dose-effect was found on the booster bactericidal antibody response. The booster response was higher than the primary response detected in mice that had received 2 doses of vaccine. However, animals that had received 4 doses responded less ($P < 0.05$) to the booster dose compared to the primary antibody response.

Noteworthy, the opsonic antibody levels did not differ after the primary immunization series (2 or 4 doses) but after the booster dose it was significantly higher in animals that had received 4 doses of vaccine.

Taken together, these results indicated important differences of the functional serological memory response of mice related to the number of doses administered during the primary immunization.

Comparison of two different assays measuring serum bactericidal activity against serogroup B meningococci

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Introduction. Serum bactericidal activity (SBA) is measured in functional assays that measure complement-mediated-lysis in the presence of serum antibody. Bactericidal antibodies in serum correlate with protection against meningococcal disease. The SBA agar-overlay assay was established at NIPH in the beginning of the 1980-ies and was used until 2003 when it was replaced by the SBA tilt assay. To investigate whether the same results were obtained with the two assays, a comparison was made.

Materials & Methods. Sera from 319 teenagers receiving 3 doses of a meningococcal serogroup B OMV vaccine, MenBvac, (N= 208) or placebo (N=111) were tested with both the agar-overlay and tilt assays, and results compared. The target strain used in the SBA assays was 44/76-SL (B:15:P1.7,16,:L3,7 with low OpcA). Two human sera were included as controls in every run of each assay. External human plasma was used as complement source.

Sera were tested in a two-fold serial dilution (starting 1:2) in microtiter plates with a 60 min incubation at 37°C of the inoculum of meningococci and complement. In the agar-overlay assay, agar was added and plates incubated overnight. The number of CFUs in the wells were counted manually. In the tilt assay, heat-inactivated sera are used. Reaction mixture from the microtiter plate are plated as running droplets onto agar plates and incubated overnight. CFUs are counted by a colony counter. The reciprocal of the highest serum dilution causing more than 50 % killing was recorded as the bactericidal titer.

Results & Discussion. Generally, titers obtained with the agar-overlay assay were higher than titers obtained with the tilt assay. 67 % of the sera showed no significant difference in titer (same or ± 1) with the two assays, whereas 27 % showed a titer difference of +2 with the overlay assay, 6 % a difference of +3 to +5. In the group receiving MenBvac, reciprocal geometric mean titer (GMT) was 15.6 with the agar-overlay assay and 5.9 with the tilt assay. 55 % of the sera showed no significant difference in titer with the two assays, whereas 37 % showed a titer difference of +2 with the agar-overlay assay, 8 % a difference of +3 to +5 two-fold titer steps.

In the placebo group where low titers were expected, GMT was 2.7 with the agar-overlay assay and 2.1 with the tilt assay. 88 % of the sera showed no significant difference in titer with the two assays, whereas 9 % showed a titer difference of +2 with the agar-overlay assay, 3 % a difference of +3 to +4 titer steps.

Conclusion. Titers obtained with the tilt assay were significantly lower than titers obtained with the agar-overlay assay. The use of heat-inactivated serum in the tilt assay may contribute to the differences observed.

Immunogenicity and safety of a trivalent *Neisseria meningitidis* ACW-135 polysaccharide vaccine.

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Background: Annual epidemics of meningococcal disease are common in the African meningitis belt. Although these epidemics are historically associated with *Neisseria meningitidis* (Nm) serogroup A and sporadic cases of Nm W135 are not uncommon, in 2002 serogroup W135 emerged for the first time as the cause of an epidemic in this region.

The emergence of W135 rendered the bivalent Nm AC polysaccharide (PS) vaccine unsuitable for epidemic response. Recognizing the emergent threat, WHO explored several strategies for providing meningitis vaccine with a W135 component. As discussed at a meeting of meningitis belt countries, existing PS vaccines were considered unaffordable at more than US$1 per dose. Appropriate conjugate vaccines were under development. A subsequent international expert consultation endorsed the strategy of working to develop a safe, effective and affordable trivalent Nm ACW-135 PS vaccine in time for the upcoming 2003 epidemic season. Discussions led to a unique public-private partnership between WHO, GSK Biologicals and the Bill and Melinda Gates Foundation. Through this partnership, an affordable trivalent vaccine was produced and licensed in time for the 2003 epidemic season.

As part of the post-licensure evaluation of the trivalent vaccine, during early 2003 assessments of effectiveness and adverse events following immunization (AEFI) were conducted in Burkina Faso where two million doses of the trivalent vaccine were used. A non-inferiority trial was also conducted in Ghana. The aim of this presentation is to provide early results of the AEFI surveillance and immunogenicity assessments.

Methods: An effectiveness study and AEFI surveillance were implemented during the mass reactive campaigns in Burkina Faso during early 2003. As part of an on-going initiative in the meningitis belt, laboratory confirmation of meningitis cases was reinforced to aid Nm case detection. To document vaccination status, vaccination cards were distributed during all trivalent vaccine campaigns. In addition, campaign safety, including AEFI surveillance, was reinforced in all campaign districts. In late 2003 a randomized, double-blind, non-inferiority trial comparing the trivalent vaccine to the tetravalent Nm ACYW-135 PS vaccine was conducted in Ghana.

Results: Preliminary results indicate that immunogenicity of the trivalent vaccine is comparable to that of the tetravalent vaccine. AEFI surveillance found reporting rates for adverse events to be comparable to or less than rates for bivalent Nm AC PS and tetravalent vaccine. Final results will be presented at the meeting.

Conclusions: An affordable trivalent Nm ACW-135 PS vaccine has been shown to be safe, effective and immunogenic when used in African meningitis belt populations. In response to an emergent threat, a unique public-private partnership met the challenge of rapidly developing and licensing a new vaccine. The challenge of addressing the future production and availability of trivalent vaccine has been successfully met with the establishment of a secure vaccine stockpile.

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Prevention of serogroup A, C and W135 meningococcal disease in Africa with outer membrane vesicle vaccines

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Epidemics of meningococcal disease in the Meningitis Belt are almost exclusively caused by clones of serogroup A subgroup III (A:4/21:P1.20,9) and partly serogroup W135 and C sequence type ST-11 (2a:P1.5,2) meningococci. Affordable vaccines providing long-term protection in all age groups are highly needed for prevention of meningococcal disease in Africa.

Serogroup A and serogroup W135 strains were cultivated separately in a synthetic growth medium, and outer membrane vesicles (OMVs) were extracted with the same technique as for the Norwegian serogroup B OMV vaccine (MenBvac). Serogroup A and W135 OMVs were then adsorbed to aluminium hydroxide and used for immunization of NMRI mice, either alone or in combination. The protein and lipooligosaccharide compositions of the vaccines were characterized, and the immunogenicity (ELISA), antibody specificity (immunoblot) and the bactericidal and opsonophagocytic activities of the antibodies generated by these vaccines were investigated. Serum bactericidal assay was performed with human complement towards representative serogroup A, W135 and C strains.

The serogroup A OMV vaccine induced high levels of anti-OMV IgG in mice, and sera showed high bactericidal titres towards serogroup A strains of both ST-5 and ST-7 from different African countries, and showed high opsonophagocytic activity. The antibodies were mainly directed towards the PorA and NadA outer membrane proteins. Serogroup W135 OMVs were also highly immunogenic in mice, and sera showed high bactericidal titres towards both serogroup W135 and serogroup C strains.

Immunization with a serogroup A+W135 OMV mixture vaccine could therefore protect against meningococcal disease caused by most serogroup A, C and W135 strains and possibly also serogroup Y strains in Africa, and be an affordable alternative to the conjugate vaccine approach. Results from preclinical trials with the combined serogroup A+W135 OMV vaccine will be presented.

Early life murine immunization with meningococcal outer membrane vesicles

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Vaccination of neonates is generally difficult due to immaturity of the immune system and potential negative interference of maternal antibodies. The challenge of neonatal vaccinology is to develop safe and effective vaccines that can be administered soon after birth. Although outer membrane vesicles vaccines against *Neisseria meningitidis* have been developed and administered to young children, little is known about the magnitude and quality of the immune response in animal models that resemble the immature immune state of human newborns.
We tested the immunogenicity of meningococcal outer membrane vesicles in neonate Balb/c mice. The influence of route, number of doses and immunization schedule was studied as well. The administration of two doses of proteoliposomes, given at 7 and 14 days after birth, by intraperitoneal route, induced the highest antibody responses observed in this study. This early life immunization was highly effective in conferring protection to 21 days-old sensitized mice challenged with meningococci.
Whereas intranasal immunization with this widely known meningococcal antigen elicits protective and long lasting antibody response in adult mice, its administration by the same route to neonatal mice merely induced low to medium levels of seroconversion. In our study, the humoral immune response against proteoliposomes was significantly increased after the use of mucosal adjuvants in neonatal immunization.

Immunogenic characteristics of recombinant MAF A protein from *Neisseria meningitidis*

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MAF A is a protein from *Neisseria meningitidis* that has been obtained by recombinant DNA technology, which allows characterizing antigens capable of stimulating a host immune response. The MAF A gene was amplified by PCR from *N. meningitidis* and cloned lacking endogenous signal peptide in the pM-232 expression vector. The protein was expressed in *Escherichia coli* as a fusion polypeptide with the N-term sequence of the P64k protein and a sequence corresponding to the "a" determinant of the hepatitis B virus surface antigen, defined as hepatitis B virus Tag (HBV-Tag). This meningococcal protein was administered in Balb/c mice in a three-dose immunization schedule. IgG concentrations in serum and IgA concentrations in saliva were measured by ELISA. The intraperitoneal administration induced higher levels of MAF A-specific immunoglobulin G (IgG) in serum compared to intranasal immunization. The elicited antibodies reacted with the native meningococcal protein on a *Western blotting* and ELISA. Animals that were immunized intranasally produced specific antibodies in saliva.

Intranasal immunization for meningococcal disease.

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A necessary first step in meningococcal disease is the colonization of the host nasopharynx by *Neisseria meningitidis*. Intranasal immunization with outer membrane vesicles (OMV)-based vaccines from group B meningococci has been shown to induce local, as well as systemic immune responses, thus being a suitable candidate for prevention of meningococcal colonization**.** Some of the most important variables to control during intranasal immunization are the volume of the vaccine inoculum, due to both the necessity to guarantee maximum absorption and to be able to extrapolate and compare results in animal models to those in human subjects; and its dosage.
In this work we study the minimal volume and schedules necessary for a successful intranasal OMV immunization in mice, by measuring immunogenicity, bactericidal activity and protection in the infant rat model. Our results show that for 50 µg of OMV, a minimum of 10 µl per nostril delivered in at least 2 doses is necessary for obtaining a functional antibody response.

Immunopotentiation of anti-HBsAg immune response by neisserial outer membrane protein complexes.

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Meningococcal outer membrane proteins (OMP) have proved to be strongly immunogenic when administered intranasally in mice. The potential of the OMP as adjuvants for other antigens was elucidated in mice with use of recombinant Hepatitis B virus surface antigen (HBsAg). These two antigens were co-administered intranasally in Balb/c mice in a three-dose immunization schedule. Immunoglobulin G (IgG) concentrations in serum and lung homogenates, and immunoglobulin A (IgA) concentrations in vaginal washes and lung homogenates were measured by ELISA. The intranasal administration of these combined antigens induced higher levels of HBsAg-specific IgG in serum compared to immunization with HBsAg alone. In addition, this intranasal immunization induced HBsAg-specific IgA responses, both in: lung homogenates and in vaginal washes. The antibody response elicited against HBsAg did not affect the anti-OMP immune response. Moreover, bactericidal antibodies were detected in the sera of mice immunized either with OMP or mixed with surface antigen.

Immunization with an expression library of *Neisseria meningitidis* serogroup B elicits specific humoral, lymphoproliferative response in mice and afford passive protection in infant rats.

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Several methods have been used for the identification of vaccine candidates against the human pathogen *Neisseria meningitidis* serogroup B (MenB), including post-genomics technologies such as proteomic, DNA microarrays and bioinformatics. Genomic immunization, also called expression library immunization (ELI), is an alternative approach never used previously on MenB that has the potential to identify new vaccine candidates. In this technology an expression library of pathogen DNA or cDNA constructed in a eukaryotic expression vector, is used as immunogen in a model that afford protection criteria. Subsequent serial immunizations reducing the complexity of this library could lead to the identification of such plasmids encoding protective antigens. In an effort to identify new immunostimulatory molecules against *Neisseria meningitidis* serogroup B (MenB) we applied ELI methodology to this bacterium. Firstly, we constructed a genomic library of strain CU385 (B:4,7:P1.19,15), comprised of 6,000 individual clones, in a new eukaryotic expression vector that allowed selection of such clones containing MenB specific DNA segments. This library was used to inject BALB/c mice and it was immunogenic, eliciting specific lymphoproliferative response and IgG antibodies against MenB antigens, compared with control mice immunized with the eukaryotic vector alone. However, functional and protective response was not detected. Subsequently, this immunogenic library was subdivided into ten sublibraries (SL), each composed of 600 individual clones, and plasmid DNA from each sublibrary were used to immunize mice. Three of the ten sublibraries (SL6, SL8 and SL9) elicited antibodies with bactericidal activity against MenB and one of them (SL8) afforded passive protection in the infant rat model of meningococcal bacteremia. One sublibrary (SL1) retained the capacity of the total library to specifically stimulate T lymphocytes, compared to mice immunized with the rest of the sublibraries and with the vector alone. Our results demonstrate that sequential division of an immunogenic ELI library of MenB could led to the identification of MenB-derived polypeptides with adjuvant or vaccine activity.

Affinity purified human serum antibodies to inner core lipopolysaccharide epitopes in *Neisseria meningitidis*.

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Introduction: Our aim is to develop a lipopolysaccharide (LPS)-based vaccine to prevent *Neisseria meningitidis* (*Nm*) serogroup B invasive disease. Our approach is to use surface-exposed and relatively conserved inner core LPS (icLPS) as an immunogen to induce high affinity, protective serum antibodies. A number of murine monoclonal antibodies (mAbs) recognising icLPS epitopes have been characterised (1). Together three of these icLPS mAbs react with 97% of a representative global collection of *Nm* strains (1). MAb L3B5 (IgG_3) protects *in vivo* (infant rats) against wild-type *Nm* strains (2), but two other mAbs (L2-16, IgG_{2b}; LPT3-1, IgG_{2a}), despite binding to whole-cells of *Nm,* lack functional activity *in vitro* and *in vivo* (1). Therefore, our current work aims to identify the characteristics of icLPS antibodies required for protection. One approach is to affinity purify human icLPS serum antibodies to determine their functional activity *in vitro* and *in vivo*.

Materials and Methods: The human immune response to a number of representative truncated and extended LPS glycoforms was determined in adult, toddler and infant serum samples by LPS ELISA. LPS glycoforms are characterised by the presence of phosphoethanolamine at 3- or 6-position of the second heptose (corresponding to L3 or L2/L4 immunotype respectively) or its absence (L5 immunotype/ L3 *lpt3* mutant). Affinity purified antibodies were prepared from healthy adult sera using a C-18 silica column coated with truncated L3 *lpt3* LPS and tested by ELISA using a number of variant LPS structures and in bactericidal (BC) and opsonophagocytosis (OP) assays.

Results: By ELISA, significant age-related differences in geometric mean titres (GMTs) of naturally occurring serum antibodies (specific to different icLPS glycoforms) between healthy adults, toddlers and infants were found. There were individual variations in the profiles of reactivity but the highest GMTs were to the extended L3 and L4, and truncated L3 *lpt3* mutant LPS. Serum antibodies were detected to a glycoform characterised by an additional glucose at the first heptose of icLPS. Affinity purified human serum antibodies were prepared using truncated L3 *lpt3* mutant LPS and were tested by ELISA, BC and OP assays against *Nm* wild-type strains. As a control, the specificity of antibodies purified using the C-18 column approach was demonstrated using truncated L3 LPS to affinity purify specific antibodies from a mixture of mAbs. Studies are underway to characterise icLPS antibodies in sera from patients with *Nm* disease.

Conclusions: Specific icLPS antibodies have been affinity purified from healthy human sera and have shown functional activity in OP and BC assays against *Nm* wild-type strains, possessing at least some truncated glycoforms. These studies provide further evidence to support the candidacy of icLPS of *Nm* as a vaccine.

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Intranasal administration of recombinant *Neisseria gonorrhoeae* transferrin binding proteins A and B conjugated to the cholera toxin B subunit induces systemic and vaginal antibodies in mice.

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The transferrin binding proteins (TbpA and TbpB) are considered potential antigens in the development of a vaccine against *Neisseria gonorrhoeae*. This is in part due to their sequence stability as well as their low antigenic variability. The importance of these antigens in virulence has also been demonstrated in a human male, experimental infection model. Furthermore, in the closely related species *Neisseria meningitidis*, vaccine studies using the transferrin binding proteins as antigens elicited bactericidal antibodies, and conferred protection upon lethal bacterial challenge. It is believed that an effective gonococcal vaccine would induce protective antibodies in the genital tract, particularly of the IgA isotype. Parenteral immunization, though effective at inducing systemic antibodies, is poor at inducing mucosal IgA. Intranasal immunization (IN) has been shown to be a promising method for the development of STD vaccines, in part due to the induction of antigen specific genital tract IgA and IgG. Furthermore, by conjugating antigens to the highly immunogenic cholera toxin B subunit (Ctb), antibody responses are enhanced in the serum and mucosal secretions following IN vaccination. In this study we explored the adjuvant properties of Ctb by immunizing mice IN with recombinant transferrin binding proteins (rTbpA and rTbpB) conjugated to rCtb. We conjugated rTbpA and rTbpB directly to rCtb using the coupling agent N-succinimidyl-(3-[2-pyridyl]-dithio)propionate (SPDP), and immunized groups of female BALB/c mice with conjugates or unconjugated Tbps. One group was immunized subcutaneously with the three unconjugated antigens rTbpA, rTbpB, and rCtb. Serum and vaginal secretions were collected, and antibodies levels to each antigen were evaluated with a quantitative enzyme-linked immunosorbant assay (ELISA). We found that both rTbpA-Ctb and rTbpB-Ctb conjugates given IN induced antibody responses in the serum and genital tract. We detected both IgA and IgG in the genital tract following IN immunization, however subcutaneous immunization mainly elicited IgG. Surprisingly rTbpA alone was immunogenic, and induced similar serum and mucosal antibody responses as the rTbpA-Ctb conjugate. However, rTbpB was much more immunogenic than rTbpA. Serum specific IgG was approximately 50-100 fold greater for rTbpB than for rTbpA. This large difference was also observed in genital tract antibody levels. This suggests methods to improve TbpA immunogenicity should be explored if TbpA is to be included in a vaccine. In conclusion, we have shown that IN immunization with Ctb conjugates is an effective strategy for the induction of serum and mucosal Tbp-specific antibodies.

Evaluation of Serogroup A Meningococcal Vaccines in Africa – a Demonstration Project

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Background: *Neisseria meningitidis* (Nm) constitutes a major public health problem in African countries of the "meningitis belt". A new serogroup A meningococcal (MenA) conjugate vaccine is being developed by the Meningitis Vaccine Project (MVP). By 2009, early introduction of the MenA vaccine will take place in selected African countries at high risk for meningococcal disease epidemics through country-wide mass vaccination campaigns among persons 1-29 years. Licensure of this vaccine will be based on immunological correlates of protection, and this vaccine will likely be the first meningococcal conjugate vaccine introduced at large scale in Africa; therefore, post-licensure evaluation of vaccine efficacy will be of increased importance.
Methods: We reviewed the literature on the use of immunogenicity criteria as a licensing strategy, examined licensing requirements for new conjugate vaccines, and reviewed precedents of vaccines against *Haemophilus influenzae* type b and Nm serogroup C, licensed using immunogenicity data, to develop plans for a post-licensure vaccine evaluation of vaccine efficacy.
Results: After MenA introduction in 2009, one country will be considered to conduct a post-licensure demonstration project during the first year of vaccine introduction. The purpose of the project will be to evaluate the performance and impact of the vaccine in a large target population under conditions of routine use. The project will primarily evaluate vaccine effectiveness, measured using a case-control design by comparing the odds of being vaccinated among laboratory-confirmed meningitis cases and controls in selected districts. A laboratory-confirmed meningitis case will be defined as a person with suspected meningitis with NmA detected by latex agglutination or isolated from cerebrospinal fluid (CSF). A suspected meningitis case will be a person with sudden onset of fever with neck stiffness, headache, purpura or bulging fontanel. Eligible persons with confirmed NmA meningococcal meningitis and matched-community controls will be enrolled, interviewed, and vaccination status will be assessed. Vaccine data will also be used to evaluate vaccine impact through cohort analysis in a larger area, preferably country-wide. Surveillance for suspected meningitis will detect changes in suspected meningitis cases, deaths and incidence rates. A series of special studies will also be implemented to provide additional information on vaccine impact, including carriage and seroprevalence studies, vaccine coverage surveys, safety evaluations, prevention effectiveness studies, risk-factors for non-vaccination and assessment of vaccine acceptability.
Conclusion: The use of serological criteria for licensure of new meningococcal conjugate vaccines has lead to a new perspective in vaccine evaluation. Field demonstration of effectiveness of new meningococcal conjugate vaccines will be a critical phase in vaccine evaluation and will constitute an important bridge between pre-licensure trials and post-licensure use and wide acceptance of new meningococcal vaccines by health authorities. Special studies will help determine the most appropriate use of the vaccine as part of national immunization programs.

Total IgG ELISA immune responses to quadrivalent polysaccharide and conjugate *Neisseria meningitidis* (A, C, Y, W-135) vaccines measured in serum and oral fluid

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Background: Antibody measurement in oral fluid is increasingly recognized as a non-invasive tool for the detection of immune responses to vaccination. A clinical trial comparing the safety and immunogenicity of quadrivalent meningococcal polysaccharide (Menomune®, PSV-4, 50 µg polysaccharide per serogroup) and polysaccharide-diphtheria toxoid conjugate (MCV-4, 4µg polysaccharide per serogroup) vaccines in 2 to 10 year-old children in Santiago was conducted to measure meningococcal serogroup-specific IgG antibodies in oral fluid and assess whether oral fluid IgG can be a surrogate for serum IgG.

Methods: Serum and oral fluid samples were collected pre- and 4 weeks post-vaccination after a single dose of vaccine from a subset of participants in the trial. Serogroup (A, C, Y, and W-135) specific IgG antibodies were measured by the standard ELISA in sera (S-ELISA) and oral fluid (OF-ELISA).

Results: Among the 1037 Chilean children enrolled, 220 participated in a nested immunogenicity study. Of these, 216 provided samples to complete all tests. For PSV-4 recipients, the pre- and post-vaccination geometric mean concentrations (GMC's) in µg /ml by S-ELISA were 0.35 and 7.7 for serogroup A, 0.42 and 3.35 for C, 0.1 and 0.87 for W-135, and 0.16 and 10.1 for Y. When measured by OF-ELISA, pre- and post-vaccination GMC's were 0.059 and 0.31for serogroup A, 0.06 and 0.18 for C, 0.04 and 0.13 for W-135, and 0.04 and 0.5 for Y. For MCV-4 recipients, the pre- and post-vaccination GMC's by S-ELISA were 0.32 and 10.3 for serogroup A, 0.37 and 2.75 for C, 0.10 and 0.54 for W-135, and 0.16 and 4.0 for Y. When measured by OF-ELISA, pre- and post-vaccination GMC's were 0.06 and 0.39 for serogroup A, 0.06 and 0.17 for C, 0.04 and 0.095 for W-135, and 0.04 and 0.23 for Y. Correlation coefficients between serogroup A, C, W-135 and Y specific IgG measured by S-ELISA and OF-ELISA pre- and post-vaccination were R=0.94, 0.93, 0.95 and 0.96, respectively.

Conclusions: There is a strong correlation between serogroup-specific IgG measured by ELISA in serum and oral fluid for all four serogroups. OF-ELISA offers a sensitive, non-invasive, safe means of measuring IgG responses among meningococcal vaccine recipients particularly in developing countries.

Development of an opsonphagocytic assay to predict protection induced by new vaccines against meningococcal disease

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Phagocytic killing of *N. meningitidis* is an important defence mechanism against meningococcal disease, whereby antibody and complement-coated bacteria are engulfed and destroyed by immune cells. Previous studies have shown that sera from convalescent patients, has a far greater opsonic activity than from unaffected individuals. Thus we have developed an *in vitro* opsonophagocytosis (OP) assay to measure this antibody-mediated phagocytic uptake using the human cell line HL60 and killed whole meningococci as targets.

In the course of developing this assay, a wide array of variables have been investigated. These include bacterial staining techniques, bacteria-to-phagocytic cell ratios and opsonin concentration. Different phagocytic cell lines have also been investigated, as well as different differentiation techniques for each. Investigations of surface marker expression were performed to assess what phenotypic effects the differentiation methods had on the phagocytic cells, and these were favourably compared to freshly extracted human granulocytes.

Briefly, the current assay methodology involves labelling bacteria internally with the fluorescent dye BCECF (Molecular Probes) , before killing with the addition of 0.2% sodium azide and 100uM PMSF. The dead bacteria are then opsonised by addition of antibody followed by Pel-freez baby rabbit complement. DMF-differentiated HL60 granulocytic cells then phagocytose the opsonised particles and the uptake measured is by flow cytometry. To date, the assay has shown good intra-assay reproducibility and shown itself to be comparable to alternate assays types, such as antibody whole-cell surface labelling and oxidative burst assays. The OP assay has so far been useful for a wide variety of studies. We have already shown that a novel vaccine based on the outer membrane vesicles (OMVs) isolated from the commensal *Neisseria lactamica,* being developed by the Health Protection Agency, induces opsonic antibodies in mice and rabbits that mediate uptake of a diverse range *N. meningitidis* strains by our phagocytic cells. The cross-reactivity observed is greater than that for animals immunised with *N. meningitidis* OMVs. The use of mutant knockout strains of *N. meningitidis* is currently being investigated to determine the role of specific surface proteins in the OP responses. In addition, the assay will be performed with live *N. meningitidis* using a contained flow cytometer. In conclusion, this opsonophagocytic assay has proved to be a robust and useful tool in the study of immune responses to new vaccines against serogroup B meningococcal disease and may help predict which candidate vaccines will be the most effective *in vivo.*

Distribution of surface protein variants among hyper-invasive meningococci: implications for vaccine design.

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Outer membrane proteins (OMPs) are candidates in the search for comprehensive meningococcal vaccines. Formulation of OMP vaccines is, however, complicated by antigenic diversity. A survey of the genes encoding three such OMPs (FetA, PorA, and PorB) was undertaken in 78 representative meningococci isolated from cases of invasive disease. The sequence diversity identified was consistent with strong positive selection acting on the three genes, which are located in different parts of the meningococcal genome.

Phylogenetic analyses of concatenated antigen gene sequences clustered isolates into groups that were largely congruent with the clonal complexes identified by analysis of housekeeping genes, but the topologies of the phylogenies differed. Further, some isolates with identical antigen gene combinations belonged to unrelated clonal complexes and identical antigen variant combinations were maintained over several decades and during global spread.

These observations violated the predictions of the clonal and epidemic clonal models of population structure, but were consistent with models of strain structure generated by immune selection. The patterns of antigenic variant combinations suggested that an OMP-based vaccine with as few as six PorA and five FetA variant sequences could generate homologous immune responses against all 78 isolates examined.

Proteomic analysis of *Neisseria meningitidis* & *Neisseria lactamica* outer membrane vesicle vaccines.

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A vaccine prepared from outer membrane vesicles (OMVs) of *Neisseria lactamica* protects mice against lethal challenge with a range of virulent meningococcal strains. The protection conferred by this vaccine may resemble the natural immunity that is believed to arise following colonisation with commensal *Neisseria spp*. in early childhood. In order to identify the protective components of the *N. lactamica* OMV vaccine, we have undertaken a proteomic analysis.

The protein profiles of OMVs from *N. lactamica* isolate Y2-1009 and *N. meningitidis* isolate K454 have been compared using 1D PAGE separation of 15 µg samples followed by nanocapillary LC-MS/MS analysis of in-gel–generated tryptic peptides. The measured peptide masses were searched against the masses of theoretical tryptic peptides from the *N. meningitidis* MC58 genome, giving unambiguous identification (• 3 peptides per protein) of 57 proteins in *N. meningitidis* OMVs and 31 proteins in *N. lactamica* OMVs, with peptides covering from 2·6% to 35% of each protein after pooling of triplicate data sets. A further 69 proteins (*N. meningitidis*) and 101 proteins (*N. lactamica*) were matched by either 1 or 2 peptides. Fifteen outer membrane proteins predicted from the *N. meningitidis* genome were not detected. Cytosolic proteins were more abundant in OMVs prepared from *N. lactamica* than in those from *N. meningitidis*. The total number of proteins detected was consistent with the number of spots detected by 2D electrophoresis. Immunoblotting of 2D gels indicated that several minor components of *N. lactamica* OMVs are strongly immunogenic.

Since the mass fingerprints were searched against a meningococcal genome, this strategy identified specifically those *N. lactamica* proteins that have close meningococcal orthologues. The same mass fingerprints will be of use in identifying differences between OMVs of commensal *vs.* pathogenic *Neisseria spp* when *N. lactamica* genomic sequence data becomes available. The conserved proteins in OMVs from both species include at least 13 known or predicted surface-exposed proteins; and also a number of periplasmic/inner-membrane proteins. The *N. lactamica* IgA protease fragments represented transmembrane and surface-exposed regions in similar proportions, suggesting that the *N. lactamica* IgA protease is not subject to autocatalytic release.

The use of 1D PAGE as a pre-fractionation prior to LC-MS/MS of in-gel–generated trypic peptides, puts hydrophobic membrane-associated antigens within the range of proteomic analysis. In combination with genomic data, this has demonstrated the complexity of OMV vaccines and the importance of comprehensive characterisation in standardising these vaccines.

Evaluation of Batch Consistency and Antigenic Complement of an Outer Membrane Vesicle Vaccine Using 2D Differential In-Gel Electrophoresis

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To date the most widely studied and arguably the most successful vaccine candidates with the potential to offer protection against meningococci, including group B organisms, consist of outer membrane vesicles (OMVs). Isolate specific OMV vaccines for hyper-endemic situations have been used in Norway and Cuba and are currently in use in New Zealand. By incorporating different antigens, it is hoped that in the future similar vaccines may be developed that offer broader protection against meningococcal disease.

OMV vaccines present the host immune system with a complex mixture of outer membrane protein antigens, many of which remain poorly characterised in terms of their importance for protective immunity. In addition, many of these antigens are highly variable, a number showing phase variation being either up or down regulated in response to the environment. The consistency of vaccine manufacture, therefore, assumes a particular importance for OMV vaccines because of the potential for changes in antigen complement to have a critical impact on the immune response.

Previously we have shown it possible to characterize OMVs using two dimensional gel electrophoresis and to apply this technique to the analysis of different vaccine batches (1). However, using conventional methods, the detection and quantification of differences between 2D gels has been notoriously difficult due to a high degree of variability between gels. The reliability of protein pattern comparisons can be improved using the new technique of differential in-gel electrophoresis, DIGE (2,3). In this method different protein samples are labelled with different mass and charge matched fluorescent dyes and can therefore be compared on the same gel. Accuracy of sample comparisons is further improved by the inclusion of an internal standard which consists of an equal mixture of the samples to be tested which is labelled with a third fluorescent dye. Gel images are analysed using specialist DeCyder imaging analysis software.

In this study we applied the DIGE technique to compare two different batches of an OMV vaccine. Spots were identified that differed in quantity between the two vaccine samples and these were excised from the gel and the corresponding proteins were identified by mass spectrometry. In addition, one dimensional gel electrophoresis was used in combination with mass spectrometry to define the protein complement of a typical vesicle vaccine.

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Inferring V region gene usage for antibodies to meningococcal group A polysaccharide by MALDI-TOF mass spectroscopy

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Depending on the age of immunization, group A meningococcal polysaccharide (PS) vaccination can elicit bactericidal or non-bactericidal anticapsular antibodies. In contrast, group A PS-protein conjugate vaccination elicits protective anticapsular antibodies at all ages. The basis for these age-dependent and vaccine-dependent disparities in group A anticapsular antibody functional activity is unknown. Traditional approaches to investigating these questions require preparation of human monoclonal anticapsular antibodies and sequencing of variable (V) region genes. Alternatively, V region usage can be inferred from limited N-terminal amino acid sequencing of group A anticapsular antibodies clonally-purified from sera. However, neither of these approaches is suitable for investigating V region usage of populations. In this study, we investigated the application of matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS) to determine the V regions encoding group A anticapsular antibodies. As a model for investigating polyclonal human serum, we mixed two murine group A anticapsular monoclonal antibodies (mAbs 13.1 and 22.1). After affinity purification using group A PS coupled to magnetic beads, the antibodies were eluted and separated by isoelectric focusing under non-reducing conditions in order to retain intact heavy and light chain pairing. In a second dimension SDS-PAGE, performed under reducing conditions, heavy and light chains were separated and subjected to in-gel tryptic digestion in preparation for MALDI-TOF. The peptide mass data generated from MALDI-TOF for the light chains were analyzed in conjunction with protein fragment mass databases to identify related antibody sequences and, in turn, putative germline light chain variable (VL) region gene families. The respective VL genes inferred for mAbs 13.1 and 22.1 were IgKV1-117 and IgKV3-12, identical to the corresponding VL genes determined by sequencing of cDNA prepared from the hybridomas. Heavy chain analysis is complicated by the large number of Cys residues present in the constant region, which results in incomplete tryptic digestion and suppression of variable region peptides. However, by eliminating the constant region, we were able to generate peptide mass data from the Fab fragments for heavy chain variable regions. The results demonstrate the feasibility of using MALDI-TOF MS to infer VL, and possibly VH region gene usage from less than 10 micrograms of mixtures of antibodies. Determination of expressed variable region genes by mass spectroscopic analysis of serum group A anticapsular antibody responses of immunized children and adults will provide information on whether or not certain V regions predominate the expressed repertoire, and whether V region gene utilization correlates with the functional activity of antibodies elicited at different ages and/or by different vaccines. The results may explain why some group A anticapsular antibodies have high protective activity against encapsulated organisms, while others do not.

Antibody specificities induced by three doses of the New Zealand or Norwegian outer membrane vesicle vaccines.

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Introduction. In a phase I/II clinical trial (V60P1) performed in New Zealand, groups of 25 adults were immunised three times 6 weeks apart with 25 microgram doses of the MeNZB (B:4:P1.7b,4; NZ98/254 strain) or MenBvac (B:15:P1.7,16; 44/76 strain) outer membrane vesicle (OMV) vaccine. Paired sera, drawn before vaccination and 6 weeks after the third dose, were immunoblotted against OMVs from both vaccine strains. The aim of the study was to compare IgG antibody binding intensities to different antigens with the serum bactericidal activity (SBA) and to identify cross-reacting antigens.

Materials and Methods. Each laboratory blotted paired sera from one half of each vaccine group, while 10 paired sera were analysed by both laboratories to compare the reproducibility of the blotting results. From each blot, three strips incubated with a reference serum served as an internal standard for later scanning of IgG binding. Antibody patterns obtained with 0.05% Tween 20 throughout the incubations were compared to those without, and the additional effect of 0.15% Empigen BB was also studied. A range of monoclonal and polyclonal antibodies was used to identify various antigens. SBA was determined with human complement using the tilt method. IgG binding to LPS and NspA, respectively, was also analysed in ELISA with purified meningococcal LPS and OMVs from *E. coli* expressing recombinant NspA.

Results and Discussion. Both laboratories obtained similar IgG binding patterns for the 10 paired sera. Tween 20 reduced the background on the blots and increased IgG binding to several antigens, including some unknown low molecular weight antigens, whereas LPS and a few other antigen bands disappeared.

Generally, a good correspondence between SBA and distinct IgG binding to the PorA, OpcA and LPS antigens, believed to induce bactericidal antibodies, was observed with the homologous strains. Most preimmune sera from the MeNZB and MenBvac groups without detectable SBA against the homologous strains lacked such bands. A general increase in several antibody specificities was observed after the third dose. The majority of postvaccination sera with SBA $\geq$ 4 against the homologous strains showed reactions with PorA, PorB**,** OpcA and/or LPS, whereas those without SBA lacked these specificities. Judged by the distinct antibody binding of preimmune sera to different meningococcal antigens in the two OMVs, many vaccinees were likely to have carried meningococci.

Comparison of antibody binding patterns to the two OMVs showed a high level of crossreactive antigens in the range of about 5-100 kDa, but not for the two serotype proteins. Although the MeNZB vaccine contained a significant level of NspA, only few MeNZB vaccinees reacted with this antigen; some of them also had this specificity before vaccination.

Immunogenicity of an investigational quadrivalent *Neisseria meningitidis* polysaccharide-diphtheria toxoid conjugate vaccine in 2 year-old children

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New quadrivalent A, C, Y and W-135 conjugate (MC-4) vaccines are under development. We recently described group C responses of 2 year-old children given one dose of an investigational MC-4 vaccine (Aventis Pasteur) in a randomized comparative trial (Granoff et al, Pediatr Infect Dis J, in press). Although the 3-year old controls given one dose of a licensed meningococcal quadrivalent polysaccharide vaccine (MPS-4) had higher group C anticapsular antibody responses, the MC-4 vaccine elicited higher antibody avidity and evidence of avidity maturation between 1 and 6 months after vaccination. The result was higher group C bactericidal responses in the MC-4 group, and greater passive protective activity in the infant rat bacteremia model.

In this report, we have extended these studies to measure serum bactericidal responses to the three other capsular groups, and serum concentrations and avidity of group W-135 anticapsular antibodies by a radioantigen binding assay. The comparator group for W-135 and Y responses was 3-year old children given MPS-4 vaccine (N=40), and for antibody responses to group A was 2-year olds given MPS-4 (N=48). We chose 3-year olds for the W-135 and Y comparators because of lower immunogenicity and unknown efficacy of these capsular groups at age 2-years, and 2-year olds for the group A comparator because group A PS is immunogenic and efficacious below age 2 years.

One month after vaccination, the geometric mean group W-135 anticapsular antibody responses were higher after MPS-4 vaccine ($P<0.01$) but the reverse was true for bactericidal titers, being higher at 1 ($P=0.10$) and 6 months ($P<0.01$) after MC-4 vaccination. At 6 months, the percentage of children with W-135 bactericidal titers • 1:4 (human complement) also was higher after MC-4 vaccine (48% vs. 15% $P<0.005$). The higher W-135 bactericidal responses after MC-4 vaccination reflected higher mean anticapsular antibody avidity ($P<0.02$), and avidity maturation between 1 and 6 months in the MC-4 group ($P<0.04$) but not in the MPS-4 vaccine group.

At both 1 and 6 months, group Y bactericidal antibody titers were not significantly different in the two vaccine groups ($P=0.12$ in favor of MC-4 at 6 months). At 1 month, the group A bactericidal GMT was higher in the MC-4 vaccine group than in controls given MPS-4 vaccine ($P<0.05$) but there were no significant differences in the group A GMT at 6 months after vaccination.

Conclusions: The MC-4 vaccine has superior immunologic properties in 2 year-olds for groups A, C, and W-135, as evidenced by higher levels of functional antibody than those elicited in controls by the licensed MPS-4 vaccine. The two vaccines elicit equivalent group Y bactericidal responses. These data predict that one dose of MC-4 vaccine will confer protection against meningococcal disease in children 2 years of age or older.

Neisseria meningiditis porin activates a specific subset of human B cells

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Neisserial porin (PorB) is currently being explored for use as a general adjuvant in vaccines. It has recently been shown that PorB is a Toll-like receptor 2 ligand (TLR2) and thus, its ability to stimulate antigen presenting cells is the focus of our laboratory. We sought to determine whether PorB stimulated human B cells. B cells were isolated from peripheral blood by magnetic bead separation and incubated for 24 hours with PorB, other TLR2 ligands, or LPS and evaluated by flow cytometry. Three distinct populations of B cells were found. Memory B cells were distinguished from naïve B cells by CD27 expression. Naive B cells (CD27 negative IgD+) were electronically separated by forward/side scatter profiles into small, naïve and large naïve B cells. Small, naïve B cells expressed TLR2 and upregulated CD54, CD69, TLR2, and TLR1 but downregulated class II in response to PorB and other TLR2 ligands. On the other hand, large, naïve B cells and memory B cells did not respond to TLR2 ligands, including PorB. Furthermore, small, naïve B cells did not respond to Neisserial LPS or Salmonella LPS whereas memory B cells responded strongly to LPS. These results suggest that small, naïve B cells might be involved in initiating immune responses to TLR2 but not TLR4 ligands. To further investigate the role of these B cells, we evaluated expression of molecules associated with germinal center formation. PorB induced upregulation of CD77 as well as CD95 suggesting that these cells may interact with other B cells but are short-lived. Finally, we assayed homing receptors. Small, naïve B cells upregulated CLA, but not alpha-4 beta-7, in response to TLR2 ligands indicating that they home to skin. Overall, these data suggest that these B cells have a specific function in generating immunity to TLR2 ligands.

Evaluation of the purified recombinant lipidated P2086 protein as a vaccine candidate for group B *Neisseria meningitidis* in a murine nasal challenge model

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Prevention of group B meningococcal disease represents a particularly difficult challenge in vaccine development due to the inadequate immune response elicited against type B capsular polysaccharide. An outer membrane protein, P2086, has been identified as a vaccine candidate that elicits broad cross-reactivity against multiple strains of meningococci. In this study, the potential of purified recombinant lipidated P2086 (rLP2086) as a vaccine candidate was further evaluated in a newly developed murine nasal challenge model. Groups of six-week old, out-bred Swiss Webster mice were immunized subcutaneously at weeks 0 and 4 with purified rLP2086 formulated with or without an attenuated cholera toxin (CT-E29H) as an adjuvant. The mice were then challenged intranasally with 2 x 10^7 CFU of *N. meningitidis* strains H355 or M982 and the colonization of nasal tissue was determined by quantitative culture 24 hours post-challenge. We demonstrated that immunization with rLP2086 significantly reduced nasal colonization of mice challenged with two different strains of group B *N. meningitidis*. We also determined the levels of serum IgG and IgA antibodies specific to rLP2086 in a protein ELISA, and against heterologous strains of group B *N. meningitidis* in a whole cell ELISA. The biological activity of the immune sera was evaluated using a bactericidal assay against multiple strains of *N. meningitidis*, and an infant rat protection model. Mice immunized with rLP2086 produced a strong systemic IgG response and the serum antibodies were cross-reactive with heterologous strains of group B *N. meningitidis.* The antibodies have functional activity against *N. meningitidis* as demonstrated via bactericidal and infant rat protection assays. These results suggest that rLP2086 is a potential vaccine candidate for group B *N. meningitidis.*

Human Bactericidal Antibody Response to a Core LOS Determinant

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We have identified three strategies for vaccine presentation of outer membrane antigens in a native membrane or artificial membrane environment: 1) use of native outer membrane vesicles (NOMV) from a *synX(-)* mutant strain as an intranasal vaccine, 2) use of liposomes containing purified outer membrane proteins (OMPs) and detoxified lipooligosaccharide (dLOS) as a parenteral vaccine, and 3) use of NOMV from a *synX(-) lpxL2(-)* mutant strain as a parenteral vaccine. We conducted a phase 1 clinical study designed to evaluate the effectiveness of liposomes for presenting OMPs and dLOS to the immune system (strategy 2). Purified OMPs from strain 9162(B:15:P1.7-2,3: P5.10,11) and purified L8v (variant of L8) dLOS were combined with and without incorporation into liposomes. A third formulation consisted of the purified OMPs combined with L7 dLOS and relatively small amounts of liposomal lipids.

Ten volunteers were vaccinated with each of the three vaccine formulations. Two doses were given six weeks apart, and serum samples were obtained at 0, 2, 6, 8 and 14 weeks. Volunteers were evaluated for reactogenicity at 30 min and at 1, 2, and 14 days after each vaccination. Laboratory safety tests were run at 0, 2 and 6 weeks. Sera were analyzed for bactericidal antibodies against four strains differing in LOS or OMP expression. Serum antibody levels were measured by quantitative ELISA and by bactericidal assay. The specificity of bactericidal antibodies in selected sera was investigated using a solid phase antibody depletion assay.

The vaccines were well tolerated. Bactericidal assays against a homologous strain showed a four-fold or greater increase in titer in 6 of 7 volunteers in group one, 9 of 10 volunteers in group two, and 5 of 10 volunteers in group three. Homologous geometric mean (GM) reciprocal titers in group 1 increased from 6.5 to 23.4; from 3.5 to 68.6 in group 2; and from 2.8 to 8.6 in group 3. Increases in antibody against both OMPs and LOS antigens were observed by ELISA. The specificity of bactericidal antibodies in group 2 sera was investigated. Between 50% and 79% of the bactericidal activity was removed from the volunteers' sera with purified L8v LOS.

A murine monoclonal antibody, 25-1-LC1, which recognizes the L8v LOS core determinant was shown to be bactericidal against strains expressing that determinant and having either short or full-length alpha chain oligosaccharide. On western blots the monoclonal antibody bound to multiple bands corresponding to LOS structures of different size.

We conclude that 1) the liposome vaccine formulation was an effective way to present LOS as a vaccine component, 2) the L8v LOS induces a strong bactericidal antibody response in humans, and 3) liposomes did not enhance the immunogenicity of the OMPs.

Characterization of a Native Outer Membrane Vesicle Vaccine Prepared from a *synX(-) lpxL2(-)* Double Mutant Strain of *Neisseria meningitidis*

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We are evaluating two strategies for using native outer membrane vesicles (NOMV) as a meningococcal vaccine: 1) use NOMV from a *synX*(-) mutant as an intranasal vaccine, 2) use NOMV from a *synX(-) lpxL2(-)* double mutant strain as a parenteral vaccine. Presentation of outer membrane antigens to the immune system as part of intact outer membrane insures that the antigens will be in their native conformation and have surface exposure similar to that on viable cells. We have prepared and evaluated for safety and immunogenicity in mice and rabbits a clinical grade lot of NOMV vaccine prepared from strain 44/76 (-:15:P1.7,16:P5.5,C:L7) *synX(-) lpxL2(-)*.

NOMV vaccine was prepared from inactivated cells grown in a 300 L fermenter on modified Catlin's medium with low iron. The vaccine was analyzed by SDS-PAGE and by western blotting with a panel of monoclonal antibodies. The safety of the vaccine was investigated by LAL assay, rabbit pyrogen test, and TNF-alpha release from human monocytes. Immunogenicity of the vaccine was tested in mice and rabbits with and without adjuvant. Animals were immunized with two doses of vaccine given 4 weeks apart. Blood was taken at 0, 4 and 6 weeks. Antibody response was measured by ELISA and by bactericidal assay using normal human serum as the complement source.

NOMV prepared from the double mutant had a normal profile of outer membrane proteins, but expressed much reduced levels of lipooligosaccharide (LOS). Compared to a control NOMV with wild type LOS, the mutant NOMV was only slightly less active in the LAL assay, but was >200-fold less active in the rabbit pyrogen test and in the TNF-alpha release assay. The vaccine was immunogenic in rabbits and mice without added adjuvant. The geometric mean (GM) reciprocal bactericidal titer in rabbits after two doses of 25 mcg was 98.7 and after two doses of 50 mcg was 154. Bactericidal titers increased less than two-fold when adsorbed to aluminum hydroxide. A dose response study was done in CD-1 mice with and without adjuvant. After two doses of between 0.1 mcg and 3.0 mcg of vaccine without adjuvant, the GM reciprocal bactericidal titer increased from 6.7 at 1 mcg to 83 at 3 mcg; and when adsorbed to aluminum hydroxide, the GM titer increased from 69.8 at 0.1 mcg to 197 at 1 mcg. Control NOMV from an isogenic strain with wild type LOS induced a GM reciprocal bactericidal titer of 362 at 1 mcg/dose.

The vaccine was non pyrogenic at a level consistent with safety in humans. In spite of the *lpxL2(-)* mutation, the vaccine had good immunogenicity in both mice and rabbits. These results suggest that the vaccine is suitable for further evaluation in a clinical study.

Poster Session II

Antibiotic Resistance

Type I secretion mediated resistance to antimicrobial agents in *Neisseria meningtidis*

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Introduction

Resistance to certain antibacterial agents in *N. gonorrhoeae* is mediated by type I secretion systems. Both the *mtrCD* and the *farAB* systems utilize the same outer membrane channel-tunnel, MtrE. In the *N. gonorrhoae* genome, MtrE is the only channel-tunnel homolog present. In contrast, genome analyses of *N. meningitidis* indicate the presence of two channel-tunnel encoding genes: a *mtrE* homolog and NMB1737/*natC*.

Aim

The aim of this study was to assess the activity and specificity of the two channel-tunnel proteins in *N. meningitidis* with respect to export of antibacterial agents.

Methods

Knock-out mutants of *mtrE*, NMB1737/*natC* and a double knock-out mutant were constructed in *N. meningitidis* H44/76 via allelic replacement. Sensitivity to various antimicrobial agents was assessed by E-test and disc diffusion tests.

Results

A major increase in sensitivity to clarithromycin, penicilline, tetracycline, chloramphenicol, metronidazole, nalidixic acid, novobiocine, polymixine B, ethidiumbromide, triton X100, Tween 20, Tween 80, Na-deoxycholate, and crystal violet was observed for the *mtrE* mutant and the *mtrE*-NMB1737/*natC* double mutant. The NMB1737/*natC* mutant did not show increased sensitivity to any of the antibacterial agents tested.

Conclusion

In *N. meningitidis* H44/76, the channel-tunnel protein MtrE is involved in resistance to several antimicrobial agents. The NMB1737/NatC channel-tunnel is not involved in export of the tested antimicrobial agents, suggesting a different role for this protein, e.g. in protein secretion.

Efficacy of oral alternative therapies for gonorrhoea in view of increasing quinolone resistance

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In recent years clinical failure to quinolones has been detected in a high proportion of patients with gonococcal infections. Therefore in our hospital alternative regimens like ceftriaxone or azithromycin or cefexime are being used for treatment of uncomplicated gonococcal infections with good clinical response. The present study was carried out to validate these clinical observations and to generate baseline data regarding in-vitro susceptibility to alternative oral antimicrobials.

N. gonorrhoeae isolates from cases of urethritis in males & cervicitis in females were subjected to antimicrobial susceptibility by disc diffusion method for ciprofloxacin, ceftriaxone, spectinomycin, tetracycline, penicillin, azithromycin and cefexime. Penicillinase production was determined by chromogenic cephalosporin method.

Thirty two strains of *N. gonorrhoeae* were isolated from 48 cases. All gonococcal isolates were found to be susceptible to ceftriaxone, cefexime and spectinomycin, and showed large zones of inhibition to azithromycin (• 30mm, 84.4%; • 26mm, 100%). A high proportion (90.6%) of isolates were fully resistant to ciprofloxacin while, 9.4% strains were less sensitive. 37.5% of the isolates were PPNG, while 9.4% isolates were TRNG. Antimicrobial susceptibility of gonococcal isolates over the last decade will be presented.

Identification of a fifth resistance gene from the chromosomally mediated resistant *Neisseria gonorrhoeae* strain FA6140. ZHAO S and NICHOLAS RA. Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA.

Chromosomally mediated resistant *Neisseria gonorrhoeae* (CMRNG) strains have acquired mutations in endogenous genes/loci that increase resistance to penicillin. Resistance can be transferred from a resistant strain to a sensitive strain by stepwise transformation and selection. At least four resistance genes/loci exist in FA6140 (a CMRNG strain), although only the first three (*penA*, *mtr*, and *penB*) are easily transferred to FA19 by transformation. The *penA* gene encodes altered forms of penicillin-binding protein 2 (PBP 2) that have lower rates of penicillin inactivation, *mtr* is a deletion in the promoter region for the MtrCDE efflux pump that increases expression of the pump, and *penB* encodes altered forms of porin IB that decrease the flux of antibiotics across the outer membrane. The fourth resistance gene in FA6140, *ponA**, encodes an altered form of PBP 1, the other lethal target for beta-lactam antibiotics in the gonococci. Unexpectedly, it is not possible to transform FA19 *penA mtr penB* (PR100) to higher resistance with DNA from FA6140. Moreover, we have not been able to transform PR100 to higher resistance with the *ponA** gene, suggesting that additional genes are required for *ponA** to increase resistance.

We hypothesized that the genetic background of the recipient strain might determine whether additional resistance genes from FA6140 could be transferred. Thus, we constructed a third level transformant of RM11.2, a derivative of FA1090. Consistent with our hypothesis, penicillin-resistant colonies were readily obtained (frequency = 1 x 10e-5) following transformation of RM11.2 *penA mtr penB* with FA6140 DNA. No changes in the first three resistance genes/loci were detected. We examined whether this new resistance gene might be *ponA**, but interestingly only about half of the colonies examined harbored the *ponA** gene. Transformation of RM11.2 *penA mtr penB* with the *ponA** gene did not result in resistant colonies, while transformation with genomic DNA from one of the resistant colonies containing the *ponA** gene resulted in 12% of the colonies with the wild type *ponA* gene. These data suggest that penicillin resistance is not mediated by the *ponA** gene itself, but instead by a fifth resistance gene, tentatively called *penD*, that is located near the *ponA* gene on the chromosome. We are currently pursuing the molecular identity of this new resistance determinant.

Identification and Characterization of *gohT*, a Loci Involved in Tolerance of Iron Protoporphyrin IX and Other Hydrophobic Agents in *Neisseria meningitidis*

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Abstract: Several genetic systems that allow the use of protoporphyrin IX (heme) have been described for the pathogenic bacterium *Neisseria meningitidis*. However, many questions about the process of heme acquisition and utilization remain to be answered. In order to isolate and analyze unidentified genes that play a role in heme-iron uptake and utilization, a *Himar 1* transposon mutant library was created in *N. meningitidis* serogroup A. One locus identified by transposon mutagenesis conferred protection against heme toxicity. A mutant with a deletion in this locus has a 10-fold higher susceptibility to heme compared to the parental strain. In addition to heme, the deletion strain is hypersensitive to other hydrophobic agents (HAs) including the heme precursor protoporphyrin IX (PPIX), the detergent Triton X-100, and the antibiotic rifampicin. In light of these findings, the gene was named *gohT* for **g**ene **o**f **h**ydrophobic agent **T**olerance. Transcriptional analysis indicated that *gohT* is co-transcribed with an upstream open reading frame designated *gohS*. Uncharacterized orthologues of *gohT* were identified in many other Gram-negative bacteria. Here we present genetic evidence for the importance of *gohT* in resistance to hydrophobic agents and its potential role in interaction with other hydrophobic agent resistance mechanisms within *N. meningitidis*.

Dissecting the relative contributions of the Asp-345a insertion versus C-terminal mutations in decreasing the rate of penicillin inactivation of PBP 2 from *Neisseria gonorrhoeae*.
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Penicillin-binding protein 2 (PBP 2) from *Neisseria gonorrhoeae* is the primary lethal target for beta-lactam antibiotics targeted against this pathogen. Mutations in the gene encoding PBP 2 (*penA*) have been reported in chromosomally mediated resistant *N. gonorrhoeae* (CMRNG) strains and acquisition of an altered *penA* gene is the first event in the stepwise transfer of resistance from CMRNG strains to penicillin-sensitive strains. We have determined the crystal structure of this enzyme to 2.4 Å resolution and now aim to delineate the structural mechanisms by which the alterations in PBP 2 decrease the rate of inactivation by penicillin. The majority of altered *penA* genes contain an amino acid insertion (Asp-345a) as well as several mutations near the carboxyl terminus. In PBP 2 derived from the CMRNG strain FA6140, the effect of these mutations is a 10-fold decrease in the k_2/K' rate constant for penicillin acylation. Previous studies have shown that the Asp-345a insertion is the main determinant of *penA*-mediated penicillin resistance, and that an aspartate is the only amino acid capable of decreasing penicillin inactivation. PBP 2 containing this single insertion (PBP 2-D345a) showed a 4-fold decrease in the k_2/K' rate constants. This amino acid is located on a loop near to the active site but efforts to crystallize PBP 2-D345a have been unsuccessful. To assess the effect of the remaining mutations we constructed *penA* genes containing only the C-terminal mutations from the FA6140 *penA* gene (PBP 2-6140CT). Unexpectedly, the k_2/K' constant for this protein was decreased ~5-fold compared to wild type, suggesting that the C-terminal mutations by themselves were as effective as the D345a insertion in decreasing penicillin acylation. PBP 2 from FA6140 contains four mutations in its C-terminus; inspection of the native crystal structure of PBP 2 suggested that of these, G516A and P551S, were most likely to impact the active site of the enzyme. Therefore, we made PBP 2 constructs with each individual mutation and one containing both mutations, and determined their k_2/K' constants. The P551S mutation caused a 3.5-fold decrease in the k_2/K' constant, whereas the G516A mutation caused only 1.6-fold decrease. PBP 2 with both mutations showed no further decrease in k_2/K' compared to PBP 2-P551S. Incorporation of these mutations into the *penA* gene of FA19 conferred increases in the MIC for penicillin that were consistent with their effects on k_2/K'. Taken together, these data demonstrate that C-terminal mutations, in particular P551S, are as effective as the D345a insertion in decreasing the rate of inactivation of PBP 2 by penicillin. Recently we have obtained crystals of PBP 2-FA6140CT and this structure will reveal the effect of these mutations that leads to the altered k_2/K' constants.

Identification of genes influencing polymyxin B resistance in *Neisseria meningitidis*

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Cationic antimicrobial agents, such as defensins and polymyxin B (PxB), are important for defense against microbial infections. Phosphoryl groups linked to the lipid A head groups have been implicated in the formation of a stable outer membrane network enabling adjacent LPS molecules to be cross-linked via divalent cations. It is believed that PxB primarily interacts with negatively charged phosphorylated head groups of lipid A, and modification of the lipid A head groups with aminoarabinose or phosphoethanolamine (PEA), which may reduce electrostatic interaction between polymyxin and LPS, correlates with an increased resistance to polymyxin. The meningococcus is intrinsically highly resistant to PxB (strain NMB, MIC ≥ 512 μg/mL); however, the mechanisms by which meningococci confer resistance have not been characterized in detail. To ascertain PxB susceptibility, a library of mariner transposon mutants in a meningococcal serogroup B strain NMB was generated. Thirty-two PxB sensitive mutants with increased sensitivity of at least 4-fold were identified from a total of ~4200 mutants by patch screening. Additionally, five PxB resistant mariner mutants were identified by replica-plating from a library constructed in a nonencapsulated derivative of strain NMB (M7, *synA::Tn916*, MIC ≥ 128 μg/mL) containing ~10,000 independent mutants. Mutants contained single insertions as determined by Southern hybridization. Using single specific primer (SSP)-PCR, chromosomal locations of mariner transposition of PxB-sensitive mutants were mapped to *porB*, *NMB0596* encoding an integral membrane protein, *gshB* encoding glutathione synthetase, *fur*, and *NMB0204* encoding a putative lipoprotein. Three sensitive mutants contained insertions in fructose 1,6 bisphosphatase (*fbp*). Interestingly, more than half of the PxB sensitive mutants have various insertions within the *mtrCDE* operon, which encodes proteins forming a well-characterized efflux pump. All five PxB resistant mutants were mapped to contain insertions within the *pilMNOPQ* operon involved in pilin biogenesis. To investigate directly the contribution of lipid A modification, a specific *lptA::Ω* insertional mutant that inactivated the PEA transferase responsible for modification of the lipid A head groups yielded the most PxB sensitive mutant with an MIC of 2 μg/ml. Consistently, PxB neutralized the TLR-4 mediated inflammatory response (TNFalpha, NO induction) elicited by lipooligosaccharide purified from the *lptA* mutant more efficiently than the response induced by wild type LOS. Taken together, these data indicated that meningococci utilize multiple mechanisms including the Mtr efflux pump and lipid A modification to confer PxB resistance.

Poster Session II
Bacterial Genetics, Physiology and Metabolism

Functional characterisation of GNA1870, a novel lipoprotein of *Neisseria meningitidis* identified by genome analysis.

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GNA1870, is a novel meningococcal antigen identified by reverse vaccinology approach. GNA1870 is a surface-exposed lipoprotein that is able to induce bactericidal antibodies and represents a promising vaccine candidate against *Neisseria meningitidis*. The antigen is expressed in all *N. meningitidis* strains tested and is present as three variants. Antibodies against a recombinant form of the protein elicit bactericidal antibodies against strains expressing the same variant and induce passive protection in the infant rat model.
In order to characterise the biological role of GNA1870 in meningococcus an isogenic mutant was generated in MC58 and characterized *in vitro*. No significant difference is observed between the wildtype MC58 strain and the mutant GNA1870 strain in their colony morphology or growth rate, suggesting that GNA1870 does not play a role on colonies morphology and growth. The ability of the mutant strain to adhere to epithelial Chang cells was investigated. The results obtained indicate that GNA1870 does not play any role in the adhesion of meninigococci to epithelial cells.
Preliminary DNA microarray data shows that deletion of the g*na1870* gene results in the up-regulation of 87 different genes. Most of the up-regulated genes are known to be involved in the defense of the bacterium from oxidative stress or from the action of antimicrobial peptides. Experiments are ongoing to define the role of GNA1870 in different growth conditions.

Identification of a mobile genetic element of *Neisseria meningitidis* associated with virulent clones

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Neisseria meningitidis, a common inhabitant of the human nasopharynx, nevertheless has the capacity in a small proportion of cases, to invade the host tissue and cause life-threatening disease, meningitis and septicaemia. The reasons for this breaking down of the normal saprophytic relationship are not well understood, but are certainly dependant on host and on bacterial factors.
In searching for the genetic determinants of the elevated pathogenic potential of certain strains of meningococus, we firstly use DNA arrays to compare the genomes of 30 strains belonging to recognised invasive sequence types (ST) with 20 strains belonging to ST having no association with disease. A single group of genes of 8 kb was significantly associated with invasive isolates. This gene cluster, the "Meningococcal Disease-Associated island" (MDA) was present in 97% of the invasive isolates and absent from all the non invasive isolates.
In order to confirm the association of this element with the invasive lineages in a wider range of strains, and further, to confirm that it was associated with disease independently of its epidemiological distribution, we investigated a collection of asymptomatically carried and disease-causing meningococci made in 1993 around Prague (Czech republic). The presence of the island was examined in 293 strains by PCR. The results confirmed the association between the presence of MDA and the hypervirulent lineages and moreover demonstrated a highly significant association with disease ($p < 0,0001$).
The island contains nine open reading frames, one of which encodes an outer membrane protein whose expression is stimulated under conditions of iron limitation, as would be encountered during systemic infection. Further sequence analysis shows similarities to bacteriophages of the type M13 of *Escherichia coli* and CTX of *Vibrio cholerae*, and molecular investigations showed that the MDA island has indeed the characteristics of a mobile genetic element. Bacteria harbouring the island secrete a circular form of the element in a nuclease-resistant state.
This work demonstrates that meningococci possessing the island are more likely to cause disease than strains lacking it. A molecular explanation is suggested by the coding of an iron-regulated outer membrane protein. Furthermore this island may be transferred from one meningococcus to another, carrying determinants important in the genesis of disease, and thus be a key factor in the development of new epidemic clones of *Neisseria meningitidis.*

Type IV pilus biogenesis in *Neissseria meningitidis:*PilW is involved in a maturation step essential for fiber stability an function

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Although type IV pili (Tfp) play a critical role in the pathogenic lifestyle of pathogenic *Neisseria* species, notably by facilitating bacterial attachment to human cells, our understanding of their biogenesis, during which the fibers are formed in the periplasm, then stabilized and finally emerge onto the cell surface, remains fragmentary. We therefore identified the genes required for Tfp formation in *N. meningitidis* by screening a genome-wide collection of mutants for those that were unable to form aggregates, another phenotype mediated by these organelles. Fifteen proteins, of which only 7 were previously characterized, were found to be essential for Tfp biogenesis. One novel component, named PilW, was studied in more detail and was found to be an outer-membrane protein that probably interacts with the PilQ multimers. We found that PilW is necessary for the stabilization of the fibers since Tfp could be restored in a *pilW* mutant by a mutation in the twitching motility gene *pilT*. However, Tfp-linked properties, including adherence to human cells, were not restored in a *pilW/T* mutant, which suggests that PilW also participates in the functional maturation of the fibers. Together, our results extend the current model for Tfp formation by suggesting that a multi-protein complex in the outer-membrane, probably centered around PilQ, is involved in the terminal stage of Tfp biogenesis during which growing fibers are not only stabilized, but also maturated, which is of primary importance for their functionality.

Capsule synthesis genes of meningococcal serogroups 29E and Z: identification and characterisation

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Meningococcal capsular polysaccharides are classified into 13 distinct serogroups as defined by antibody reactivity and structural analysis. The genes essential for capsule expression are clustered on the *cps* locus with conserved regions for capsule modification and capsule transport, and a variable serogroup-specific region A for capsule synthesis. For the clinically relevant serogroups A, B, C, W-135, and Y, the capsule synthesis genes are known and are used for non-culture serogrouping. Non-culture serogrouping is not yet possible for serogroup 29E and Z meningococci, which are mostly found in carriers and rarely cause disease because no sequence data are available. The capsular polysaccharides of both serogroups are composed of D-galactosamine linked with 2-keto-3-deoxyoctulosonate (KDO) in serogroup 29E and with glycerol and phosphate in serogroup Z, respectively (1, 2). The identical component, i.e. D-galactosamine, results in immunological cross-reactivity affecting reliability of serogrouping. Therefore, we cloned and sequenced the capsule synthesis genes of serogroups 29E and Z. Sequence analysis revealed a high A+T content typical for all known region A genes in meningococci. In serogroup 29E meningococci, eight genes were found in the region A, three of which might be involved in capsular KDO synthesis. In serogroup Z, four genes were detected, three of which showed high homologies to capsule synthesis genes of serotype 2 *Actinobacillus pleuropneumoniae*. The capsule polysaccharide of serotype 2 *A. pleuropneumoniae* also harbours glycerol and phosphate. We currently perform insertional inactivations of the genes to provide proof, whether they are indispensable for capsule synthesis. Furthermore, specific PCR assays are designed for non-culture serogrouping.

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Genome maintenance in *Neisseria meningitidis*: a role for base excision repair in mutator activity?

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Genome alterations due to horizontal gene transfer and subsequent recombinational events are causes of constant strain on the gene pool of *Neisseria meningitidis*, the causative agent of meningococcal disease. The meningococcus is naturally competent throughout its entire life-cycle, and transformation is dependent on the presence of DNA uptake sequences (DUS). A significantly higher density of DUS was found within genes involved in DNA repair and other genome maintenance genes than in any other annotated gene group[1]. This over-representation of DUS in genome maintenance genes might reflect facilitated recovery of genome preserving functions.

In addition to the highly recombinogenic genome and an elevated spontaneous mutation rate, the meningococcus is exposed to DNA damaging agents from a potent immune system as well as endogenous sources. Thus, this bacterium faces extensive and unusual challenges in terms of genome maintenance. However, the meningococcus is well equipped for DNA repair and expresses components representing all known DNA repair pathways. The base excision repair pathway is one of the major lines of defence against the detrimental effects of DNA damage. The DNA glycosylases initiating this pathway recognise and excise different kinds of DNA damage.

We have cloned and over-expressed the meningococcal DNA glycosylases MutY and Fpg of the base excision repair pathway in *Escherichia coli*. DNA glycosylase activity was monitored towards a range of DNA substrates using purified proteins. In addition, meningococcal strains with mutations in the genes encoding MutY and Fpg were constructed, and wildtype and mutant strains were compared with regard to mutagenicity, fitness and survival under varying stress conditions. Our findings show that meningococcal DNA glycosylases clearly play a role in genome maintenance. More importantly, this allowed us to search for novel mutator genes and one gene was indeed identified mediating a hypermutable phenotype in both *N. meningitidis* and *Neisseria gonorrhoeae*. This is relevant in the light of the recent findings by Stojiljkovic *et al*. of co-modulation induced by mismatch repair on phase variation and transformation[2].

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Examination of TbpB surface exposure in *Neisseria gonorrhoeae*

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Transferrin utilization by *Neisseria gonorrhoeae* is dependent on the expression of the transferrin receptor, which consists of two transferrin binding proteins (Tbp) A and B. TbpA is responsible for transport of iron into the cell. TbpB is a lipid-modified protein, for which a precise role within the receptor complex has not been completely elucidated. Both TbpA and TbpB show exquisite specificity for human transferrin and TbpB preferentially binds holo-transferrin. TbpB is not necessary for the acquisition of iron from transferrin although it has been suggested that TbpB increases the efficiency of iron uptake. Little is known about the mechanism of lipoprotein processing to the outer membrane surface. To gain insight into this process, export of TbpB was examined in three previously created TbpB mutants containing deletions of all or part of the structural *tbpB* gene. Two of the mutants, FA6815 and FA6819 contain the N-terminal 250 and 290 amino acids of TbpB, respectively. FA6905 contains a deletion of the entire *tbpB* structural gene. All of these strains are incapable of binding transferrin by Western blot. FA6815 and FA6819, however, produced a small TbpB product that was detectable by Western blot. In a dot blot assay, we determined that both of these small N-terminal TbpB products were present on the surface of the gonococcus. The data obtained from these experiments suggest that only the extreme N-terminus of TbpB is necessary for efficient export to the cell surface. For the second phase of this study, we hypothesized that TbpB was completely surface exposed and tethered by its N-termiuns to the outer membrane through a lipid moiety. To test this hypothesis, the hemagglutinin (HA) epitope was inserted into TbpB with the dual purpose of determining cellular location of the insertion site as well as examining effects on protein function. The epitope sequence was inserted using a two-step PCR technique, followed by cloning and sequencing of the resulting HA-encoding PCR products. After gonococcal transformation, the mutagenized copy replaced the wild-type *tbpB* gene by homologous recombination. Two insertionally mutagenized *tbpB* genes have been created, placing the HA-epitope insertion downstream of the N-terminus of the mature gene product. Both of the mutants were analyzed for surface exposure of the epitope, for transferrin binding on Western blot and for transferrin utilization in the presence and absence of TbpA. Based on these analyses the two mutants were phenotypically wild type. The epitope was surface accessible in both of these mutants, demonstrating that TbpB is surface exposed from just beyond the mature N-terminus. We are currently constructing and characterizing other HA epitope insertions with the goal of understanding surface topology and structure-function relationship in this lipoprotein.

Paying the Price for Phase Variation: the Homopolymeric Tract in the *lgtA* Gene from *Neisseria meningitidis*

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Phase variation describes a process of reversible on/off switching of genes that occurs at a high frequency. In pathogenic bacteria phase variation is a feature of genes associated with virulent determinants such as: pili, lipooligsaccharide, capsular polysaccharide and outer membrane proteins. *Neisseria meningitidis* and *Neisseria gonorrhoeae* both display an extensive repertoire of variant LOS structures. The complexity and heterogeneity of these LOS structures is due to the presence or absence of the LOS biosynthesis genes found in the *lgt*ABCDE locus and the unlinked *lgt*G, and/or the ability of these genes to phase vary. In *N. meningitis* the *lgt*A gene encodes for a eta-1,3-N-acetylglucosaminyltransferase, which is involved in the biosynthesis of the terminal LOS structure lacto-N-neotetraose and contains a homopolymeric tract of guanosine residues. The number of guanosine residues (Gs) in the *lgt*A homopolymeric tract can vary from between 5Gs to 17Gs, resulting in a range of 1 to 5 glycine residues in the protein. In *N. meningitidis* strain MC58 phase variation of *lgt*A, which has 14 Gs, is responsible for the switching between L3 and L8 immunotypes and occurs at a rate of 1 in 200. This modulates the sialylation of LOS where L3 is sialylated and is non-invasive, while L8 is non-sialylated and is invasive. Hence, this immunotype switching is most likely important in pathogenesis.

Previous studies have shown that there is less potential for a lower number of G-residues in the homopolymeric tract of *lgt*A to phase vary and that the higher the number of G-residues the greater the frequency for phase variation. Analysis of the enzymatic activity of purified LgtA variants with a range of homopolymeric tract lengths has confirmed that alteration in tract length effects LgtA specific activity. These results indicate that in the case of homopolymeric G tract in LgtA the acquisition of the phase variation mechanism is not neutral with respect to enzyme activity.

The Gonococcal Genetic Island of *Neisseria gonorrhoeae*: A Mobile Genetic Element?

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A large genetic element of low G+C content, differing dinucleotide frequencies, and relatively few DNA uptake sequences exists in most strains of *Neisseria gonorrhoeae.* This gonococcal genetic island (GGI) is inserted in the chromosome at the *dif* site and flanked by a direct repeat, an incomplete *dif* site. In *E. coli*, XerCD, a site-specific recombinase, recognizes the *dif* site and resolves chromosomal dimers during DNA replication. The presence of both a *dif* site and a near perfect *dif* site, along with the evidence for horizontal acquisition and the absence of the GGI from some strains, suggest that the GGI may be gained or lost by site-specific recombination. However, mobility of the GGI may not be limited to site-specific recombination. Because gonococci are naturally competent for transformation, gain or loss of the GGI may occur via transformation, as well.

In order to elucidate whether the GGI is a mobile element, we have examined both site-specific recombination, as well as homologous recombination. We have created strains and constructs that should enable the detection and quantification of the gain or loss of DNA at the *dif* site via site-specific recombination. To determine whether GGI loss can occur by transformation, the region flanking the GGI was cloned from a GGI deficient strain and transformed into a strain containing a productive mTn*CmPhoA* insertion within the GGI. A blue/white screen was performed and white colonies, indicating the loss of alkaline phosphatase activity, were obtained. The loss of the GGI was verified by PCR, indicating that the GGI can be lost via homologous recombination.

This GGI deletion mutant strain was further characterized for its interactions with host cells. As depicted by electron microscopy, primary cervical cells infected with the GGI deletion strain showed hyper-aggregation of the gonococci to the host cells, an altered attachment phenotype relative to wildtype infection. Although the GGI deletion mutant displayed an altered attachment phenotype, quantitative analysis of infection by the GGI deletion mutant in the ME180 cervical cell line showed no significant difference in the number of gonococci associated with host cells. However, an 85-fold reduction in invasion of ME180 cervical cells was demonstrated, suggesting that the GGI may have a role in invasion of the host epithelium. The *in vitro* effects of the GGI in conjuction with the mechanisms of its transfer, site-specific recombination at the *dif* site and/or homologous recombination, indicate that the GGI may be a mobile genetic element affecting survival within the host.

Characterization of Gonococcal MinE Reveals Two Critical Functions for Proper Cell Division Site Selection in Prokaryotes

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The tripartite Min protein system specifies proper cytokinetic site selection in dividing bacteria. MinC, MinD, and MinE, are found in many Gram-negative bacteria, including the coccus *Neisseria gonorrhoeae* (Ng) and the bacillus *Escherichia coli*. MinC and MinD are responsible for inhibiting cell division. In rod-shaped bacteria such as *E. coli*, MinE acts as a topological sensor by inducing the MinCD complex to oscillate from pole-to-pole to restrict their activity to these regions through a coil that winds internally within. This allows FtsZ and other cell division proteins to initiate cytokinesis at the midcell. The role of MinE in round bacteria is not clearly known. By studying site-directed mutants of conserved gonococcal MinE ($MinE_{Ng}$) residues, the objectives of this study were to identify regions of $MinE_{Ng}$ that interacted with $MinD_{Ng}$ and imparted topological specificity to $MinD_{Ng}$. We previously showed that $MinE_{Ng}$ (87 aa) is functional in *E. coli*, thereby creating a biological model for determining the functionality of this protein. Amino acid substitutions of highly conserved $MinE_{Ng}$ residues were generated to examine their effects in bacterial cytokinesis. We found that $MinE_{Ng}$ has two primary domains, a N-terminal $MinD_{Ng}$-binding domain, and a C-terminal domain which is implicated in the oscillation of intracellular GFP-$MinD_{Ng}$. N-terminal $MinE_{Ng}$ mutants failed to bind to $MinD_{Ng}$ and did not induce GFP-$MinD_{Ng}$ oscillation, produce a minicell phenotype, or stimulate $MinD_{Ng}$ ATPase activity. However, $MinD_{Ng}$ did interact with C-terminal mutants of $MinE_{Ng}$, although at reduced levels. These mutants were able to induce GFP-$MinD_{Ng}$ oscillation, despite their different cell division phenotypes and $MinD_{Ng}$ ATPase activities. These findings suggest that the binding strength of $MinE_{Ng}$ to $MinD_{Ng}$ is critical for $MinE_{Ng}$ to properly function in bacterial division site selection. Thus, a heterologous model for MinE/MinD function has been developed. The N-terminus of $MinE_{Ng}$ is primarily implicated in $MinD_{Ng}$ binding, while the C-terminus is involved in a minimum interaction with $MinD_{Ng}$ which is required for $MinE_{Ng}$ to stimulate sufficient $MinD_{Ng}$ ATPase activity to properly regulate normal cell division. Finally, interaction between $MinD_{Ng}$ and $MinE_{Ng}$ recruits $MinD_{Ng}$ to a coiled protein scaffold. A model of cell division site selection in cocci based on these results has been developed. Ultimately, subtle differences or commonalities in MinE from different bacteria can be used as a basis for specialized or broad range novel antimicrobial design.

The *Neisseria gonorrhoeae* lactate permease is required for resistance against complement mediated killing and survival *in vivo*. EXLEY RM[1], SHAW J[2] , WU H[3], SMITH H[4], JERSE A [3], TANG CM[1]. [1] The Centre for Molecular Microbiology and Infection, The Flowers Building, Imperial College London, Armstrong Road, London, SW7 2AZ, UK. [2]Divison of Genomic Medicine, F-floor, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX, UK [3] Department of Microbiology and Immunology, F. Edward Herbert School of Medicine, Uniformed Services University, Bethesda, Maryland 20814, USA. [4]The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

Neisseria gonorrhoeae causes a sexually transmitted disease that elicits an intense inflammatory response within the human genital tract, leading to transcytosis of polymorphoneutrophil leukocytes and exudation of serum components into the genital tract lumen. *N. gonorrhoeae* must survive within this hostile environment, and evade killing by the innate immune response. The major mediator of killing in serum is complement activation and deposition on the microbial surface, with assembly of the membrane attack complex, and cell lysis. Lactate, even when added at millimolar concentrations to cultures containing glucose, enhances serum resistance of the gonococcus through sialylation of lipopolysaccaride (LPS). Lactate is present with glucose on human mucosal surfaces and in serum, and is one of the few effective carbon sources for *N. gonorrhoeae.*

To further elucidate the impact of lactate on the pathogenesis of gonococcal infection, we constructed a strain of the bacterium that is specifically unable to utilise lactate. A gonococcal sequence (designated *lctP)* was identified that shares 21% identity to the lactate permease from *E. coli.* Lactate uptake studies were performed on the gonococcal isolate, F62 and the corresponding *lctP* mutant (F62delta*lctP*). F62 rapidly accumulated ^{14}C lactate assimilating 5.2 nmol of lactate per mg of cellular protein/min., whereas uptake by F62delta*lctP* was negligible, demonstrating that this gene is necessary for lactate uptake by *N. gonorrhoeae*. After growth with lactate and glucose, measurement of 2-keto-deoctonate demonstrated that F62delta*lctP* expresses less LPS than F62. Next, we compared the survival of F62delta*lctP* against the wild-type in the presence of a range of dilutions of human serum. At all dilutions of human serum up to 1:32, the wild-type bacterium was recovered in higher numbers than F62delta*lctP*; the survival of the strains was identical in serum that had been heated to 56°C to eliminate complement activity. Finally, we found that F62delta*lctP* was markedly attenuated for survival in the murine vaginal tract, demonstrating the importance of lactate availability *in vivo*.

Pathogenesis and diagnosis of human meningococcal disease using immunohistochemical and PCR assays. GUARNER J, GREER PW, WHITNEY A, SHIEH WJ, FISCHER M, WHITE EH, CARLONE GM, STEPHENS DS, POPOVIC T, ZAKI SR. Centers for Disease Control and Prevention, Atlanta, GA; and Emory University School of Medicine, Atlanta, GA.

Worldwide, *Neisseria meningitidis* remains the leading cause of fatal sepsis. Cultures may not be available in fulminant fatal cases or may not useful to the diagnosis in patients receiving antimicrobials. An immunohistochemical (IHC) assay for *N. meningitidis* was applied to formalin-fixed autopsy or biopsy tissue samples from 14 patients with meningococcal disease. Significant histopathologic findings in 12 fatal cases included interstitial pneumonitis, hemorrhagic adrenal glands, myocarditis, meningitis, and thrombi in glomeruli and choroid plexus. Meningeal inflammation was observed in 6 patients. Skin biopsies of 2 surviving patients showed leukocytoclastic vasculitis and cellulitis. By using IHC, meningococci and granular meningococcal antigens were observed inside monocytes, neutrophils, and endothelial cells or extracellularly. Thirteen patients had disseminated meningococcal infection as evidenced by presence of meningococci in blood vessels, capillaries, or sinusoids of a variety of tissues. By using real-time polymerase chain reaction (PCR) on formalin-fixed tissues, meningococcal serogroup determination was possible in 10 of 14 cases (8 serogroup C, 1 Y, and 1 B). In summary, by using IHC and real-time PCR on formalin-fixed tissues and cells, *N. meningitidis* can be diagnosed and serogrouped. IHC determined the distribution of meningococci and meningococcal antigens in tissues during meningococcemia and meningococcal meningitis, allowing better insights into *N. meningitidis* pathogenesis.

Mutations affecting peptidoglycan acetylation in *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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Neisseria gonorrhoeae and *Neisseria meningitidis* both acetylate their peptidoglycan (PG) by addition of O-linked acetate to the C-6 position on the N-acetylmuramic acid. In *N. gonorrhoeae* this acetylation is thought to make the bacteria more resistant to host lysozyme. Also the acetylated PG is more active in inducing arthritis in rats as compared to non-acetylated gonococcal PG. Examination of the gonococcal genome sequence of strain FA1090 identified a putative O-acetylase of the membrane-bound O-acyltransferase (MBOAT) family. We have designated this gene *pacA*, for peptidoglycan acetylation gene A.

Using an HPLC-based assay for acetate on the PG, we found that an insertion mutation in *pacA* eliminated acetylation. Insertion of a wild type copy of *pacA* at a distant location on the chromosome did not restore acetylation, suggesting that genes downstream of *pacA* might be required. RD5 is a naturally occurring strain that does not acetylate the PG. Sequencing of *pacA* in RD5 revealed a 26 bp deletion in the coding sequence, resulting in a frameshift and a premature stop codon. When this mutated *pacA* was introduced into wild type strain MS11, the mutation was found to eliminate acetylation, and this mutation was complemented by a wild type *pacA*. We also made mutations in the three genes downstream of *pacA*. The gene directly downstream of *pacA* was required for acetylation and we designated this gene *pacB*. The other two genes were not required for acetylation. Analysis of PG turnover showed that the MS11 *pacA* mutant is not reduced in overall PG fragment release. However, further analysis will be necessary to determine if the profile of fragments released is different from that of wild type strains.

The *pacA* mutants were not defective in growth and showed no difference in sensitivity to penicillin G. However, treatment with 1 mM EDTA revealed that the *pacA* deletion mutant is more than 100 fold more sensitive to this compound than the wild type parent. As EDTA is known to trigger autolysis, this result may indicate that acetylation of the PG protects against autolysis.

Using PCR we were not able to detect the *pacA* gene in several commensal *Neisseria* species including *N. flavescens*, *N. subflava*, *N. perflava*, *N. cinerea*, and *N. mucosa*. HPLC analysis of a *N. flavescens* strain showed no acetate on the PG. However, *pacA* was present in two *N. lactamica* isolates and HPLC analysis confirmed acetylation in an *N. lactamica* strain. *pacA* and *pacB* are also found in the published *N. meningitidis* sequences. An insertion mutation in *pacA* in a meningococcal serotype C strain eliminated acetylation. The analysis of different species indicates that PG acetylation is present in the pathogenic neisseria and the slightly pathogenic *N. lactamica*.

Characterization of TonB-dependent iron acquisition of *Neisseria gonorrhoeae*

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Pathogenic *Neisseriae* have evolved a repertoire of high-affinity iron acquisition systems to facilitate essential iron uptake in the human host. This requires both specific outer membrane receptors and energy-harnessing cytoplasmic membrane proteins. The Ton complex is capable of coupling cytoplasmic membrane energy to the outer membrane, permitting high-affinity transport into the periplasm. *N. gonorrhoeae* has evolved at least four characterized TonB dependent receptors enabling iron acquisition from a variety of iron sources including transferrin, lactoferrin, hemoglobin and the xenosiderophore, enterobactin. In this study, we further investigated additional iron sources that may be acquired during gonococcal infection, and examined whether uptake was dependent on a functional Ton system. To address the role of TonB in uptake of both exogenous and intracellular iron sources, a Ton system knockout was constructed by insertion of a streptomycin resistance encoding cassette into the *AgeI* site of the *tonB* gene. This mutant was tested in a plate-bioassay to determine the role of TonB in uptake of heterologous siderophores and inorganic iron sources. Gonococcal growth was promoted by the siderophores aerobactin, coprogen, neocoprogen, ornibactin, and fusigen; however the detected plate growth was independent of the characterized Ton system as well as proteins involved in transferrin (Tbp) and lactoferrin (Lbp) uptake. Additionally, gonococcal growth was promoted by various inorganic iron sources in a TonB independent manner, as evaluated in the plate bioassay. The role of TonB in intracellular survival was addressed by conducting a gentamicin-killing assay in which eukaryotic cells were infected with either wild-type or mutant gonococci for 24 hours. Extracellular bacteria were then removed by a gentamicin treatment, and intracellular gonococci are enumerated over a 24-hour period. As expected, iron acquisition was critical to intracellular survival of the gonococcal strain FA19 within A431 human endocervical epithelial cells as treatment with Desferal inhibited intracellular survival of the gonococcal wild type strain. This implies that survival within these cells is dependent upon iron availability. By use of a strain that lacks functional Tbp and Lbp systems in this assay, we determined that neither transferrin nor lactoferrin serve as the intracellular iron source for the gonococcus, as a double mutant strain exhibited nearly identical survival patterns as the wild-type. With respect to the role of TonB, it appears that this protein may provide a benefit to survival at earlier time-points, however infection with the mutant strain yielded a similar number of gentamicin-resistant colonies by the end of the 24-hour intracellular growth assay. We conclude that TonB provides some benefit in intracellular survival and continue to evaluate the TonB dependence of acquisition of other xenosiderophores produced by a variety of microorganisms.

Preliminary analysis of the *Neisseria gonorrhoeae* stringent response

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Analysis of the various *Neisseria* genome sequences reveals a distinct lack of two-component regulatory systems as well as a restrictive number of different sigma factors (e.g., there is no apparent RpoS-mediated stationary phase regulon). However, one of the few regulatory systems that the gonococcus does seem to possess in order to combat stressful conditions is the ability to engage in a stringent response mediated by the *relA* and *spoT* gene products. This adaptive response involves the rapid accumulation of the regulatory nucleotide guanosine-3'5'-(bis) pyrophosphate (ppGpp) and the 3'-diphosphate, 5'-triphosphate analog, pppGpp whose accumulation shuts down macromolecule synthesis within the cell. Moreover, an active stringent response has also been shown to contribute to the pathogenicity of several intracellular and extracellular pathogens. Our initial assessment of the gonococcal stringent response involved the construction of gonococcal *relA* and *spoT* null mutants. Following starvation of gonococci for the amino acid serine using a modified minimal medium, wild type cells exhibited a rapid (within minutes of encountering starvation conditions) robust accumulation of (p)ppGpp nucleotides, which was nullified in a Δ*relA::kan* isogenic strain. In contrast, (p)ppGpp accumulation was not affected through the inactivation of the *spoT* gene. Thus, the presence of the RelA protein alone is sufficient for (p)ppGpp production when gonococci are starved for the amino acid serine. Therefore, we propose that given the potentially adverse conditions that the organism is likely to encounter on an infected mucosal surface, the gonococcal stringent response, and, presumably, an associated stringent response regulon, is likely to be invoked during an active infection.

A novel transcription factor from *Neisseria gonorrhoeae*, forming a subfamily of the MerR Family of bacterial regulators.

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NmlR from *N. gonorrhoeae* (Neisseria *merR*-like Regulator) is a prototype for a subfamily of the MerR Family of bacterial regulators. The function of this transcription factor is examined and a model for its role in cellular response to environmental stresses such as oxidative stress is presented. NmlR regulates expression of *adhC* (a glutathione-dependant alcohol dehydrogenase). This gene is positioned divergent to *nmlR*. The target operator/promoter is overlapping and divergent to that for *nmlR* in a MerR arrangement. NmlR is proposed to also regulate a CPx-type P-type ATPase (a metal ion pump), upstream of which exists a MerR-like target operator/promoter. Promoter activity studies indicated that NmlR repressed both these operator/promoters and that repression was Zn(II) dependent. An *nmlR*$^-$ *N. gonorrhoeae* strain had an altered physiology, indicatively being highly sensitive to diamide and cumene hydroperoxide. Real time PCR has highlighted NmlR regulation of *adhC* and the CPx-type P-type ATPase and the gene divergent to this latter gene, a thioredoxin reductase. Under normal culture conditions NmlR represses these genes, but when stressed by diamide it acts as their activator. Assaying for AdhC with a range of substrates revealed the *N. gonorrhoeae nmlR*$^-$ possessed no significant activity.

Analysis *Cis*- and *Trans*-Acting Factors Involved in Recombination and Repair in *Neisseria gonorrhoeae*

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In *Neisseria gonorrhoeae*, homologous recombination is critical for the processes of recombinational DNA repair, genetic exchange via DNA transformation, and pilin antigenic variation. Antigenic variation at *pilE* occurs through a unidirectional, homologous recombination event between a *pilS* silent copy and the *pilE* expression locus. Genes encoding the recombination factors RecA, RecX, RuvABC, and RecG have been shown to be involved in all homologous recombination-mediated processes in *N. gonorrhoeae*. Genes encoding RecBCD and RecN are involved DNA repair and DNA transformation, while genes encoding RecO, RecR, RecQ, and RecJ are involved in DNA repair and pilin antigenic variation. In addition, several *cis*-acting factors of the *pilE* expression locus have been shown to be important for antigenic variation.

In order to analyze the role of origin-independent replication restart in the homologous recombination processes of *N. gonorrhoeae*, the effects of *priA* or *rep* mutations on homologous recombination were examined. PriA or Rep are proposed to initiate replication restart. The genes encoding both PriA and Rep are important for DNA transformation. The gene encoding PriA is crucial for mediating DNA repair caused by UV light or by hydrogen peroxide. The gene encoding Rep is involved in, but not required for, antigenic variation, whereas the gene encoding PriA is not involved in this process. These studies identify a novel role for *rep* in the recombination leading to antigenic variation. In addition, these findings are the first demonstration of a requirement for the replication restart pathway in response to oxidative damage in any organism.

In order to identify additional factors involved in pilin antigenic variation, we investigated whether sequences 5' to the *pilE* expression locus act in *cis* on this process. Transposon mutagenesis and deletion analysis of the region spanning approximately 1kb upstream of *pilE* were performed. Transposon insertions spanning a 50bp region 300-350bp upstream of the *pilE* start codon, or deletion of this region, block pilin antigenic variation. These results suggest an essential role for these sequences in antigenic variation. Transposon insertions in a 50bp region 250-300bp upstream of the *pilE* start codon impair, but do not abrogate, antigenic variation. These data suggest that these upstream sequences are an accessory site important for antigenic variation or interfere with upstream sequences essential to this process. Experiments are currently being carried out to determine how these upstream sequences contribute to pilin antigenic variation.

AtlA functions as a peptidoglycan transglycosylase that is required for function of the *Neisseria gonorrhoeae* type IV secretion system

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The gonococcal genetic island (GGI) is a variable 57kb genetic island found in about 80% of *Neisseria gonorrhoeae* isolates and encodes a type IV secretion system (T4SS). The T4SS genes of the GGI are similar to the genes necessary for F-plasmid transfer during *Escherichia coli* conjugation. T4SS in other bacteria including *Agrobacterium tumefaciens*, *Bordetella pertussis,* and *Helicobacter pylori* secrete protein, or DNA and protein substrates. Strains of *N. gonorrhoeae* carrying the GGI have been shown to secrete DNA into the culture supernatant. All characterized T4SS include peptidoglycan transglycosylases that are thought to create a space in the bacterial peptidoglycan so that the T4SS machinery can assemble. The GGI encodes a putative peptidoglycan transglycosylase, AtlA, which is homologous to the bacteriophage lambda *R* gene product. The *R* gene product is a peptidoglycan transglycosylase involved in lysing infected bacteria. It was previously shown that an insertion mutation in *atlA* eliminated T4SS function, although the function of AtlA has not been demonstrated.

In order to examine AtlA function, a point mutation was made in its putative catalytic site. This site is shared by all peptidoglycan transglycosylases and is essential for peptidoglycanase activity. A strain with this mutation was deficient in DNA secretion, and complementation with a wild type copy of *atlA* restored DNA secretion. This result indicates that AtlA is required for type IV secretion and functions as a peptidoglycan transglycosylase.

Because *atlA* is not present in all versions of the GGI, we investigated the importance of the presence of *atlA* and variations in the genes surrounding it. Directly upstream of *atlA* is the gene *traG,* which can be present as a variant allele called *sac-4*. The presence of the *sac-4* allele is associated with disseminated infection isolates. In one variation of the *traG* locus, *atlA* is missing and sequence encoding a peptidoglycan endopeptidase domain is fused to *traG.* When the *traG-atlA* region of the GGI was replaced by the peptidoglycan endopeptidase version of *traG*, DNA secretion was significantly reduced. Strains with the *sac-4* version of *traG* were able to secrete DNA at a level similar to that of strains without the *sac-4* allele of *traG*.

The similarity between AtlA and the bacteriophage lambda *R* gene product suggests that AtlA may, like R, be a peptidoglycan transglycosylase. To examine this possibility, we replaced the *R* gene with *atlA*. Induction of the wild type lambda lysis genes resulted in bacterial lysis, as did induction of the lysis genes with *atlA* replacing *R*. The ability of AtlA to functionally replace R confirms the function of AtlA as a peptidoglycan hydrolase. The necessity of AtlA for DNA secretion suggests that it plays a role in degrading peptidoglycan to allow for T4SS assembly or function.

Potential role of neisserial NMB0419 gene in adherence of human epithelial cells and pilus formation: functional characterisation in *Escherichia coli* and transcriptome profiling of NMB0419 knockout in *Neisseria meningitidis*

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NMB0419 of *Neisseria meningitidis* strain MC58 is a 198 aa putative periplasmic protein, three-quarters of which comprises four 34-aa (tetratricopeptide) repeats, potentially forming a domain of antiparallel alpha-helices characteristic of the TPR family of protein-folding chaperones[1]. A review of other meningococcal strains and other *Neisseriae* revealed that an *NMB0419* homologue is present in all strains, though the number of TPR repeats varies. NMB0419 is 42% identical in sequence to BPF001, a protein of the Brazilian Purpuric Fever clone of *Haemophilus influenzae* biogroup *aegyptius* implicated in respiratory epithelial invasion[2]. We have accordingly sought a role for NMB0419 in meningococcal adherence and invasion of human epithelial cells.

Recombinant NMB0419 was expressed in *Escherichia coli* K-12 from the plasmid pET0419 to study association of the transformant with monolayers of the human bronchial epithelial cell line 16HBE14. There was no increase in epithelial invasion compared to a control *E. coli* strain bearing the empty vector (pET), but *E. coli* (pET0419) was significantly more adherent. This adherence was abolished by in-frame deletion of DNA encoding the TPR domain, and also by addition of D-mannose to the tissue culture medium, suggesting that NMB0419 may facilitate formation of MS pili. The involvement of MS pili in this adherent phenotype was supported by light microscopy of infected cells. An "aggregative adherence" pattern was observed for *E. coli* (pET0419) but not *E. coli* (pET). Rigid structures resembling type I pili were seen on the surface of *E. coli* (pET0419) by electron microscopy, which were absent on *E. coli* (pET).

Inferring from our bioinformatic analysis a potentially wide repertoire of protein-protein interactions involving NMB0419, we have begun to explore the consequences on global gene expression of deletion of this gene. Using broth-grown organisms initially, we have compared the transcriptome of an in-frame deleted NMB0419 knockout of *N. meningitidis* MC58 to the parental strain using a whole genome neisserial DNA microarray. 10 genes were up- and 26 down-regulated >1.7 fold in the knockout mutant (using a cut-off of $p < 0.05$). 6/10 up-regulated genes encode putative membrane-associated proteins, including NspA and a component of the L-lactate permease. One of the most down-regulated genes (at least 3-fold) was *pilE*, the major component of the meningococcal pilus. 13/26 other down-regulated genes encode putative membrane proteins.

Our results so far suggest that NMB0419 is a periplasmic chaperone with broad target specificity, including components of pili that are involved in host-pathogen interactions and may be important in virulence.

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Sialylation of lacto-*N*-neotetraose lipooligosaccharide in gonococci, but not meningococci, results in enhanced factor H binding: the modulatory role of gonococcal porin

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Sialylation of gonococcal lacto-*N*-neotetraose (LNT) lipooligosaccharide (LOS) uniformly results in enhanced resistance to the direct bactericidal action of nonimmune normal human serum (NHS). We have previously shown that sialylated gonococci bind factor H, the key soluble phase regulatory protein of the alternative complement pathway, which may constitute a mechanism of serum resistance. Meningococci require capsular polysaccharide for virulence and serum resistance, and the role of LOS sialylation in disease pathogenesis is not fully understood. In contrast to gonococci, sialylation of meningococcal LNT LOS, rather unexpectedly, did not result in enhanced binding of factor H. LOS sialylation in unencapsulated meningococci resulted in only low level of serum resistance (survival in <5% NHS), and not all strains showed enhanced resistance to serum upon LOS sialylation.

We sought to explain the differences in behavior of LOS sialic acid on the two pathogenic neisserial species with respect to factor H binding and complement regulation. Porin (Por) is the most abundant outer membrane protein on the neisserial surface and is closely associated with LOS. We have previously shown that gonococcal Por1A can bind factor H. Por1B gonococcal strains bind factor H only when their LOS is sialylated. We hypothesized that the Por molecule of gonococci may be a critical determinant in modulating factor H binding to sialylated gonococci. To test this hypothesis, we replaced the PorB2 molecule of meningococcal strain Y2220 *siaD* (unencapsulated by insertional inactivation of the polysialyltransferase, or *siaD* gene, LOS fully sialylated, and does not bind factor H even when LOS is sialylated) with the Por1B molecule of gonococcal strain F62 (binds factor H only when LOS is sialylated).

Flow cytometric analysis demonstrated that the resultant meningococcal mutant (Y2220 *siaD* PorF62), bound factor H to the same extent as the gonococcal strain F62 grown in the presence of 5′-cytidinemonophospho-*N*-acetylneuraminic acid. Y2220 *siaD* PorF62 meningococcal mutant survived (0% killing) in 5% NHS, while the recipient gonococcal strain Y2220 *siaD* was killed (~80%) in as low as 1.25% NHS. A Y2220 *siaD* PorF62 meningococcal mutant with a hybrid porin containing the N-terminal loops of Y2220 porin and the C-terminal loops of F62 porin did not bind factor H, suggesting that the presence of the N-terminal loops of the gonococcal porin are required for factor H binding.

While meningococci require capsular polysaccharide, but not LOS sialylation, for virulence, gonococci rely on LOS sialylation to resist the killing effect of serum and for pathogenesis. Our data may serve to explain the contrasting behavior of LNT LOS sialylation on the surface of the two pathogenic neisseria species.

Replacement of the porin of serum-sensitive *Neisseria meningitidis* with the porin from *N. gonorrhoeae* regulates the classical pathway of complement and confers serum resistance

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This study was undertaken to examine the importance of complement classical pathway regulation by bacterial surfaces in mediating serum resistance and virulence. *Neisseria meningitidis* capsular polysaccharide (Cap) is an essential virulence factor that confers resistance to direct complement-mediated killing. Meningococcal Cap-negative strains are sensitive to the bactericidal action of non-immune normal human serum (NHS) and are avirulent. The other pathogenic neisseria, *N. gonorrhoeae*, uses its porin protein (Por) to evade complement by binding serum complement regulatory molecules such as C4b-binding protein (C4bp) and factor H.

Using homologous recombination, we replaced the PorB (C4bp non-binding) protein of two Cap-negative meningococcal strains (H44/76 Cap- and Y2220 Cap-; both with unsialylated lipooligosaccharide) with the C4bp-binding Por1A protein of gonococcal strain FA19.

Akin to gonococcal strain FA19, the resultant meningococcal strain mutants (H44/76 Cap-/PorFA19 and Y2220 Cap-/PorFA19) were resistant to serum (100% survival in 50% and 10% NHS, respectively). This was in contrast to H44/76 Cap- and Y2220 Cap-, which were completely killed (0 % survival) in even 10% NHS. Flow cytometric analysis confirmed that H44/76 Cap-/PorFA19 and Y2220 Cap-/PorFA19 meningococcal mutants bound C4bp. Bound C4bp was functional as evidenced by its ability to degrade C4b to the hemolytically inactive C4d fragment. Hybrid mutants lacking loop 1 of PorFA19 (H44/76 loop 1 with FA19 loop 2-8) did not bind C4bp and were serum sensitive, confirming that loop 1 is essential for C4bp binding and the complement resistant phenotype. A hybrid mutant containing the first two loops of FA19 (FA19 loop 1-2 with Y2220 loop 3-8) did not bind C4bp, suggesting that loop 1 alone was not sufficient for C4bp binding.

The ability of unencapsulated meningococci to survive direct complement-mediated killing was regained by the acquisition of the gonococcal porin. The virulence of these meningococcal mutants in an animal model remains to be tested.

Plasmids in commensal *Neisseria*

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Naturally occurring plasmids have been identified in the genus *Neisseria*, in particular in the pathogenic species *Neisseria gonorrhoeae*[1]. In addition to these native plasmids, a number of derivatives replicating in *Neisseria* have been developed in the laboratory as genetic tools for *Neisseria* research. One of these, the hybrid plasmid pMIDG100[2] (GenBank accession number AY174111) has been successfully transferred by conjugation into a wide range of commensal and pathogenic *Neisseria*, but failed to pass to any member of a diverse collection of *N. lactamica*[3]. We are seeking to make plasmids which replicate in *N. lactamica* and may be used to express antigens with vaccine potential. Two approaches are being pursued: the first, to modify pMIDG100; and the second to identify, and modify as necessary, novel plasmids from commensal *Neisseria.*

Several new neisserial plasmids have been identified. A survey of *N. lactamica* strains found plasmids in five out of eight. Four of these plasmids were sequenced completely: two were found to be essentially the same as pJS-A[4], a previously described plasmid found in *N. meningitidis*, while the remaining two were novel. All were cryptic plasmids of approximately 2 kb, with 2 major open reading frames, the largest of which showed homologies suggesting function as a replication protein. These plasmids do not appear to replicate in *E. coli* and are currently being modified to make *E. coli – N. lactamica* shuttle vectors. The complete nucleotide sequence of a small (approx. 2 kb) cryptic plasmid, pMIDG2830, from *N. flavescens* has also been determined. This plasmid also appears to be novel and was not found in any members of a collection of commensal neisserial strains screened by Southern hybridisation. The *aph*A-3 gene conferring kanamycin resistance has been inserted into pMIDG2830 to act as a selectable marker, along with a multiple cloning site and the neisserial uptake sequence. This plasmid replicates successfully in *E. coli* and is being used in attempts to transform *N. meningitidis* and other commensal *Neisseria*, in particular *N. lactamica*.

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Biochemical and Evolutionary Analysis of Phosphoethanolamine Addition to Lipooligosaccharide of Pathogenic *Neisseria*

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Lipooligosaccharide (LOS) is an important neisserial virulence determinant consisting of a series of oligosaccharide chains (OSs) attached to a core region (consisting of Lipid A, KDO and heptose). Variation in the expressed OS structure occurs as the result of alterations in the expression of *lgtA, C, D* or *G* genes. Core region variability consists of differences in the location and number of phosphoethanolamine (PEA) decorations and acyl chain composition. Additional variation occurs if LOS is decorated with acetate or sialic acid. These differences are important in neisserial biology because specific LOS structures affect a strain's ability to invade human cells or to elicit differential immune responses. Heptose II (HepII) of the core can be modified with PEA at the 3, 6, or 7 positions. The specific decoration site affects the ability of *Neisseria* to survive in human serum, with cells expressing the 6-HepII modification binding complement component C4b more efficiently than cells adding PEA at 3-HepII. We have hypothesized that a single gene encoding a PEA transferase exists in the *Neisseria* and that this gene product is responsible for the location of the PEA decoration onto HepII. We identified a homolog of *N. meningitidis* MC58 *lpt-3*, previously shown to be involved in PEA decoration of *N. meningitidis* LOS, in *N. gonorrhoeae* FA1090 through a chromosomal DNA database search. We PCR amplified this gene and cloned it into vector pUC19. The transposon Tn5 was inserted into the coding region of *lpt-3*, and this recombinant gene was introduced into gonococcal strain F62delta*lgtA*, a strain containing an alpha-chain consisting of lactose and a PEA at 3-HepII, generating F62delta*lgtAlpt-3::Tn5*. LOS isolated from F62delta*lgtAlpt-3::Tn5* lost the ability to bind Mab 2-1-L8, a Mab that requires the presence of PEA at 3-HepII and lactose composing the alpha-chain. After purification of Lpt-3 over-expressed from pET15b in *Escherichia coli*, we developed a biochemical assay that demonstrated that gonococcal Lpt-3 could transfer PEA to LOS isolated from F62delta*lgtAlpt-3::Tn5* in vitro. The DNA sequence of *lpt-3* from several different meningococcal and gonococcal strains was determined and analyzed to study the extent to which it is conserved across the pathogenic *Neisseria*. *lpt-3* was PCR amplified and sequenced from *N. meningitidis* 53415, 53418, 13102, 13113, and 89I as well as *N. gonorrhoeae* FA1090, MS11, and F62. A high degree of DNA sequence variation was found at the nucleotide level, and these differences result in some significant changes in the amino acid sequence of Lpt-3. The nature of the differences in these coding sequences suggests that multiple alleles of *lpt-3* exist, and the presence of a particular allele will determine if the strain has the genetic capacity to add PEA to the 3, 6 and or 7 position of HepII.

A Novel 'Clip-and-Link' Activity of RTX Proteins from Gram-negative Pathogens: Covalent Protein Cross-linking by an Asp-Lys Isopeptide Bond upon Calcium-dependent Processing at an Asp-Pro Bond

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Clinical isolates of *Neisseria meningitidis* produce an RTX protein, FrpC, of unknown biological activity. Here we show that physiological concentrations of calcium ions induce a novel type of autocatalytic cleavage of the peptide bond between residues Asp^{414} and Pro^{415} of FrpC that is insensitive to inhibitors of serine, cysteine, aspartate and metalloproteases. Moreover, as a result of processing, the newly generated N-terminal fragment of FrpC can be covalently linked to another protein molecule by a novel type of Asp-Lys isopeptide bond that forms between the carboxyl group of its C-terminal Asp^{414} residue and the epsilon-amino group of an internal lysine of another FrpC molecule. Point substitutions of negatively charged residues possibly involved in calcium binding (D499K, D521K, D510A and E532A) dramatically reduced the self-processing activity of FrpC. The segment necessary and sufficient for FrpC processing was localized by deletion mutagenesis within residues 400 to 657 and sequences homologous to this segment were identified in several other RTX proteins. The same type of calcium-dependent processing and cross-linking activity could, indeed, be observed also for the purified ApxIVA protein of *Actinobacillus pleuropneumoniae*. These results define a protein cleavage and cross-linking module of a new class of RTX proteins of Gram-negative pathogens of man, animals and plants. In the calcium-rich environments colonized by these bacteria this novel activity is likely to be of biological importance.

Differential Expression of alpha -2,3-Sialyltranseferase of *Neisseria gonorrhoeae* (*Ng*) and *Neisseria meningitidis* (*Nm*) Clinical Isolates.

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Lst is an outer membrane alpha-2,3-sialyltransferase that monosialylates the lipooligosaccharide (LOS) of pathogenic Neisseria, facilitating their resistance to complement-mediated killing during infection. Although Lst expression is constitutive, Triton extracts of *Neisseria gonorrhoeae* (*Ng*) clinical isolates contain on average 3-4 fold more sialyltransferase (stase) activity than those of *Neisseria meningitidis* (*Nm)* clinical isolates. To investigate the hypothesis that these differences occur at the transcriptional level, *lst* transcript levels were examined by Northern blot analysis of total RNA isolated from 4 *Ng* strains (F62, A4, B9, B23) and 4 *Nm* strains (X3, X4, X5, MC58⊄3). A single band of approximately 1.3 kb was 3 times more prominent in total RNA of the *Ng* strains than *Nm* strains. To confirm and quantitate this result, *lst* transcript levels were quantitatively measured in 6 isolates of *Ng* (A4, B9, B23, FA1090, F62, B25) and 6 isolates of *Nm* (X3, X4, X5, X8, C1, MC58⊄3) using real-time PCR. Lst mRNA was 5 ± 0.8 times more abundant in the 6 *Ng* stains than in the 6 *Nm* strains. To further investigate the differences in the *lst* expression by *Ng* and *Nm*, stability of *lst* mRNA of *Ng* F62 and *Nm* MC58 was examined using Northern blot analysis and real-time PCR after treatment of *Ng* F62 and *Nm* MC58 cultures with Rifampicin. Results revealed that *lst* mRNA of *Nm* MC58 has a shorter half life ($t_{1/2}$ ~ 40 sec) than *Ng* F62 ($t_{1/2}$ ~ 90 sec). Comparative sequence analysis of the upstream (5′*lst*) region of *Nm* and *Ng* revealed three prominent differences: 1) a 13 bp (base pair) element that is found as a single copy in all *Nm,* but as a single copy or tandem repeat in *Ng* strains, 2) a 105 bp insertion element present exclusively in strains of *Nm*, and 3) a conserved 9 bp tandem repeat found in *Ng* strains which is highly varied in *Nm* strains. The effect of these elements on the differential expression of *lst* was studied using LacZ reporter fusions. Results suggested that neither the 13 bp element nor the 105 bp insertion element but the 9 bp element plays a significant role in the differential expression of *lst*. We conclude that the differential expression of *lst* results from the differential transcription of *lst* and differential degradation of *lst* transcripts.

Quantification of *Neisseria gonorrhoeae* Pilin Antigenic Variation

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Pilin antigenic variation (Av) in *Neisseria gonorrhoeae* (the gonococcus) involves the transfer of a silent loci (*pilS*) sequence into a portion of the expressed locus (*pilE*). We have developed a semi-quantitative real-time RT-PCR assay (SQ-PCR) to measure the frequency of *pilS* to *pilE* recombination from each silent copy. We designed 18 pairs of hybridization probes for the hypervariable loop (HV_L) portion of the 18 silent pilin copies of FA1090, with one set detecting total *pilE* transcript levels. This assay allows us to measure the contribution of individual silent copy sequences to pilin Av.

To measure pilin Av, total RNA was isolated from a gonococcal strain grown for 18, 22, 26 and 30 hours. RNA levels were normalized, reverse transcribed, and real-time PCR performed with the SQ-PCR assay. Fluorescent acquisition curves demonstrated a different slope for the isolated *pilE* template compared to the purified standards for most of the HV_L probes making quantitation impossible. However, mixtures of the standard for the silent copy being detected and the standard carrying the starting *pilE* sequence, which is the majority *pilE* template, provided curves which more closely match the experimental data. Thus, using mixed standards allows a semi-quantitative analysis of pilin Av. In order to validate the results of the SQ-PCR data, we performed several Southern colony blots of 1055 total colony isolates and isolated colonies which had altered in the HV_L portion of *pilE*. Sequence analysis of the entire *pilE* gene of varied isolates demonstrated that the representation of silent copies were similar to those found with results of the SQ-PCR assay.

Initial investigations demonstrated that with a specific starting *pilE* sequence, only a subset of the silent pilin copies were prevalent at all time points in two separate cultures. Furthermore, with a different parental silent copy, a different representation of silent copies were detected recombining into *pilE*. The basis behind the *pilE*-directed non-uniform distribution of silent copies is being investigated. These data demonstrate that a real-time RT-PCR assay can be used to investigate the mechanisms leading to Av of pilin in *N. gonorrhoeae*.

Defences against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*; distinctive systems for different lifestyles

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Mechanisms for coping with oxidative stress are crucial for intracellular survival of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Reactive oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) are encountered by pathogenic *Neisseria* during endogenous respiratory processes as well as during interactions with host cells. While *N. gonorrhoeae* and *N. meningitidis* are closely related and highly similar organisms, they possess distinct oxidative stress defence mechanisms. The oxidative stress response of the pathogenic *Neisseria* was investigated using oxidative killing assays. The susceptibility of mutant strains of *N. gonorrhoeae* and *N. meningitidis* to oxidative stress was determined using paraquat (generates intracellular $O_2^{\cdot-}$), xanthine/xanthine oxidase (generates extracellular $O_2^{\cdot-}$ and H_2O_2) and hydrogen peroxide (provides extracellular H_2O_2) killing assays.

In earlier work we established that *N. gonorrhoeae* relies on accumulation of Mn(II) via an Mn uptake system (MntABC) for protection against superoxide radicals, while its superoxide dismutase (SodB) does not appear to play a role in protection against superoxide killing. We have now determined that a distinct situation is seen in *N. meningitidis.* A homologue of the *mntABC* locus was identified in *N. meningitidis*, and similar to the situation in *N. gonorrhoeae,* the *N. meningitidis mntC* mutant strain was also susceptible to oxidative killing *in vitro*. However supplementation of growth media with Mn did not enhance resistance to oxidative killing in *N. meningitidis*. *N. meningitidis* possesses two superoxide dismutases, SodC and SodB. We have shown that SodB of *N. meningitidis* is highly active compared to the SodB of *N. gonorrhoeae*, and plays a role in protection against oxidative killing. However, while the SodC of *N. meningitidis* is also highly active, a *sodC* mutant strain was no more sensitive to oxidative killing than the wild type as measured by *in vitro* killing assays.

The pathogenic *Neisseria* were also found to differ in their defences against hydrogen peroxide. The cytochrome c peroxidase (Ccp) of *N. gonorrhoeae* was found to be involved in protection from hydrogen peroxide killing. Investigations of a *ccp* mutant strain and a *ccp/* catalase (*katA*) double mutant strain revealed a role for cytochrome c peroxidase in protection from hydrogen peroxide killing. Investigation of a broad range of *N. gonorrhoeae* and *N. meningitidis* strains revealed *ccp* to be a *N. gonorrhoeae* specific gene, that is located downstream of *katA*. Previous work by other groups has highlighted higher levels of catalase in *N. gonorrhoeae* than in *N. meningitidis*, as well as the presence of a glutathione peroxidase only in *N. meningitidis*.

The oxidative stress defence mechanisms of *N. gonorrhoeae* and *N. meningitidis* have been investigated in model systems in order to gain a better understanding of these processes in Neisserial pathogenicity.

Genetic changes within the meningococcal ST-8 complex/cluster A4 strains in Scotland

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Neisseria meningitidis is an important cause of meningitis and bacteraemia worldwide. Our laboratory holds a unique collection of invasive meningococcal isolates dating back to 1964. MLST was used for the retrospective characterisation of invasive meningococci in Scotland from the 1980s and 1990s. Nucleotide sequences of seven housekeeping genes (*abcZ*, *adk*, *aroE* ,*fumC, gdh*, *pdhC*, *pgm*) were determined. Alleles and sequence types were assigned using the MLST database (http://neisseria.org/nm/typing/mlst/). The results of these were then analysed to examine the population structure of the organism.

It was found that meningococci of ST-8, which were predominant during the 1980s, were replaced by similar strains of the same complex in the early 1990s.These included ST-153, ST-1349 and ST-66. ST-153 and ST-66 differed from ST-8 by one nucleotide in the *gdh* gene and five nucleotides in the *fumC* gene, respectively; all of these nucleotide substitutions were synonymous. However, ST-1349 differed from ST-8 by seven nucleotides in the *fumC* gene and 32 nucleotides in the *pgm* gene, Some of these nucleotide substitutions were non-synonymous although it is unclear whether these changes are of benefit to the organism for its survival.

Analysis of DNA replication in *Neisseria gonorrhoeae* and identification of a putative origin of replication

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DNA replication is a fundamental process that has been well characterized in *Escherichia coli*, but very little is known about this process in *Neisseria*. Bacteria possess a single origin of replication per chromosome that is typically located in the region of *dnaA-dnaN-recF-gyrB*; though *E. coli*'s origin has undergone a translocation away from *dnaA* and is linked to *gidA*. Bacterial origins contain binding sites for DnaA and A·T rich sequences required for formation of an open complex to which the replication machinery can be recruited. The location of the putative *Neisseria meningitides* origin has been determined to be in the region of *pilE* using oriloc, a di-nucleotide skew based computer program; however, a specific origin of replication has not been identified. In addition, both sequenced strains of *N. meningitides* contain a *dif* site, a sequence associated with the replication terminus. Using quantitative PCR and microarray analysis, we have identified the origin of replication for *N. gonorrhoeae* to be within a 39 kb region on the FA1090 genome, which does not contain any genes previously associated with bacterial origins and is located across the chromosome from the *dif* site. Sequence analysis of intergenic sequences within this region has revealed a putative origin of replication, containing three DnaA boxes and an A·T rich sequence, linked to *pilT*.

Most bacteria are haploid organisms with a single chromosome per cell, which can contain multiple replication forks, as is the case with exponentially growing *E. coli*. Our studies of *N. gonorrhoeae* (Gc), using flow cytometry, indicate that exponentially growing gonococci have four to eight Gc genome equivalents per cell, which was verified using fluorescent microscopy. Of the gonococcal cells examined, on average 50% were monococci and 40% were diplococci, suggesting that each coccal unit has on average four Gc genome equivalents. In order to determine whether multiple replication forks or multiple, complete chromosomes are present per coccal unit, quantitative PCR was employed. Hybridization probes in the region of the terminus were used to determine the number of completely replicated chromosomes present. Our quantitative PCR results support the idea that exponentially growing *N. gonorrhoeae* cells contain multiple, completely replicated chromosomes (on average three chromosomes per coccal unit). These results along with the novel location of the origin reveal that DNA replication in Gc may differ from *E. coli* and the other bacteria for which DNA replication has been examined.

Genetic and functional characterization of a lipooligosaccharide (LOS) glycosyltransferase gene, *lgtH*, in *Neisseria meningitidis*

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A genetic locus with five LOS glycosyltransferase genes, ***lgtABCDE,*** responsible for the biosynthesis of variable lacto-*N*-neotetraosyl chain of *Neisseria* LOS was first described in *Neisseria gonorrhoeae* (1). The gene cluster was also found in *Neisseria meningitidis* but with fewer genes at this locus, mostly ***lgtABE*** (2). We examined the ***lgt*** locus in over 30 strains of *N. meningitidis* and revealed a gene, ***lgtH***, which encoded for galactosyltransferase but was distinguishable from two characterized homologous genes, ***lgtB*** and ***lgtE***, by phylogenetic analysis. The mutual exclusion of ***lgtH*** and ***lgtE*** suggested that they might serve the same function (3).

In order to characterize the function of ***lgtH***, a recombinant plasmid of the gene was constructed by insertion of kanamycin resistance cassette into the coding region of ***lgtH***. A *N. meningitidis* LOS prototype strain, 6275 (***lgtABH***, L3 immunotype) was transformed by electroporation for construction of the ***lgtH*** isogenic mutant. The ***lgtH*** mutant was confirmed by PCR and by Southern blot hybridization. The LOS in the mutant showed a reduction in molecular size on SDS-PAGE gel and lost its reactivity with an L3 antibody on immunoblot. Sugar analysis showed that Gal was missing in the mutant LOS when compared to the wild-type LOS. The molecular mass of hydrazine-O-deacylated LOSs was analyzed by MALDI-TOF mass spectrometry. The O-deacylated mutant LOS had a major component with a mass of 2389 corresponding to Glc.Hep_2.GlcNAc.PEA_2.Kdo_2.lipid A. The wild-type one had two major components with mass of 3330 and 3039 corresponding to Gal.GlcNAc.Gal.Glc.Hep_2.GlcNAc.PEA_3.Kdo_2.lipid A $\pm$ NeuNAc. These results show that the ***lgtH*** gene encodes a galactosyltransferase that uses the LOS acceptor truncated at Glc of the lacto-*N*-neotetraosyl chain.

In conclusion, the function of the ***lgtH*** gene in *N. meningitidis* has been characterized to be beta-1,4-galactosyltransferase using Glc-Hep **….** lipid A as acceptor in the LOS biosynthesis. Thus, the function of ***lgtH*** and ***lgtE*** is the same although the former has a higher homology to ***lgtB***, a different galactosyltransferase using GlcNAc-Gal-Glc-Hep **….** lipid A as acceptor.

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Natural competence for transformation in *Neisseria lactamica*

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Neisseria lactamica is a common human pharyngeal commensal. In contrast to its close relatives, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, *N. lactamica* only very rarely causes disease. It is however an important immunostimulatory agent in children and young adults[1].

N. lactamica is naturally competent for transformation, a feature that has been poorly investigated in this species despite the important role of transformation in horizontal gene transfer. Natural competence in the pathogenic *Neisseria*, however, has been extensively studied and shown to depend on the presence of a 10 bp DNA uptake sequence (DUS) as well as the type IV pilus machinery. The secretin PilQ and its substrate, the pilin, are two major components of this machinery. Secretins belong to a large family of bacterial outer membrane proteins associated with translocation of single proteins and macromolecules. A subset of this family is termed PilQ proteins. Meningococcal PilQ differs from many secretins due to its high abundance in the outer membrane in addition to a unique polymorphic region in the N-terminal part of the protein containing repetitive elements (small basic repeats, SBRs). *N. lactamica* pili have previously been shown to be most similar to meningococcal class II pili[2].

To characterize the transformation process in *N. lactamica* we quantified the transformation efficiency by time limited exposure to genomic *N. lactamica* DNA, meningococcal DNA and *E. coli*-dervied plasmid DNA. Interestingly, only genomic DNA could be transformed into piliated *N. lactamica* strains, however, both homologous and heterologous *N. lactamica* DNAs were accepted. Meningococal DNA and plasmid DNAs were not transformed, indicating the presence of well-developed restriction-modification systems. Furthermore, the expression of the PilQ complex and pili was monitored by immunoblotting. An abundance of *N. lactamica* PilQ dodecamer was found in whole-cell lysates. Large amounts of pilin from shearing fractions indicated the presence of pili on the *N. lactamica* cell surface. In addition, we have monitored the number of SBRs in the *N. lactamica pilQ* gene by PCR and sequencing due to their possible involvement in the interaction between the PilQ complex and the pilus. SBRs were identified and their organisation was different from the meningococcal PilQ SBRs. In addition, the presence of DUS in selected homologs of meningococcal DUS-rich genes was assessed.

This work is critical to understanding the transformation process in *N. lactamica*. Our aim is to detail the dynamics of the exchange of DNA by transformation within and between entities in the genus *Neisseria*. These studies are also relevant for any *N. lactamica*-based meningococcal vaccine development.

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Impact of vaccination against serogroup C meningococci on the epidemiology of meningococcal disease in the Netherlands

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Introduction Since 1999 the incidence of meningococcal disease increased from 3.6 cases to 4.5 cases per 100,000 inhabitants in 2001. The proportion of serogroup C meningococcal disease rose from 14% in 1999 to 39% in 2001. The increase of meningococcal disease in 2001 was exclusively due to the increase of serogroup C. Nation-wide vaccination of the population in the age group 0-9 year had been accomplished in the period June - November 2002 and one shot vaccination at the age of 14 months has been implemented in the national vaccination program since September 2002.
Materials and Methods Meningococcal isolates were characterised in the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) by serogrouping, serotyping, and sequencing of the variable regions of *porA*, encoding the PorA epitopes. The number of cases of meningococcal disease of the period between January 2001 - April 2002 (pre-vaccination period) was compared with that of the period January 2003 - April 2004 (post-vaccination period).
Results and Discussion In 2003, the incidence of meningococcal disease decreased to 2.0 cases per 100,000 inhabitants, while in 2002 the incidence was 3.8 cases per 100,000 inhabitants. During the pre-vaccination period 593 isolates of serogroup B meningococci were received by the NRLBM. The number of cases of serogroup B meningococcal disease decreased by one third to 400 cases during the post-vaccination period. In contrast, during the pre-vaccination and post-vaccination periods, the number of isolates of serogroup C meningococci received by the NRLBM was reduced by 88% from 417 to 50 cases, respectively.
Among persons with age between 14 month and 18 years the number of cases of serogroup C disease reduced from 266 cases in the pre-vaccination period to only 2 cases (0.8%) during the post-vaccination period. These two patients had not been vaccinated. Remarkably, the number of serogroup C isolates among persons > 18 years was reduced by 68% (from 151 to 48 cases) in the post-vaccination period. This reduction of cases of serogroup C disease among non-vaccinees might be indicative for herd immunity. However, the bimonthly distribution of cases of serogroup C disease showed that the incidence already declined before the introduction of the vaccine independent of the age group.
The NRLBM received no isolates of serogroup C meningococci during the months April-May 2004.
Conclusions In 2003 the incidence of meningococcal disease in the Netherlands showed a decrease of 48%. This decline of meningococcal disease is partly caused by the natural fluctuation in the incidence of serogroup B as well as serogroup C meningococcal disease. Nevertheless, the vaccination against serogroup C meningococcal disease was very effective; after the introduction of the vaccine, cases of serogroup C meningococcal disease were no longer observed among vaccinees by the NRLBM.

The roles of LuxS in *Neisseria meningitidis*

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Neisseria meningitidis is a human pathogen that causes potentially lethal meningitis. Little is known about how this bacterium undergoes transition from the harmless commensal organism of the human nasopharynx to a life threatening, bloodstream-disseminated pathogen. Previous studies have shown that a mutation of $luxS_{Nm}$ attenuates *N. meningitidis in vivo*, but the basis of this attenuation is not yet known. LuxS is proposed to have two roles, one in cell-to-cell communication (as the synthase of the quorum sensing signal AI-2), and the other as part of the activated methyl cycle (AMC).

To understand the stage of infection influenced by LuxS, we examined the initial processes of infection, the adhesion to epithelial cells. The *luxS* mutant shows a significant reduction in the ability to adhere Chang epithelial cells whereas this defect is restored in the complemented strain, suggesting a contribution of LuxS early in pathogenesis.

In vitro, LuxS synthesizes AI-2 from S-ribosylhomocysteine which it itself generated by the detoxification of S-adenosylhomocysteine by an enzyme encoded by the *pfs* gene. We were able to demonstrate that a similar chain of reactions occurs *in vivo* since a pfs_{Nm} mutant no longer produces AI-2. A major role of the AMC is to provide activated methyl groups, therefore we examined the $luxS_{Nm}$ and pfs_{Nm} mutants for defects in methylation status. No alteration in DNA methylation was observed in the $luxS_{Nm}$ and pfs_{Nm} mutant strains compared to their parent strain using Dam (DNA Adenine Methylase) negative (MC58) or Dam positive (B16/B6) strains. However, there may be a reduction in rRNA methylation of these mutants as they exhibit lower resistance to thiostrepton, a 'methylated target'-sensitive antibiotic.

All organisms require sulphur for growth and since the AMC recycles the sulphur-containing metabolites, methionine and homocysteine, we also looked at the fitness of these mutants under sulphur-limiting conditions. Compared with *N. meningitidis* wild type strain, the $luxS_{Nm}$ mutant has impaired growth in sulphur-limited conditions which can be restored with additional sulphur, but not with exogenous AI-2. This indicates the integral contribution of LuxS to bacterial metabolism, specifically in the AMC which together with the methylation defect may account for its role *in vivo*.

Mechanisms for loss of encapsulation in 166 polysialyltransferase gene positive meningococci isolated from healthy carriers

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Neisseria meningitidis isolates obtained from healthy carriers are frequently unencapsulated due to a number of genetic mechanisms including slipped strand mispairing, gene inactivation by insertion sequences, deletions, and point mutations (Hammerschmidt et al. 1996; Hammerschmidt et al. 1996; Dolan-Livengood et al. 2003; Sadler et al. 2003). An analysis of a representative and genetically diverse set of 166 unencapsulated, but sero-genogroupable isolates obtained in a cross-sectional carriage study was undertaken. Insertion sequence (IS) elements (IS*1016*-like, IS*1106*, IS*1301*) were responsible for the loss of encapsulation in (28%) of the isolates, slipped strand mispairing in the *siaA* and in polysialyltansferase genes in (23%). Novel sites of IS insertion as well as novel phase-variable homopolymeric tracts were identified. Irreversible mutations (insertions, deletions, base exchange) were found in 30% of the strains. Eight isolates exhibited one or two of four non-synonymous mutations in the UDP GlcNac 2-epimerase gene, necessary for sialic acid biosynthesis, which may have rendered these meningococci unencapsulated. The mechanisms of loss of encapsulation were not associated with clonal complexes as determined by the analysis of six clonal lineages represented by at least eight isolates. The study demonstrates that, besides strains with a capsule null locus, as many as 30% of meningococci isolated from carriers are irreversibly mutated within the capsule synthesis operon.

Expression of the MntC Mn transporter is controlled by PerR

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In previous studies we have shown that the accumulation of manganese (Mn) in *Neisseria gonorrhoeae* protected against killing by superoxide anion, and was independent of superoxide dismutase activity. Mn dependent resistance to superoxide in *N. gonorrhoeae* is dependent on the accumulation of Mn(II) ions involving the ABC cassette transporter MntABC. We have identified and characterised MntC, the periplasmic binding protein of the MntABC transporter. MntC belongs to a group (cluster IX) of divalent cation binding proteins. We have shown the *N. gonorrhoeae* strain 1291*mntC* mutant was hypersensitive to superoxide killing, and is also more sensitive to H_2O_2 killing than the wild-type. This correlated with a lower level of catalase activity expressed by the *mntC* mutant. Thus, the *mntC* mutation appeared to have a more complex phenotype and may, along with Mn, have other regulatory roles.

The control of Mn and iron uptake in bacteria is mediated by members of the DtxR and Fur families. MntR, a DtxR-like protein, in *Bacillus subtilis* and *Escherichia coli* are transcriptional regulators of genes that encode Mn(II) uptake systems. However, we could not find the *mntR* homologue in *N. gonorrhoeae* genome. The Fur protein acts as an Fe(II)-dependent transcriptional repressor of genes encoding iron uptake proteins. Many bacteria, including *B. subtilis*, have two additional Fur homologues called PerR and Zur. PerR, a manganese-dependent repressor, regulates the peroxide defence response in *B. subtilis* and *Staphylococcus aureus*. A homologue of PerR was identified in the *N. gonorrhoeae* genome and its role in oxidative stress was assessed by construction and phenotypic analysis of a *N. gonorrhoeae* strain 1291*perR* mutant. Unlike the situation in *B. subtilis*, the *perR* mutant had no effect of catalase (KatA) expression, consistent with our previous findings that KatA is regulated by OxyR in *N. gonorrhoeae*. Expression of MntC in the *perR* mutant was increased several fold, indicating that this component of the *N. gonorrhoeae* Mn-dependent oxidative stress response system is repressed by PerR. In wild-type cells MntC expression is influenced by Mn concentration. This Mn dependent regulation is no longer seen in the *perR* mutant, indicating that the Mn regulation of MntC expression is via PerR.

In investigations on a wider regulatory role for Mn, we observed that the key virulence factor pili had reduced expression when cells were grown in the presence of Mn. Pili have a crucial role in both colonization of the host and adhesion to host cells. Also, this Mn-dependent repression of pilin expression was independent of PerR, and was not at the level of transcription.

The difference in adhesion of a *Neisseria meningitidis pilT* mutant to human cells is due to an effect on the level of PilC

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Introduction: The type IV pili (Tfp) of *Neisseria meningitidis* play a critical role in meningoccal virulence, natural genetic competence and twitching motility. Tfp are also subject to retraction which relies on the PilT protein, a membrane associated nucleotide-binding protein. *pilT* mutants are heavily piliated, and adhere to fixed monolayers of T84 epithelial cells, while the wild type organisms adhere inefficiently (Pujol et *al*., 1999, PNAS **96**). Among the other components of the Tfp machinery, PilC an outer membrane, pilus-associated protein is important for pilus biogenesis, genetic competence and adhesion. On a living cell monolayer, up-regulation of PilC is necessary for adhesion. However, in the model of the fixed T84 cell monolayer, adhesion does not induce expression of *pilC*. The aim of this work was to gain insight into the mechanisms involved in the hyper-adhesive phenotype observed in a *pilT* mutant on fixed T84 epithelial cell monolayers.

Results and Discussion: Using DNA arrays, we performed an analysis of relative expression levels in wild type and *pilT* mutant meningococci. Statistical analysis of the data, using the SAM software package, revealed 40 differentially expressed ORFs. Interestingly, one of the 26 up-regulated genes in the *pilT* mutant strain was *pilC*. We confirmed this result using quantitative Real-Time PCR and by western blot. We postulated that the level of PilC may be responsible for the hyper-adhesive phenotype of the *pilT* mutant. To investigate this hypothesis, we performed adhesion assays on fixed T84 epithelial cell monolayers using isogenic meningococcal strains, wild type or *pilT* mutant, harbouring an IPTG-inducible *pilC* gene. We obtained differences in adhesion levels, which responded to the level of induction of *pilC* regardless of the wild type or *pilT* genetic background. At medium levels of *pilC* induction, the adhesion was similar to that observed in wild type strain, while at maximal induction of *pilC*, the level of adhesion was similar to that observed in the *pilT* mutant. One hypothesis, which would explain this, is that the PilT product acts on the level of expression of *pilC*. However, we were unable to demonstrate a direct interaction of PilT with the *pilC* promoter sequence by gel shift assays.

Taken together, our results suggest that the hyper-adhesion phenotype observed on a fixed T84 cell monolayer with the *pilT* mutant acts by an effect on the differential expression of *pilC*. A subtle balance between PilC and PilT may play a critical role in Tfp biogenesis and its function in adhesion to human cells.

Poster Session II

Cellular Microbiology

Influence of Pili and Opa protein on the course of epithelial cell invasion and the pattern of cytokine release from Fallopian tube (FT) explants infected with *Neisseria gonorrhoeae in vitro*.

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Introduction. Salpingitis caused by *Neisseria gonorrhoeae* is associated with the local production of cytokines. The pilus and Opa proteins are virulence factors that influence the course of gonococcal infection, but little is known regarding their contribution during infection of the Fallopian tube (FT) epithelium and the nature of the resulting inflammatory response. In the current study, the attachment of gonococci to human FT explants and the subsequent invasion of the epithelium by bacteria was investigated. In addition, the roles of pili and Opa protein were examined and the nature of the inflammatory response determined.

Materials and Methods. Human FT organ cultures were challenged with phenotypic variants of *Neisseria gonorrhoeae* strain P9, differing in their expression of pilus and Opa protein: $Pil^+Opa_b^+$, $Pil^-Opa_b^+$, Pil^+Opa^- and Pil^-Opa^-. At 3, 6, 12 and 24 h after infection, the tissue was fixed and the presence of gonococci within the tissue identified immuno-histochemistry. In addition, specific immunoassays were used to quantify the levels of cytokines TNF-alpha, GM-CSF, IL-1alpha, IL-1beta, IL-6, TGF-beta, IL-10, IL-8, MIP-1alpha, MIP-1beta, MCP-1 and RANTES within the culture supernatants.

Results. Piliated organisms adhered in significantly higher numbers to the FT epithelium than non-piliated organisms. However, there was no difference in the adherence of piliated organisms expressing Opa ($Pil^+Opa_b^+$) and piliated bacteria lacking expression (Pil^+Opa^-). Significantly, the rates of bacterial invasion of the FT epithelium were similar for all the variants, regardless of their expression of pilus and Opa protein. Infection with *Neisseria gonorrhoeae* stimulated the release of 8 out of the 12 cytokines examined: TNF- alpha, GM-CSF, IL-1 alpha, IL-1beta, TGF-beta, IL-10, IL-8, MIP-1 beta. There were subtle differences in the patterns of cytokine secretion, with the Pil^-Opa^- variant stimulating the release of TNF-alpha, GM-CSF, IL-1alpha, IL-1beta,• TGF-beta, IL-10 and MIP-1beta, but not IL-8. By contrast, expression of pilus and/or Opa protein modulated secretion of these cytokines. Pil^+Opa^- bacteria increased the secretion of IL-1β only; the $Pil^-Opa_b^+$ variant induced increased secretion of IL-1beta and ¶TGF-beta, whereas the variant expressing both pilus and Opa protein ($Pil^+Opa_b^+$) stimulated the release of IL-1alpha, IL-1beta, TNF-alpha and IL-8.

Conclusions. Adherence of *Neisseria gonorrhoeae* to the FT epithelium was primarily mediated by pili and not influenced by expression of Opa protein. However, expression of pili and/or Opa protein was not essential for invasion of the oviductal epithelium by gonococci, but did influence the patterns of cytokine secretion. Secretion of cytokines appeared to be inhibited by bacteria expressing either pili or Opa_b protein, and *in vivo* the ability of invading gonococci to modulate the local inflammatory response may promote pathogen colonization of the FT epithelium.

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INTERACTIONS OF *NEISSERIA MENINGITIDIS* WITH ENDOTHELIAL CELLS : THE ROLES OF VITRONECTIN, FIBRONECTIN AND OTHER MOLECULES

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The matrix proteins such as Fibronectin (Fn) and Vitronectin (Vn) have been previously implicated in mediating adhesion to and invasion of endothelial cells by pathogenic *Neisseria*. Apparently contradictory results have been published implicating the primary role of Vn and Fn in mediating Opc-dependent *Neisseria meningitidis* (Nm) interactions with human endothelial cells[1,2]. Current studies aimed to clarify the roles played by these matrix proteins in mediating interactions of Opc-expressing derivatives with distinct endothelial cell lines which included HBMECs (brain), HMEC1 (dermal) or HUVECs (umbilical vein). First, in direct matrix binding assay, we observed that immobilised cellular fibronectin ($_C$Fn) was capable of supporting the adhesion of Nm to a greater extent than immobilised vitronectin (Vn), fibrinogen (Fb) or plasma fibronectin ($_P$Fn). For studying the roles of these host-derived proteins in bridging the bacteria to their integrin receptors on various endothelial cell lines, we used bacteria pre-coated with different purified matrix proteins. $_P$Fn was found to be the most effective followed by $_C$Fn for Nm binding to HBMECs whereas Vn was not effective confirming previous work[1]. In contrast, Vn was a better mediator of binding when using HUVECs, followed by Fn also confirming a previous report[2]. Although Fn supported binding to HMEC1 also, lower levels of binding to HMEC1 were displayed than observed for HBMECs. These results were confirmed using serum depleted of matrix proteins. The presence of RGD peptides block binding of Nm pre-coated with the matrix proteins indicating that the interactions are integrin dependent. The studies suggest that the integrin profiles on distinct endothelial cells are distinct and this determines which matrix protein will support interactions at different endothelial-bacterial interface. In addition, the integrin expression profile may also be influenced by inflammatory mediators[3] present during infections, and may result in novel integrin-bacterial interactions during disease.

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I-domain-Containing alpha Integrins Serve as Pilin Receptors for Neisseria gonorrhoeae Adherence to Epithelial Cells

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Previous studies using the immortal cervical epithelial cell line, ME180, indicate that CD46 serves as the pilus receptor in these cells. However, ME180 cells do not express complement receptor 3 (CR3, CD11b/CD18), which serves as an important mechanism by which *N. gonorrhoeae* elicit membrane ruffling and cellular invasion of primary, human, cervical epithelial cells. In these primary, cervical cells and in cervical tissue, CD46 is expressed on the basolateral surface and is not transcytosed to the apical cervical surface. Similar phenomena are observed for other, diverse, epithelial cell surfaces. Therefore, CD46 is not expected to be available to serve as the initial, primary pilin receptor. Additionally, our data indicate that CD46 does not appear to be required for the association or invasion of primary, cervical epithelial cells at early time points post-infection. The I (for inserted)-domain is an approximate 200 amino acid region found within the alpha subunit of some integrin heterodimer receptors e. g. the collagen receptor. Our previous studies indicate that gonococcal pilus binds directly to the I-domain of CR3 and that this interaction is required for gonococcal adherence to and invasion of primary cervical epithelia. Gonococcal proteins that are secreted in a contact-inducible manner augment these processes. Host cell molecules available to serve as the pilus receptor have not been examined using primary, male urethral cells or tissue. Based on previous studies we reasoned that I-domain-containing alpha-integrins might be available to serve as the pilus receptor on primary, male, urethral epithelial cells, as we have previously demonstrated for the CD11b-pilus interaction occurring on cervical epithelia. Recombinant I-domain and antibodies directed against the I-domain containing integrins, alpha1- and alpha2-integrin, inhibited the association of gonococci with primary, male, urethral epithelial cells and immortal cell lines of variable origin. By confocal microscopy we demonstrated co-localization of gonococci with I-domain-containing alpha-integrins at early time points post-infection and that this interaction dissociates with extended infection. Similarly, Western Blot analyses readily revealed that gonococcal pilin co-immunoprecipitates with the alphaı-integrin. However, studies performed in parallel and that were designed to capture CD46-pilus immune complexes revealed that a CD46-pilus interaction is only visible by chemiluminescence if the film was overexposed. Collectively, these data suggest that while CD46 might be able to bind gonococcal pilus, the alpha1-integrin is preferentially used as the initial docking site for gonococci on primary, male urethral cells.

***Neisseria gonorrhoeae* FA1090 Opa phenotypes vary 10-fold in their invasion of human fallopian tube epithelium which expresses CEACAMs and syndecans.** LUND SJ, CARLSON DJ, COLE H, LANGAN AS, SHIELDS CM, GORBY GL. Omaha VA Medical Center, Creighton University School of Medicine, University of Nebraska Medical Center; Omaha, Nebraska.

Neisseria gonorrhoeae FA1090 has the potential to produce nine antigenically distinct opacity-associated (Opa) proteins. The degree to which each one facilitates invasion of human fallopian tube cells and the receptors utilized are unknown. The relative invasiveness of each Opa phenotype in the human fallopian tube organ culture (FTOC) model was investigated using mixed infections of equal numbers of defined Opa phenotypes of *N. gonorrhoeae* FA1090 or *Escherichia coli* expressing β-lactamase/Opa fusion proteins (*E. coli* Opa^{+}). Monoclonal antibodies that recognize different FA1090 Opas (gift of Janne Cannon) were used to identify the Opa phenotype of invasive bacteria recovered from an extracellular antibiotic killing assay. Some antibodies recognize two Opas, so in some cases gonococcal phenotypes could only be narrowed to two potential Opas. However, ambiguous *E. coli* phenotypes could be disambiguated by specific PCR reactions. *N. gonorrhoeae* expressing Opa B/D and *E. coli* expressing Opa B were significantly more invasive in the human FTOC model than the other Opa phenotypes. In the case of *E. coli* Opa+ variants, Opa B was 10-fold more invasive than the least invasive phenotype, Opa A. Putative receptors for Opa proteins include heparan sulphate proteoglycans (HSPGs), e.g. syndecans, and carcinoembryonic antigen-like cell adhesion molecules (CEACAMs). CEACAMs and syndecans were variably detected in fresh non-infected human fallopian tube epithelium by RT-PCR and immunofluorescent techniques. FA1090 Opa phenotypes A, B, and C bound ^{3}H-heparin significantly better than the other FA1090 Opa phenotypes. This suggested the potential for these Opas to utilize HSPG receptors preferentially for fallopian tube invasion. However, the addition of heparin throughout the experimental infection to competitively inhibit Opa binding to HSPGs had a minimal effect on the relative proportions of Opa phenotypes recovered. This suggests that HSPGs are not the receptors used by *N. gonorrhoeae* FA1090 for the invasion of human fallopian tube epithelium or that the degree to which HSPGs are exploited was relatively low and similar among different FA1090 Opas.

The Gonococcal Genetic Island-Encoded Type IV Secretion System is Involved in Infection

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Eighty percent of *Neisseria gonorrhoeae* strains contain the 57-kb gonococcal genetic island (GGI), which encodes a type IV secretion system (T4SS). This T4SS resembles the transfer region of *E. coli* F-plasmid in its organization and by homology. Certain versions of the GGI correlate with clinical strains obtained from patients with disseminated gonococcal infection, a rare complication of gonorrhea that results in systemic spread of *N. gonorrhoeae* and often manifests as painful arthritis, dermatitis, or meningitis. We have previously shown that many genes of the T4SS are necessary for secretion of chromosomal DNA by *N. gonorrhoeae* and that this DNA can participate in natural transformation of recipient gonococci during coculture experiments. Here we study the putative role of the T4SS in gonococcal infection and DNA secretion. Insertion-duplication mutants of *traG* and *traN* as well as *traH* and *traA* deletion mutants were examined. TraG and TraN are putative inner membrane and outer membrane proteins, respectively, that may form a channel through which secreted substrates pass. TraH is a putative periplasmic protein involved in T4SS pilus biosynthesis, while TraA is the putative T4SS pilin subunit. We have shown previously that *traG* and *traH* are necessary for secretion of DNA. In addition to *traG* and *traH*, we found that *traN* is also necessary for DNA secretion by *N. gonorrhoeae*, while *traA* is dispensable. We examined the bacteria-host cell interactions of these T4SS mutants during infection of primary human cervical cells by scanning electron microscopy (SEM). In addition, quantitative infection assays examining gonococcal association with and invasion of the cervical carcinoma cell line ME180 were performed. SEM analysis revealed that few T4SS mutants are bound to the primary cells early in infection and these mutants display an aggregative phenotype compared to wildtype gonococci; however, quantitative infection assays suggest that T4SS mutants associate with and invade ME180 epithelial cells at similar levels as wildtype. This apparent disparity may be due to the inherent differences between primary cells and transformed cell lines. We also examined the role that secreted DNA plays in gonococcal infection. When wildtype gonococcal infection is allowed to proceed in the presence of DNaseI, the infection appears similar to T4SS mutants by SEM; few mutant gonococci are attached at early time points and aggregates are visible at later time points. These preliminary results suggest that T4SS and/or its secreted DNA may be involved in wildtype infection.

PilX, a pilus associated protein essential for bacterial aggregation, is a key to pilus facilitated attachment of *Neisseria meningitidis* to human cells

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The attachment of pathogenic *Neisseria* species to human cells, in which type IV pili (Tfp) play a key but incompletely defined role, depends on the ability of these bacteria to establish contacts with the target cells but also inter-bacterial interactions. In an effort to improve our understanding of the molecular mechanisms of meningococcal adherence to human cells, we screened a collection of defined mutants looking for those presenting a reduced adhesion. This analysis underscored the essential role of Tfp in this process and also led to the identification of mutants displaying an adherence as impaired as that of non-piliated mutants but expressing quantitatively and qualitatively unaltered Tfp. The corresponding gene, which was designated *pilX*, was found to encode a pilin-like protein that co-purifies with Tfp fibers and is essential for bacterial aggregation. We provide here several pieces of evidence suggesting that PilX has intrinsic aggregative but not adhesive properties and that the reduced number of adherent bacteria seen with a *pilX* mutant is a consequence of the absence of inter-bacterial interactions. These data extend the current model of Tfp-facilitated adhesion of *N. meningitidis* to human cells by suggesting that Tfp-induced cooperation between bacteria is essential in initial meningococcal-host cells interactions.

MUTATIONAL ANALYSIS OF HUMAN CEACAMS DEMONSTRATES THE POTENTIAL OF RECEPTOR POLYMORPHISM IN INCREASING HOST SUSCEPTIBILITY TO MENINGOCOCCAL INFECTION

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In order to define in greater detail the interactions of pathogenic *Neisseria* with CEACAMs, we undertook further genetic engineering of the N-domain of CEACAMs, the docking site for *N. meningitidis* and *N. gonorrhoeae* Opa proteins and for ligands of several other mucosal pathogens. In our previous investigations, individual substitutions of several residues for alanine on the non-glycosylated CFG face of the receptor, were shown to affect binding of pathogenic *Neisseria* and included Ile 91, Tyr 34, Gln 89, Gln 44, Ser 32 and Val 39. Several of the same residues were also apparently involved in interactions of *Haemophilus influenzae* with the receptor. The observations suggest the potential of these bacteria to compete for the receptor *in vivo* - a phenomenon that can be demonstrated *in vitro*. Recently, we created a new 3-D structural model of the human CEACAM1 N-domain based on the mouse CEACAM1 crystal structure[1]. This model resolved one of the discrepancies of the previous model that was based on a combination of available X-ray structures of CD2, CD4 and REI Ig folds[2]. In the previous model, Val 39, a residue involved in binding of some Opa proteins, located at a distance from other residues of importance in bacterial binding. In the current model, Val 39 locates in a closer proximity to the main bacterial binding pocket on the N-domain. A close examination of the model also indicated that some changes in residues at the core of the binding site or the adhesiotope[2], may provide a better docking site for some bacterial ligands. Therefore we created several conservative and non-conservative mutations in the three residues Ile 91, Tyr 34 and Gln 89, to assess how such changes may affect the functional affinities of various bacteria for the receptor. The investigations confirm the primary importance of Ile 91 in all bacterial binding to CEACAM1. They further define that certain substitutions particularly at positions 34 and 89, predicted to form a better binding pocket for ligands, indeed increase the avidity of bacterial binding. The binding of different bacterial ligands was differentially affected by distinct substitutions at these sites. Since increased functional affinity of interactions lead to increased cellular invasion, these studies add weight to the notion that rare polymorphisms that may occur in CEACAMs within the human population may lead to increased susceptibility not only to meningococcal infection but also to infections by other mucosal pathogens.

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Meningeal cell activation by *Neisseria meningitidis* lipopolysaccharide (LPS) and non-LPS components is toll-like receptor (TLR)4 and TLR2 independent

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Cells of the meninges play an important role in initiating and sustaining the intracranial inflammatory response that is characteristic of meningococcal meningitis. An important virulence mechanism during the disease process is shedding of outer membrane (OM) vesicles into the cerebrospinal fluid and LPS present in these OM is believed to be a major inflammatory modulin involved in activation of meningeal cells. In gram-negative bacteria it has been demonstrated that LPS-driven signalling is mediated through the cell surface molecule toll-like receptor (TLR)-4. Immune responses induced by a *N. meningitidis* LPS-deficient mutant however have been shown to be TLR2, and not TLR4, dependent. In the current study, we have investigated the interactions between human meningeal cells and i) *N meningitidis* strain H44/76 and the isogenic LPS-deficient mutant H44/76 PLAK33 and ii) the isolated OM of both these strains. The contribution of non-LPS components of the meningococcus on induction of cytokines and of toll-like receptors in mediating biological responses to LPS-replete and LPS-deficient OM preparations was examined.

Human meningeal cells, cultured *in vitro*, were infected with wild-type meningococci and LPS-deficient meningococci, or treated with OM from either organism. The LPS-deficient mutant and OM induced the secretion of IL-6, IL-8, MCP-1, RANTES and GM-CSF, clearly demonstrating that non-LPS bacterial components were involved in cellular activation.

Non-stimulated meningeal cells, as determined by RT-PCR for mRNA expression and immuno-cytochemistry with specific antibodies, expressed low levels of TLR2 and TLR4 surface molecules. Treatment of meningeal cells with LPS-replete OM did not lead to upregulation of mRNA expression for TLR4, or accessory proteins MD2 and CD14. Additionally, other LPS-accessory surface proteins, namely HSP70, HSP90-alpha, CXCR4 and GDF5, were found not to associate with TLR4 by fluorescence resonance energy transfer. Treatment of meningeal cells with LPS-deficient OM did not lead to an upregulation of TLR2 mRNA expression and did not involve any of these other accessory proteins. The absence of TLR2 and TLR4 signalling was also confirmed by the lack of CD25, a marker for NF-kappaB driven activation, expression in reporter CHO cells expressing either TLR molecule.

These data indicate that TLR 4 and TLR2 molecules are not involved in the recognition of LPS-replete or LPS-deficient OM by meningeal cells. This suggests that recognition of LPS and other inflammatory modulins is dependent on other as yet uncharacterised receptors on the cell surface. Moreover, the biological consequences of cellular activation by non-LPS modulins suggests that clinical interventions based solely on abrogating the effects of LPS are unlikely to be effective.

Mannose-binding lectin enhances the phagocytosis and killing of *Neisseria meningitidis* by human macrophages

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Deficiency of mannose-binding lectin (MBL) is the most common human immunodeficiency and is associated with an increased risk of mucosally-acquired infections including meningococcal disease. Tissue macrophages are an important first-line of defence against mucosal infections and so we determined the effect of MBL on uptake of meningococci by human macrophages.

MBL significantly increased the capture and internalisation of *Neisseria meningitidis* by human monocyte derived macrophages. Inhibition of actin polymerisation indicated that MBL exerted this effect by a dose dependent acceleration of uptake into phagosomes which was maximal within the normal physiological concentration of MBL (1500 ng/ml) and was independent of another receptor for meningococcus, scavenger receptor-A.
MBL accelerated the acquisition and subsequent loss of the early endosome marker, early endosomal antigen (EEA)-1 and enhanced the acquisition of the late endosomal marker, lysosome-associated membrane protein (LAMP)-1. MBL reduced the survival of meningococci within macrophages by more than half, despite the increased uptake of organisms, and significantly reduced the number of extracellular bacteria by 60%.

We conclude that the uptake and killing of *N. meningitidis* is enhanced by MBL. These results suggest that MBL could modify susceptibility to meningococcal disease by modulating macrophage interactions with the organism at the site of initial acquisition.

CD46 independent binding of neisserial type IV pili and characterization of the PilC pilus adhesin for human epithelial cells

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An early critical event in the neisserial infection is the type IV pilus-mediated adherence to the human mucosal epithelium. The PilC protein, located on the tip of the pilus, has earlier been identified as the major pilus adhesion (1). Here we show that piliated gonococci and purified PilC protein exhibit similar binding specificities to human epithelial cell lines, supporting the function of PilC as the pilus associated adhesin for epithelial cells.
Previous studies by Källström et al. (2) suggested the cell surface protein CD46 as a receptor for neisserial pili. Despite using a variety of approaches we could not observe a CD46 dependent interaction of piliated gonococci with human host cells. At first, cell line dependent differences in binding efficiencies of piliated gonococci as well as purified pilus adhesin PilC did not correlate with the level of surface expressed CD46. Furthermore, no binding of piliated gonococci or purified PilC protein was observed on CD46-transfected non-human cell lines CHO and MDCK. Finally, specific down-regulation of CD46 expression in human epithelial cell lines by RNA interference did not alter the binding efficiency of piliated gonococci or purified PilC protein. These data argue, that pilus-mediated gonococcal infection of epithelial cells can occur in a CD46-independent manner, questioning the function of CD46 as an essential pilus-receptor for *Neisseria*.

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Identification of Plasminogen binding proteins of Neisseria meningitidis

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For several pathogenic bacteriae surface bound activated plasminogen has been shown to be an important factor in the processes of tissue invasion and penetration of basal laminae. An increasing number of bacterial proteins has been described as plasminogen receptors. Neisseriae have also been shown to bind plasminogen, but no receptor has been identified yet.
We investigated plasminogen binding to serveral strains of Neisseria meningitidis using viable bacteria and denatured neisserial proteins. According to known data we could show binding to pathogenic strains and to apathogenic carrier isolates. Exogenously provided urokinase-type plasminogen activator converts bound plasminogen to enzymatically active plasmin. At least two proteins have been identified as plasminogen binding partners. One of them, a protein of unknown function has not been described before as a receptor for plasminogen. It is enriched in a preparation of outer membrane proteins.
Currently, we further characterize the identified neisserial plasminogen binding proteins with respect to their subcellular localizations. Recent data suggest additional potential plasminogen binding proteins in Neisseria meningitidis. Experiments to investigate the functional role of plasminogen bound to the surface of Neisseria meningitidis are planed in future.

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Experimental and theoretical studies of *Neisseria* twitching motility.

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Twitching motility is a type of spontaneous movement used by single cell organisms. We are investigating this movement in both theoretical and experimental ways. Our main interests are to study bacterial movement upon interaction with other bacteria and with host target cells. The human pathogen *Neisseria gonorrhoeae* has the ability to move because they are able to extend and retract their pili filaments.

Each pilus can extend several •m from the bacterial surface. The force needed for twitching motility in *Neisseria* is supplied by a protein motor called PilT located in the bacterial membrane. Pili filaments extend and the tip of the pili attach to the surface on which the bacterium crawls upon. Finally the attached filament starts to retract and thereby pulls the bacteria forward. It is not known if the bacteria attract or repel each other or if the presence of presumptive host cells affects the motion. To analyse this we have analysed a series of pictures and converted the sequences to movie format. The theoretical part consist of simulation of the phenomena and analysis of features such as parameters describing the motion, structure of the bacterial colonies and time scale of the process.

Our studies show that *Neisseria* motion is not a random walk on any surface investigated. Bacteria in the vicinity of epithelial cells show a distinct movement which is similar to bacterial movement on glass surfaces. Upon interactions with a target cell bacterium attract each other and form microcolonies in the medium before attaching to the cell. Cell filopodia were used for colonisation by the bacteria upon first contact with a non-confluent target cell layer. We are currently generating cell lines expressing fluorescently labelled host cells receptors such as CD46 and will use this to investigate the host cell membrane reaction and further defence against an attack by pathogenic *Neisseria.*

Twitching motility is a fundamental ability of a large family of bacteria that cause serious infections. Understanding this important property is crucial for the knowledge of the infection process. Another aspect is the possibility to use the insights into biological motors to develop artificial nano-devices, which could have many interesting technical applications.

Apoptosis pathways and role of tumour necrosis factor alpha (TNF-a) in the selective death induction of cultured human Fallopian tube epithelial cells infected *in vitro* by *Neisseria gonorrhoeae.*

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Introduction. Infection of the Fallopian tubes (FT) by *Neisseria gonorrhoeae* (*Ngo*) can lead to acute salpingitis, an inflammatory condition resulting in damage primarily to the ciliated cells, with loss of ciliary activity and sloughing of the cells from the epithelium. Little is known of the mechanisms involved in the early stages of the inflammatory response that occurs in the FT following ascending Ngo infection. Recently, we have shown that human FT epithelium constitutively expresses the cytokine receptors IL-6R, TNFR1 and TNF-RII and that expression of IL-1alpha, IL-1beta, IL-6, IL-8 as well as TNF-alpha, is observed in tissue infected with Ngo. The production of cytokines is likely to contribute to the cell and tissue damage that is observed in gonococcal salpingitis, and our previous study suggested that Ngo infection induced apoptosis in FT tissue explants. In the current study, we used cDNA microarrays to investigate gonococcal induction of apoptosis in primary epithelial cells cultured from human FT and also evaluated the role of TNF–alpha in apoptosis.

Results. Significant apoptosis was induced following infection both with bacteria and exogenous TNF-α cytokine. Infection with an MOI=1 of Ngo induced apoptosis, but this was abrogated when cultures were infected with concentrations of MOI=10-100. Microarray analysis identified the expression of several genes associated with apoptosis in control, uninfected cells: for multiple genes of the TNF receptor family (TNFRSF1B, -4, -6, -10A, -10B and –10D) and the Bcl-2 family (BAK1, BAX, BLK, HRK and MCL-1). Expression of these genes was significantly up-regulated following infection with gonococci (MOI=1). To determine whether TNF-alpha secretion correlated with the apoptotic phenotype, human FT epithelial cells were infected with Ngo (MOI=1) in the presence and absence of anti-human TNF-alpha1 and -TNF-alpha2 antibodies. Infection in the presence of anti-TNF-alpha1 and TNF-alpha2 antibodies significantly inhibited gonococcal induction of apoptosis.
Conclusions. Several genes related to apoptosis are expressed in primary epithelial cells of the human Fallopian tube. Infection with Ngo induces apoptosis and this correlates with the presence of TNF-alpha and an up-regulation of multiple genes of the TNF receptor family and the Bcl-2 family. However, increasing concentrations of Ngo abrogate apoptosis and this is possibly due to inhibition of the effects of TNF-alpha. *In vivo*, it is likely that cells of the human FT epithelium respond to the presence of low numbers of invading Ngo by undergoing apoptosis in an attempt to prevent colonisation by the pathogen. However, the presence of increasing numbers of bacteria inhibits apoptosis and provides the pathogen with an advantage in successfully colonising the mucosal epithelium.

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Interaction of *N. meningitidis* with cerebrovascular and respiratory epithelial cell lines.

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Meningococcal cellular association and invasion has the ability to potentiate bacterial pathogenesis. Using cells derived from the cerebrovascular and respiratory systems provides a suitable model of potential contact sites of *Neisseria meningitidis* during infection. Using an association and invasion assay, the number of meningococcal cells associating and invading the human cells can be quantitatively assessed.

The colony forming units (CFU) association and invasion was carried out using conventional methodology.

CFU counts from the association assay demonstrated that the peak of bacterial association for both cell lines was 4 hours post-infection with CFU counts declining over time until 10 hours post-infection, whereby at this time the cells showed pronounced cytopathic effects. In the invasion assay, the peak in CFU counts was around 8 hours post-infection, after which the counts declined. The amount of cells invading the cerebrovascular and bronchial cell lines was on a similar scale to that of HEp-2 cells, known to be invaded by meningococci. These results demonstrate the time-dependent manner of meningococcal adherence and subsequent invasion in these novel cell lines. Future work will include detailed analysis of the interaction of meningococci and these cells.

CEA RELATED CELL ADHESION MOLECULES: THE POTENTIAL OF DISTINCT SIGNALLING RECEPTORS TO SUPPORT BACTERIAL ADHESION AND ENTRY INTO TARGET CELLS

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CEACAMs comprise a number of GPI anchored and transmembrane molecules within the cell-expressed branch of the CEA family. CEACAM1, a ubiquitously expressed member of the CEA family, and CEA, an epithelial receptor, are targeted by a number of *Neisseria gonorrhoeae* (Ng) Opa proteins of strain MS11 whereas none have been reported to target CEACAM8[1]. In addition, several CEACAMs especially CEACAM3, a PMN receptor, and CEACAM6 often present on epithelial cells are targeted by fewer Ng Opa proteins[2]. In the case of Nm, in our studies CEACAM1, CEACAM3, CEA and CEACAM6 were shown to bind to several Opa proteins of strain C751. Our previous studies have also shown that CEACAM1 can serve as a receptor for other mucosal pathogens including *Moraxella catarrhalis* and typable and nontypable *Haemophilus influenzae* (THi, NTHi). For this study, we analysed whether all the mucosal pathogens that target CEACAMs have the potential to target multiple members of the CEACAM family, since such targeting has implications in the ability of various pathogens to occupy these receptors and indeed compete on the target cells in the human respiratory niche, as well as to interact with phagocytic and other effector cells. Using HeLa transfectants with distinct CEACAM expression including CEACAM1, CEACAM3, CEA, CEACAM6 and CEACAM8, we have observed selective targeting of CEACAMs by distinct mucosal pathogens. Nm and NTHi strains exhibit the broadest specificity for CEACAM family members and bind to CEACAM1, CEACAM3, CEA and CEACAM6. In addition, some Nm variants and some NTHi isolates were shown to bind also to CEACAM8. Interestingly, acapsulate derivatives of THi tend to target CEACAM1 principally and very little binding to other CEACAMs including CEACAM3 was observed. In further investigations, using Opa derivatives of Ng strain P9, we have observed that some Opa expression does not confer CEACAM-binding property to the bacteria. In contrast, in the absence of Opa expression, and only in their absence, another ligand mediates binding to CEACAM1, confirming previous observations on MS11 by several investigators. As with some Nm isolates, in Ng strain P9, some Opa proteins confer the ability to bind to CEACAM8. Thus, these studies have identified greater range of Opa protein specificities than previously recognised. The studies have also analysed invasion potential of various bacteria when targeting distinct CEACAMs and the roles of ITIM motifs in cellular invasion via CEACAM1-L.

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IS CAPSULE AN EFFECTIVE BARRIER AGAINST INTERACTIONS VIA OUTER MEMBRANE ADHESINS?

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Several studies have shown that targeting of CEACAMs by *N. meningitidis* (Nm) and *N. gonorrhoeae* Opa proteins allows adherence to, and in the case of some cell types, subsequent invasion of cells implicating this process in the pathogenesis of diseases caused by these organisms. Previous studies with Nm have shown that Opa-mediated adhesion and invasion of target cells are diminished in the presence of capsule or sialylated LPS suggesting that trans-epithelial passage *in vivo* may require down-modulation of capsule. However, CEACAM targeting by capsulate Nm via Opa proteins was observed using transfected cells expressing high receptor densities. If such an interaction were to result additionally in cellular invasion, this would provide an alternate mechanism by which Nm could invade target tissues without the need for capsule down-modulation. Since receptor levels on cells may vary in response to a number of regulatory mechanisms, and may be upregulated by inflammatory cytokines, we undertook experiments to create *in vitro* models in which receptor densities can be predictably regulated. This would allow examination of a range of potential *in vivo* situations during health and disease enabling us to predict how bacteria may interact with target tissues in a changing host environment. We used a Tet-on™ gene expression system that works by production of a double-stable Tet cell line with a gene encoding a regulatory protein based on a "reverse" Tet repressor and a response plasmid containing the gene of interest. The presence of doxycycline, a tetracycline analog, in the cell culture medium drives transcription of the regulatory gene that, in turn, allows transcription and expression of the response plasmid and the gene of interest in a dose dependent manner. CEACAM1-4-L was cloned into the response plasmid and a stable transfection was carried out using Chinese Hamster Ovary (CHO) and HeLa cells. We could demonstrate by flow cytometry that the levels of CEACAM expression in certain cell clones could be tightly controlled by varying the amount of doxycycline in the medium. Using these systems, defined derivatives of serogroup B and serogroup A Nm strains as well as *H. influenzae* type b isolates were assessed in cell adherence and invasion assays. These studies show that capsulate bacterial adherence increases proportionately with receptor levels whilst invasion occurs once a threshold level of receptor is attained and that a significant number of internalised organisms still retains the capsule. Thus whilst capsule may dampen the outer-membrane protein interactions of bacteria in the absence of pili, by steric hindrance or its negative charge, our studies demonstrate that capsule is not an effective barrier when the functional affinities of bacterial ligands for their cognate receptors are elevated due to the greater availability of receptors.

Use of Genetically Marked Opacity Protein Variants of *Neisseria gonorrhoeae* and a Translational *opa*B::*pho*A fusion to Examine *opa* Gene Expression during Murine Genital Tract Infection SIMMS AN, JERSE AE. Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland

The opacity (Opa) proteins of *Neisseria gonorrhoeae* (GC) are encoded by a family of 10-12 distinct *opa* genes, each of which undergo phase variable expression due to a reversible frame shift mutation that occurs in a repeated region within each gene. Opa proteins mediate invasion of cultured epithelial cells via binding to heparin sulfate proteoglycan (HSPG) molecules or members of the carcinoembryonic cell adhesion molecule (CEACAM) family. Opa-mediated evasion of components of the innate response has also been reported. GC can express no Opa proteins, one Opa protein or multiple Opa proteins simultaneously. Evidence that Opa proteins play a role during genital tract infection is based on the recovery of a high percentage of Opa-positive GC from male volunteers and female mice following inoculation with primarily Opa-negative GC. In both models, a high percentage of multiple Opa protein expressers was also recovered. Here we employed two genetically manipulated versions of GC strain FA1090 to examine the hypotheses that i.) selection of Opa-positive GC results in isolation of Opa-positive variants early during murine infection, and ii.) induction of *opa* gene phase variation by host stimuli contributes to Opa protein expression in vivo. To test for selection during infection, a chloramphenicol resistance (Cm^R) gene was introduced into a nonessential chromosomal locus. OpaI (HSPG-binding) and OpaB (CEACAM-binding) variants of this strain (FA1090-Cm^R) were isolated and used to follow the recovery of an OpaI or OpaB population following intravaginal inoculation of mice with mixed suspensions consisting primarily of Opa^-,Cm^S GC. Using polyclonal antiserum specific for the unique hypervariable regions of each of the Opa proteins of FA1090 to determine the Opa phenotype of inoculum and vaginal isolates, a majority of Cm^R,OpaI variants was recovered from 12 of 16 (75%) mice inoculated with primarily Cm^S,Opa^- GC and 8-11% Cm^R,OpaI GC on day 1 or day 2 post-inoculation. Similarly, a majority of Cm^R,OpaB variants was isolated from 8 of 8 (100%) mice inoculated with the Opa^-,Cm^S and Cm^R,OpaB mixture. Both OpaI and OpaB variants were associated with epithelial cells as determined by immunofluorescent staining of vaginal smears. These results are consistent with selection of the Opa-positive population; of interest is the fact that human CEACAMs are absent in mice. To examine if induction of *opa* gene phase variation occurs during infection, a translational *opa*B::*pho*A fusion was constructed and inserted into a nonessential chromosomal locus of strain FA1090. The rate of *opa*B gene phase variation during growth in iron-supplemented broth was similar to that reported for gonococcal *opa* genes expressed in *E. coli* (10^{-3} / cell/ generation). We are currently using this construct to determine the rate of *opa* gene phase variation during murine infection and under conditions designed to mimic that of the female genital tract.

Intracellular Effects of Signal Recognition Particle Pathway Modulation in *Neisseria gonorrheae*

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For a bacterial cell to grow and replicate properly, it is essential that it be capable of targeting its proteins to their proper cellular locations. Numerous proteins whose final destination is the cytoplasmic membrane (CM) in Gram-negative bacteria are transported by the Signal Recognition Particle pathway (SRP) in a co-translational manner. SRP targeted proteins have been identified to have a role in many cellular processes including nutrient acquisition, electron transport, antibiotic resistance, cell cycling, and protein export. Proper SRP function is essential for viability, as a lethal phenotype has been observed under conditions of depletion and/or overexpression of SRP components.

The prokaryotic SRP is comprised of two proteins, Ffh and FtsY, and a 4.5S RNA. Ffh and the 4.5S RNA form a complex that in turn binds to a short, hydrophobic signal sequence located near the N-terminus of a nascent polypeptide early in translation, forming a ribonucleoprotein complex. This ribonucleoprotein complex then binds the membrane-associated SRP receptor, FtsY. GTP is hydrolyzed (Ffh and FtsY are GTPases) and the peptide is released to the SecYEG translocon, which facilitates insertion of the protein into the CM concomitant with translation. We have shown that PilA, the gonococcal FtsY homolog, is able to directly bind DNA of the *pilE* promoter and hypothesize a role for this binding in protein targeting. *pilE* encodes pilin, the major subunit of pili, an important gonococcal adhesin. PilA binding to DNA has been shown to strongly enhance PilA's GTPase activity, which is essential for its function as the SRP receptor. Here, we demonstrate that several additional DNA sequences significantly stimulate PilA GTP hydrolysis, and these sequences are associated with genes encoding presumed or confirmed SRP-dependent proteins.

To understand the role of SRP in gonococci, we have constructed a strain conditionally expressing *PilA*. *pilA*, under control of the tightly regulated *tac* promoter, was inserted into the chromosome along with the *lac* repressor and a selectable marker (Em^R) by allelic exchange, such that *pilA* expression is under control of IPTG. Our data show that at intermediate levels of PilA expression (50 micromolar IPTG), bacteria grow similar to the wildtype parent, MS11. However, growth in absence of IPTG or at high concentrations (500 micromolar IPTG) results in a dramatic reduction in growth rate. This is consistent with observations that depletion and/or overexpression of SRP components is deleterious to the cell. Furthermore, immunoblot analysis using mAb against pilin showed an accumulation of pilin in the cytoplasm of the gonococci either over- or underexpressing PilA, implicating the SRP in proper export of pilin.

Infection of human cervical cells does not select for subpopulations of virulent gonococci

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Neisseria gonorrhoeae is a highly adapted human-specific pathogen that initiates infection at the mucosal epithelia by using multiple adhesins to interact with a variety of host cell receptors. Colonization begins at the cell surface with a multi_step adhesion cascade. *In vitro*, the gonococcus is able to adhere to and invade many tissue culture cell lines. Adhesion is mediated by an assortment of phase variable surface antigens of the gonococcus (Opa protein, lipooligosaccharide, pili). Given the high rate of phase variation that occurs in the expression of each of these cell surface structures, bacterial cells present in a colony actually represent a mosaic of cell surfaces. We tested the hypothesis that in any given adherence and invasion experiment, a subset of gonococci would be selected for that expressed the "right" cell surface to allow it to efficiently attach to a specific tissue culture line. *N. gonorrhoeae* strains MS11, F62 and FA1090 were incubated with human cervical epithelial ME180 cells for three hours (MOI 10:1, bacteria to host cell). Bacteria that failed to attach during this time were collected from media. Adherence bacteria were recovered from lysed ME180 cells. The number of attached and unattached bacteria were quantified and incubated with afresh batch of ME180 cells for three hours. In all attachment assays, the percentage of bacteria attaching to the ME180 cells was about 0.1%. The degree of adherence of each bacterial cell type was visualized by confocal microscopy. Opa protein and LOS expression were determined before and after each attachment experiment. The data indicate that in these reinfection assays, viable count, confocal microscopy images and Opa and LOS profiles did not support the hypothesis that a more adherent population was selected for in the readherence assay.

Ultrastructural analysis of the pathogenesis of gonococcal endometrial infection

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Neisseria gonorrhoeae is a strict human pathogen transmitted through sexual contact. In the female, the endometrium can become infected and acts as a transition zone between uncomplicated cervical infection and infection of the fallopian tube and pelvic inflammatory disease. *N. gonorrhoeae* may persist in the endometrium despite monthly shedding of this tissue. We have developed an endometrial organ culture infection model to study the initial interactions between *N. gonorrhoeae* and endometrial epithelium by microscopy; these results confirm our previous observations in primary human endometrial epithelial cells. We have observed a minority of gonococci internalized into endometrial epithelial cells; internalization may facilitate persistence or spread into basal endometrial stroma or glands. Gonococci have diverse interactions with endometrial epithelia, including intimate association, microvillus engagement, lamellipodia and ruffle formation. Gonococci could be found in intracellular vacuoles in secretory epithelial cells. Gonococci attached to cilia but were not observed associated with the membrane of ciliated epithelial cells or internalized into ciliated epithelial cells. These studies indicate that gonococci utilize multiple mechanisms to associate with endometrial epithelial cells and can associate with both ciliated and secretory cells. After prolonged infection, *N. gonorrhoeae* were attached to glandular epithelial cells of endometrial tissue. These cells regenerate surface and glandular epithelial cells following menstruation and may facilitate gonococcal persistence in the endometrium and eventual spread to fallopian tubes. These studies suggest that endometrial epithelium acts as a dynamic interface between cervical and fallopian tube gonococcal infection.

Poster Session II

Genomics and Gene Expression

Expression of Gonococcal *fur* and *tonB* Genes During Infection of Epithelial Cells from the Lower Female Genital Tract. AGARWAL S[1] and GENCO CA[1,2]. Department of Medicine[1] and Department of Microbiology[2], Boston University School of Medicine, Boston, MA 02118, USA.

A microarray analysis of the Neisseria genome indicates that nearly 10% of gonococcal genes have the potential to be regulated by the ferric uptake regulatory protein, Fur. A number of these genes contain a Fur consensus binding sequence in their promoter region and electro mobility shift assays have shown that Fur binds to the promoter regions of a subset of these genes including *recN*, *secY*, *tonB* and *fur*. We have recently demonstrated, in a clinical study, that iron- and Fur-regulated *fbpA*, *tbpA*, *tbpB* and *fur* genes are differentially expressed in mucosal samples from *Neisseria gonorrhoeae* infected male subjects. Because of difficulty in obtaining samples from gonococcal infected female subjects, as a high number of infected women are asymptomatic (~80%), we have initiated studies to mimic in vivo conditions by using immortalized normal human endocervical, ectocervical and vaginal cell lines. To investigate these pathogen host-cell interactions we first created transcriptional fusions of the gonococcal *fur* and *tonB* genes with the green fluorescent protein (GFP). The promoter region of the *tonB* and *fur* genes, spanning the –10, and –35 regions, and the putative Fur box region, as confirmed by DNaseI footprinting, were used to construct GFP fusions in *N. gonorrhoeae*. The promoter region of *rmp*, a constitutively expressed gene was utilized as a control. These fusions were constructed upstream of the promoterless GFP in pLES 99, a suicide vector for *Neisseria*, and subsequently transformed into *N. gonorrhoeae* strain F62. Sequencing, PCR and fluorescent microscopy confirmed the proper insertion of the fusions in the gonococcal chromosome. The activity of the promoters examined by FACS analysis indicated that transcription of the *fur* GFP fusion increased 1.8 fold in the first hour to 3.7 fold in the fourth hour during growth under iron-deplete conditions. Similar results were obtained in the *tonB* GFP transcriptional fusion strain. As expected no difference in fluorescence was observed in *rmp* GFP transcriptional fusion. These results were further confirmed by RT-PCR analysis using internal fragments of *fur*, *ton*B and *rmp* genes. Differential regulation in immortalized endocervical, ectocervical and vaginal cell lines when infected with the *rmp*, *fur* and *tonB* GFP transcriptional fusions was studied using FACS and RT-PCR analysis. Similar upregulation of *fur* and *tonB* was observed during adherence of gonococci to all three cell lines when compared to the *rmp* transcriptional fusion (ranging from 2- to 5-fold in the first hour of adherence). In addition, we observed increased expression of *fur* and *tonB* GFP fusion strains during intracellular growth at different time points of infection. These results demonstrate that expression of the gonococcal *tonB* and *fur* genes are increased during both adherence to and invasion of female epithelial cells, and together with our in vivo studies suggest that these Fur-regulated genes are expressed during gonococcal disease in both males and females.

Global responses to differing atmospheric conditions in *N. gonorrhoeae*.

AHMAT, N, SAUNDERS, NJ

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Introduction:
N. gonorrhoeae is generally handled as an aerobe, which requires CO_2 for growth, but also grows both microaerophilically, and anaerobically in the presence of nitrite as an alternative terminal electron receptor. During infection it is exposed to different atmospheric conditions in different sites and stages of infection. This includes a possible intracellular stage, and survival in the lower gastrointestinal tract and in the vagina, which are likely to be associated with relatively low oxygen tensions and/or anaerobic conditions. It is possible that such environmental conditions may provide general signals for niche-associated behaviour. We have therefore investigated the role of atmospheric conditions on gene expression using a microarray-based strategy.

Methods:
Neisseria gonorrhoeae strain FA1090 was grown under different atmospheric conditions on solid supplemented GC media; aerobically (with 5% CO2, and this was used as the reference sample), microaerophilically, and anaerobically (with nitrite). The expression profiles for each condition were determined using the pan-*Neisseria* (version 2) microarray. Slides were printed using Genetix QArray microarrayers, onto Genetix Amine slides. RNA was extracted using standard methodology, and labelled by direct incorporation of Cy3 and Cy5 dyes. Slides were scanned using Perkin Elmer ScanArray, spots were identified and data extracted using BlueGnome BlueFuse software, and data analysis was performed using standard tools including GeneSpring.

Results:
Compared to growth in normal atmosphere with 5% CO_2, approximately 40 genes are up- and down-regulated more than 2 fold in microaerophilic condition. About twice as many genes show more than 2 fold changes in anaerobic growth with nitrite. Strikingly, very different sets of genes are differentially regulated in the different growth conditions, consistent with a role for atmospheric conditions as important niche-indicating signals.

Generally growth of FA1090 under different atmospheric conditions shows differential expression of some regulators, including: in several iron manipulating/metabolising genes and regulators such as *marR*, *rho*, and *lrp*. Notably, there are changes in the patterns of expression of genes associated with iron acquisition, as well as metabolic processes recognized to influence important host interactive phenotypes such as lactate and pyruvate. The genes that have been reported to be associated with anaerobic growth in gonococci, such as *pan1* and *nar* were not significantly expressed, perhaps in part representing differences in expression between solid and liquid culture conditions.

Comparative *Neisseria* genomics

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The 2.2 megabase genome of *Neisseria meningitidis* serogroup C FAM18 has been sequenced and annotated at the Sanger Institute. The sequence is from an ET-37 complex strain isolated in North Carolina in the 1980s. We will present our comparative analysis of FAM18 with the genomes of the serogroup A (Z2491) and serogroup B (MC58) *N. meningitidis* strains and *N. gonorrhoeae* FA1090. This type of analysis can determine a core set of *Neisseria* genes as well as highlighting which genes unique to each strain. Variation in gene complement may be correlated with phenotypes and sequence alignments can indicate mechanisms of evolution. The project to sequence the genome of *N. lactamica* is underway at the Sanger Institute. *Neisseria lactamica* is a commensal of the human throat. It is closely related to, and resides in the same niche as, *N. meningitidis*. Comparison of the *N. lactamica* genome with those of its pathogenic relatives will be very useful in identifying virulence-associated genes. The *N. lactamica* genome is estimated to be around 2.3 Mb with a G+C content of around 52%. Progress and preliminary analysis from this project will also be presented.

Use of 2DE/MS annotated maps of Neisseria meningitidis serogroup A proteomes for analysis of microevolution during epidemic spreads.

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A characteristic general feature of the Neisseria genomes is a remarkable degree of variability. However, *Neisseria meningitidis* serogroup-A (menA), which caused major pandemic waves, notably differs from other serogroups by having a fairly stable clonal population structure. (see: Zhu, P., *et al., Proc. Natl. Acad. Sci. USA* 2001, 98, 5234-5239). We considered that comparative mapping of proteomes expressed by menA strains could be usefully adopted for microevolution studies which would look at phenotypes in conjunction with the well known genotyping methods.

We first established a MALDI-TOF annotated, 2DE reference map of a whole-cell protein extract from menA strain Z4970, a clinical isolate classified as 'ancestral' with respect to several pandemic waves. Total protein samples from bacteria grown on GC-agar were separated by two-dimensional electrophoresis; proteins in individual spots were digested with trypsin and identified by MALDI-TOF spectrometry coupled with in silico searches of genomic databases. We so identified 273 gene products, covering several functional classes, and including 94 proteins so far considered as hypothetical.

Both the map and the annotation data will be made available through the European Bacterial Pathogen Project database web site hosted by the Max-Planck Institute for Infection Biology at http://www.mpiib-berlin.mpg.de/2D-PAGE/index-2DPAGE.html

In parallel we also prepared protein maps of similar extracts from other 8 menA isolates belonging to diverse genoclouds identified within 3 pandemic waves which occurred between the years 1960 and 2000. We then tested if in silico comparative analysis of the 9 proteome maps could identify phenotypic variations presumably resulting from epidemiological fitness selection during the epidemic host passages. We shall present examples of such methodological approach showing that it can provide information on gene-product variation patterns which can be correlated to indipendently assessed filogenetic grouping.

The role of sigma factors in *Neisseria gonorrhoeae* interactions with epithelial cells

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The initial step of a gonococcal infection is colonization of mucosal tissues. Gonococci bind to mucosal epithelial cells via specific receptors on both the bacterial and host cell surfaces, enter the cells, and then traverse across them to exit to the subepithelial space where an acute inflammatory response is generated. A goal of our research is to identify regulatory phenomena that characterize the early response of gonococci to colonization of a new host, and hypothesize that attachment to epithelial cells can transmit signals that result in a modulation of the expression of genes necessary for proliferation in the host.
To examine this at the genome-wide level, we employed DNA microarrays. Gene expression in strain MS11 (P^+, Opa^-) following adherence to A431 cells (a human epithelial cell line of cervical origin) was compared to that of MS11 grown in cell-free culture. Our results indicated that several genes were differentially regulated upon host cell contact, including *rpoH*, which encodes a homolog of the heat shock sigma factor, σ^{32} (RpoH), as well as two probable RpoH-regulated genes, *groES* and *groEL*, which encode the molecular chaperones GroEL and GroES. In *E. coli* and other gram-negative bacteria, the expression of *rpoH* is under the control of the extra-cytoplasmic sigma factor, ECF/RpoE/σ^E, (encoded by *rpoE*) when subjected to extracytoplasmic stress, as well as several other regulatory systems which utilize other sigma factors. In contrast to the many sigma factors utilized in *E. coli* and other bacteria to regulate gene expression, there are only four putative sigma factors encoded in *N. gonorrhoeae*: RpoE, RpoH, RpoN (σ^{54}) and the housekeeping/vegetative sigma factor, σ^{70} (RpoD). One of these, RpoN, has been shown to be non-functional in gonococci, with the *rpoN* gene containing a large deletion that results in a frame-shift[1]. Little else is known about functions of RpoE and RpoH in GC. Since we observed an upregulation of *rpoH* and RpoH-regulated genes in GC upon adherence to epithelial cells, we hypothesized that gonococcal heat shock regulon might have a role in infection. We have examined this possibility using DNA arrays, tissue culture and mutagenesis approaches. Our results indicate that *rpoE* is not essential in gonococci, and that RpoE does not appear to be involved in regulation of *rpoH* and its regulated genes either in standard laboratory medium or upon host cell contact. In contrast, we determined that *rpoH* is essential for growth and viability of GC. A gonococcal strain conditionally expressing *rpoH* strain was constructed to explore the role of RpoH and its regulon in the interaction between GC and host cells in culture.

[1]Laskos, L., Dillard, J. P., Seifert, H. S., Fyfe, J. A. M., Davies, J. K. (1998) *Gene* 208:95-102.

Identification and characterisation of genes required for the interaction of *Neisseria meningitidis* and the human nasopharynx.

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Neisseria meningitidis is an obligate human pathogen and a major cause of septicaemia and meningitis. Although the systemic disease is fatal in up to 20% of cases, the bacterium can be isolated from the nasopharynx of 10-40% of the healthy population where bacteria are frequently found in a sub-epithelial location. Colonisation in itself is asymptomatic and the interaction of the meningococcus and the human host during this process is incompletely understood. Therefore identifying bacterial genes required for colonisation should provide insights into the mechanisms that allow the meningococcus to successfully inhabit the human nasopharynx. In addition, crossing the cellular layers and reaching the systemic circulation is critical for establishing infection and studying colonisation should reveal important information for understanding the pathogenesis of meningococcal disease.

We have used an air-interface organ culture model consisting of human nasopharyngeal tissue to analyse a library of signature tagged mutants of serogroup B *N. meningitidis*, with the aim of identifying genes required during the colonisation process. This model provides a complex, multicellular system with active cilia, mucous producing cells, and also includes sub-epithelial tissue and thus constitutes a biologically relevant model. A total of 10 mutants which failed to infect nasopharyngeal tissue were identified by negative selection. The gene disrupted in each of these mutants was isolated by maker rescue, and the transposon insertion site mapped by sequence analysis. Two of the 10 mutants are affected in the biogenesis of Type four pili, providing validation for the screening method. Of the remaining genes, three are predicted to encode proteins with membrane-spanning domains and therefore predicted to be surface located. The mutations were back-crossed into the parental strain and the resulting mutants were found to be defective when compared directly against the wild-type parental strain. To establish whether these genes affect the expression of known adhesins, whole cell lysates of the mutants have been analysed by Western blotting using mAbs against pili (SM1), Opa and Opc, and rabbit polyclonal sera against PilC. The expression of these adhesins by all the mutants is unaltered compared against the wild-type. Further experiments are in progress to determine the role of these three genes in colonisation and the potential of using the corresponding proteins as candidates for vaccines against meningococcal infection.

Comparison of the repeat lengths associated with phase variable genes in the four main experimental strains of *Neisseria gonorrhoea.*

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Phase variation is the mechanism by which bacteria can reversibly switch between alternate phenotypes. In *Neisseria* spp. this process is usually mediated by alterations in the length of simple sequence repeats either within the promoter or the coding regions of genes. Variation in the length of these repeats leads to altered promoter strength or premature translational termination, respectively.

The phase variable genes are important for virulence and also for niche adaptation. The majority of these genes encode surface exposed products, or affect surface exposed structures, and are therefore frequently targeted by the immune system. A list of all the potential phase variable genes was created looking for repeats associated with phase variation by genome analysis of the three sequenced neisserial genomes (Snyder et al 2001). This identified 65 genes in *N. gonorrhoea* strain FA1090 that were potentially phase variable.

Using targeted PCR and sequencing we are determining the conservation of the phase variable repertoire in the commonly used experimental strains and determining their expression status.

Results indicate that the majority of FA1090 repeats are of same length in our separately passaged FA1090 strain indicating a great degree of stability in this strains phenotype during *in vitro* passage. The longer repeats show differences indicating diversity within the cultures, but these are still predominantly of the previously defined lengths. Within the other genomes there are observed differences in ~40% of the genes. This provides evidence that these genes are under phase-variable control, and that these genes may contribute to the strain-dependent behaviours associated with these experimental strains. The genes associated with different phenotypes include: pilin glycosylation proteins, LPS biosynthesis proteins, restriction modification systems, as well as hypothetical proteins.

Capsular Switch from serogroup C to serogroup W135 of the ET-37 Clonal Complex *in vitro* and *in vivo*.

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Neisseria meningitidis serogroup W135 has been upgraded to the rank of new epidemic variant, after the outbreaks that occurred during the Hajj pilgrimage to Mecca in 2000 and sub-Saharan Africa since 2001, among populations vaccinated against *N. meningitidis* A and C. W135 strains incriminated in these outbreaks belong to the ET-37 clonal complex. We aimed to study capsule switching in a defined biological system and to analyze the role of host immune response in the selection of transformants. We first constructed a serogroup C strain harboring a transcriptional fusion *pilC:lacZ:aphA3'* that was kanamycin resistant and produced β-galactosidase. A serogroup W135 strain–was also constructed by insertion of *ermAM* erythromycin resistance cassette into *Bcl*I site of *siaD* gene of the serogroup W135. Transformations using these two strains then permitted to obtain transformants that were Km^R :$LacZ^+$ and Ery^R and had lost the serogroup C capsule but demonstrated agglutination capacity with W135 sera. Based on these *in vitro* results, we used the animal model of sequential influenza A virus/*N. meningitidis* respiratory infection in six week-old female BALB/c to attempt to obtain *in vivo* transformation. At day 7 after IAV primary infection a mix was administrated by the intra-nasal route that was composed of $1x10^8$ cfu/mouse of *N. meningitidis* C Km^R :$LacZ^+$ strain (C37KZ) and 5µg of purified DNA of W135:Ery^R (NM03.01). At 24h the mice were sacrificed and Km^R:$LacZ^+$:Ery^R transformants were recovered from the lungs and tested for their agglutination capacity. From this transformation two isogenic mutants were obtained. One had lost capsular serogroup C expression and had acquired serogroup W135 capsular antigen, whereas another transformant expressed both capsular antigens. These results demonstrated the *in vivo* transformation capacity of *N. meningitidis* that may lead to capsule switch.

The *irg* genes of *Neisseria gonorrhoeae*

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The sexually transmitted disease gonorrhea is caused by the Gram negative diplococcus *Neisseria gonorrhoeae*. The pili of *N. gonorrhoeae* play a vital role in the establishment of infection. The pilus is composed of pilin protein monomers. The genes encoding pilin can under go homologous recombination. *Moraxella*, like *Neisseria*, is capable of variation of its pili. This variation is due to the inversion of the DNA which turns pilus expression on and off in *Moraxella*. This inversion is mediated by the site specific recombinase Piv. In the chromosome of *N. gonorrhoeae*, eight genes have been identified to have similarity to Piv. We have named the pivNG copies *irg1-8* for invertase related gene family.

In order to address the role of the *irg* genes in *N. gonorrhoeae*, we cloned and mutated each gene, either creating a deletion/insertion mutation or by making an in-frame deletion. We constructed a strain in which all eight *irgs* were disrupted. We also created an Irg over expressing strain in which one *irg* copy was placed under control of *lac* promoter. Neither the full *irg* mutant strain nor the over expressing strain affected gonococcal antigenic variation at *pilE*, DNA repair, or DNA transformation. Furthermore, we found that the *irg* genes are not involved in DNA rearrangement as analyzed by Southern Blot. However, sequence analysis suggested that these *irg* genes are transposases contained in insertion sequences specific for the pathogenic *Neisseria.*

The iron-regulated proteome and transcriptome of *Neisseria meningitidis*

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The pathogenic Neisseria species produce a number of iron regulated proteins that are important in virulence. A central role in iron regulation plays a ferric uptake regulator (Fur) protein which can act as a transcriptional repressor or activator.
We analyzed the iron-regulated proteome and transcriptome of the *N. meningitidis* isolate 10/96 (C:2a:P1-2,5) on whole genome level. Steady-state proteome of meningococci grown under iron-depleted and iron-replete conditions was analyzed by 2-D electrophoresis and proteins exhibiting significantly enhanced or reduced expression levels were identified by MALDI-TOF MS analysis of their tryptic digests. In parallel, total RNA was isolated from the same cultures and screened for iron-regulated mRNA expression profiles using commercial spotted whole-genome meningococcal DNA microarrays (Eurogentec). In total we found 85 genes significantly up-regulated under iron-replete condition and 114 genes up-regulated under iron-depleted condition. Only 18 genes were found to be significantly iron regulated by both proteomic and transcriptomic approaches.

Analysis *in vitro* and *in vivo* of the transcriptional regulator CrgA of *Neisseria meningitidis* upon contact with target cells

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Contact between CrgA, a LysR-like regulatory protein in *Neisseria meningitidis*, and DNA is involved in the repression of several bacterial genes upon contact with epithelial cells. We used a defined *in vitro* system containing *crgA* promoter, purified RNA polymerase (RNAP) and purified CrgA protein to demonstrate that CrgA was directly responsible for this transcriptional repression. Interaction between the C-terminal domain of CrgA and the RNAP led to the production of short abortive transcripts, suggesting that CrgA may act by preventing RNAP from clearing the promoter. We probed the regulation by CrgA of its own production by analyzing CrgA-DNA contacts during cell-bacteria interaction by assaying *in vivo* protection against dimethylsulphate (DMS) methylation. Comparison of DMS footprints *in vitro* and *in vivo* suggested that CrgA repressed transcription through specific base contacts, probably in the major groove of the DNA double helix, resulting in DNA looping. Upon contact with target cells, CrgA was released from the DNA, allowing transcription of the target gene to proceed to elongation and facilitating tight control of the expression of genes regulated by CrgA.

Transcriptomic analysis of wild-type and PhoP mutant of the meningococcus.

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PhoP is the regulator component of a two-component system that, in salmonella, controls the expression of genes involved in virulence. We have constructed a *phoP* mutant of the meningococcus and have shown that the mutant is completely avirulent in a mouse model of disease. This finding suggests that, as for the salmonella, PhoP controls virulence gene expression in the meningococcus. However, the identity and function of genes controlled by PhoP in the meningococcus is completely unknown. As a first step towards characterising the PhoP regulon in the meningococcus, we have performed transcriptome analysis of wild-type and phoP mutant strains grown on blood agar and also in defined media at 2mM and 10mM magnesium. More than 40 genes were significantly down-regulated in the phoP mutant (normalised expression less than 0.5 of wild-type, with t test p value < 0.005) when grown on blood agar. These genes included many that are known to play a role in pathogenesis but also many unknown genes. Genes that were upregulated in the phoP mutant also included known virulence genes but also a large number of genes involved in oxidative phosphorylation, suggesting that, like the *E. coli* ArcAB two-component regulatory system, the meningococcal PhoP may be involved in respiratory control.

Detection of beta-barrel outer membrane proteins in genome-wide screenings.

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ABSTRACT
Motivation: Recent studies in genomics, proteomics, structural biology and genetics have begun to reveal the versatility and ubiquity of the beta-barrel membrane proteins. Their distribution extends well into many families of organisms, all of which have about 3% of beta-barrel membrane proteins in their respective proteomes. It is therefore urgent to develop methods capable of detecting this class of outer membrane proteins in large sequencing projects and use this information as aiding tool in protein annotation, and test the identified proteins as targets for vaccine or drug design.
Results: We defined two variables primarily based on the statistical bias in amino-acid composition, as predictors of the potential to form a beta-barrel structure, and we fitted Linear and Quadratic discriminant models with a training set comprising 1001 positive and negative beta-barrel proteins for the analysis of microbial proteomes. Leave-one-out cross validation showed that LDA correctly classified 98.2 % of the 1001 proteins in the training set while QDA performed for a 97.5 %. The correspondent proteomes of several gram-negatives bacteria were analyzed and the results of the predictions are discussed.

Availability: The program BBP is available on request from the authors.

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Transcriptional regulation of the *pilE* gene of *Neisseria gonorrhoeae*

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The *pilE* gene in *Neisseria gonorrhoeae* encodes the subunit of the major virulence factor, type 4 pili. Understanding the regulatory processes associated with expression of this gene may provide an insight into the progression of the infection caused by this pathogen. The control of expression of *pilE* is complex, with several upstream sequences potentially involved in its regulation. Upstream of *pilE*, two potential UP elements, found on either side of a confirmed IHF binding site (IHFBS) (Hill *et al*., 1998), have been shown to bind the alpha subunits (RpoA) of RNA polymerase (RNAP). UP elements are AT-rich regions commonly found in promoter regions of genes transcribed from sigma 70 promoters. Their interaction with RpoA appears to stabilise the RNAP interaction with the promoter resulting in a boost in transcription levels. In some instances this level of regulatory control requires accessory proteins. A theory for the mechanism of *pilE* regulation has been proposed that suggests that binding of IHF may allow RpoA/RNAP to shift from using a relatively weak UP element (downstream of the IHFBS but upstream of the promoter), to a stronger UP element upstream of the IHFBS resulting in increased expression levels. Experiments were performed to investigate the importance of phasing (i.e. helical face and distance between the identified DNA binding sequences) in *pilE* regulation. The results support our theory, showing that if the phasing between the IHFBS and either of the UP elements is altered, transcription levels generated from the *pilE* promoter region significantly decrease. This finding also argues against the involvement of DNA structural transitions in the transcriptional regulation of *pilE*. Consequently, three-dimensional modelling has been used to look at the interactions between IHF, RpoA and the *pilE* promoter region in the wild type and various mutant promoters.

Fur and iron mediated regulation of the *aniA* gene of the pathogenic Neisseria.

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In most bacteria, regulation of genes required for acquisition and regulation of iron are controlled by the ferric uptake regulator (Fur). In the presence of iron, Fur binds to a specific DNA sequence (Fur box) in the promoter region of these genes and in its most basic form represses transcription. Recent studies indicate that in addition to the regulation of iron transport genes, Fur can also control the transcription of a wide variety of genes. We recently reported that a remarkably large number of genes in the pathogenic Neisseria are activated during growth in presence of high iron. We also observed that a large percentage of these iron-activated genes were regulated by the transcriptional regulatory protein Fur in support of the proposed dual role of the Fur protein as both a negative and positive regulator of gene transcription. While the molecular mechanisms of Fur mediated transcriptional repression have been thoroughly investigated, the mechanism of activation by this global regulator has not been well defined. In previous studies we demonstrated that the Neisserial *aniA* and *norB* genes, encoding nitrite reductase and nitrite oxide reductase respectively, are activated through a Fur- and iron-mediated mechanism. In this study, we have further confirmed the interactions of Fur with the promoter region, that encompasses the putative Fur box, by EMSA and DNase I footprint analysis. Studies of the protected region of the iron-activated *aniA* and *norB* genes revealed perfect hexameric repeats flanked by repeats with varying degrees of homology. In addition, we demonstrate that the *aniA* gene is transcribed during aerobic growth when the organisms are grown in the presence of iron. To define the role of the AniA protein in Neisseria pathogenesis, we have constructed and characterized *aniA* mutants in both *N. meningitidis* and *N. gonorrhoeae.* Disruption of the *aniA* gene did not appear to alter expression of iron- and Fur-regulated *fur*, *sodB*, *fbpA*, or *tonB* genes. The *N. meningitidis* and *N. gonorrhoeae aniA* mutants were incapable of growth under anaerobic conditions in the presence of NO_2. We are currently examining the growth and virulence of the *N. meningitidis aniA* mutant in a meningococcal animal model.

Strain-to-strain diversity and newly identified genes within over 60 examples of Minimal Mobile Elements of the *Neisseria* spp.

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In the *Neisseria* spp., natural competence for transformation and homologous recombination allow the generation of mosaic genes, such as *opa*s, antigenic variation through recombination with silent cassettes, such as *pilE*/*pilS*, and the horizontal exchange of whole genes or groups of genes in Minimal Mobile Elements (MMEs). A MME is defined as a region between two conserved genes within which different genes are found in different strains. These elements are described as 'minimal' reflecting the fact that they do not have features suggesting mobilization by associated transposases, nor do they currently appear to have features by which remotely encoded systems would facilitate mobilization. These were initially identified through comparisons of the available *Neisseria* spp. genome sequences, on which basis the MME*pheS-pheT* was the first to be studied in an extended set of strains (Saunders & Snyder, 2002). This observation has now been expanded to a study of the complete repertoire of MMEs in the four neisserial genome sequences, revealing over 60 examples of MMEs containing strain-specific genes and present in strain-specific combinations.

The genome sequences were assessed using the whole-genome graphical interface ACEDB to identify regions where conserved genes flank strain-specific genes. Regions that were obviously the result of the activity of mobilizing elements, such as transposases, bacteriophage insertion, or chromosome rearrangement, were excluded unless other evidence suggested an MME in some strains. Primers were designed within the flanking conserved genes such that they would anneal in any of the four sequenced strains, thus optimizing for annealing in other strains. A set of 11 *N. meningitidis*, *N. gonorrhoeae*, *N. lactamica*, and *N. polysaccharea* strains were selected for PCR amplification of the MMEs, based on results from the MME*pheS-pheT* study. PCR products were assessed by size and restriction digest pattern. Those that were different from the genome sequences were sequenced and further analyzed to identify new genes and new MME sequences. Of the over 60 MMEs revealed through comparative genome analysis, just over two-thirds of the regions had further variation within the MME in a set of 11 diverse strains, compared to the genome sequences. While not all of these variations in sequence were due to different MME-associated genes, we have identified over 20 new strain-specific genes. In addition, variation within the sequences of MMEs, the combinations of genes within the MMEs, and the presence of intact genes in MMEs varies considerably from strain-to-strain. Polymorphisms in sequences from within the conserved flanking genes are indicative of the points of homologous recombination between the imported DNA and the native DNA. Further, the sequences within each MME are present in different combinations in different strains, supporting the model that these are horizontally, rather than vertically, transferred elements.

Additions to the pan-*Neisseria* microarray to include genes from a fourth neisserial genome sequence and genes not represented in the genome sequences.

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Introduction. The first version of the pan-*Neisseria* microarray was printed in the Spring of 2002, including probes for *N. gonorrhoeae* strain FA1090, and *N. meningitidis* strains MC58 and Z2491. In addition, probes were generated for an island of genes from *N. gonorrhoeae* strain MS11. This microarray is designed so that it can be used for analysis and comparison of both *N. gonorrhoeae* and *N. meningitidis*. To expand the scope of this tool, we have identified additional complete gene sequences in a fourth neisserial genome sequence, in GenBank submissions, and in Minimal Mobile Elements (MMEs), for which probes were designed and included on the second version of the pan-*Neisseria* microarray.

Materials & Methods. The complete genome of *N. meningitidis* strain FAM18 was obtained from the Sanger Institute and was assessed against pan-*Neisseria* microarray probes and the other three neisserial genome sequences using ACEDB. GenBank entries were accessed through NCBI. Individual genes were subjected to TBLASTN searches against the neisserial genome sequences. Those identified as not present in the genome sequences were extracted from GenBank for re-annotation and probe design, through importation into ACEDB. Genes identified through the sequencing of divergent MMEs were also assessed for inclusion on the microarray. Sequences were further analyzed using the GCG software package and primers were designed using Primer3 and ordered from Sigma Genosys or Operon. PCR products were amplified from chromosomal DNA using QIAGEN HotStar *Taq*, visualized on 2% E-Gels, and were diluted for use as templates for a second round of PCR. Second round products were precipitated, visualized on 2% E-Gels, and combined with Genetix Spotting Solution for printing onto Genetix Amine slides using Genetix QArray Mini microarray printers.

Results & Discussion. From the *N. meningitidis* strain FAM18 genome sequence, 78 new genes and 19 divergent genes were identified, against which probes were generated. With over 2000 annotated features in this genome sequence, relatively few new probes needed to be added to the pan-*Neisseria* microarray to include all the genes from this fourth genome sequence. This shows that our design strategy allows for the ready incorporation of probes from new and additional genome sequences without the need for extensive re-design or modification of the existing probes. From GenBank and MMEs, 70 additional genes were identified and probes against these were added as well. The utility of this expanded microarray was tested against DNA extracted from *N. meningitidis* strains of various serogroups and from clinical isolates of *N. gonorrhoeae*.

Conclusion. Inclusion of these genes broadens the utility of the pan-*Neisseria* microarray, the second version of which now includes 2741 probes to the genes within the four sequenced neisserial genomes and 123 probes to other genes that are not present in the genome sequences.

Microarray analysis and characterization of gonococcal genes responding to oxidative damage by hydrogen peroxide

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The interaction of *Neisseria gonorrhoeae* (Gc) with activated polymorphonuclear leukocytes (PMNs) is an important aspect of gonococcal infection and pathogenesis. PMNs evolve a potent respiratory burst, generating a number of reactive oxygen species (ROS) that include superoxide, hypochlorite, hydroxyl radical, and hydrogen peroxide (H_2O_2). The ability of Gc to survive this interaction with PMNs suggests that this bacterium has evolved many defenses to protect against oxidative stress.

We performed a microarray analysis to identify *Neisseria gonorrhoeae* genes that were differentially expressed after exposure to hydrogen peroxide using the Pan-neisseria microarray. Among the 78 genes found to be up-regulated more than 2.5 fold were several chaperone genes, putative transcriptional regulators, *msrAB*, a gene whose product is known to be important for survival to oxidative insult by Gc, many hypothetical proteins, as well as genes predicted to be involved in iron acquisition and/or members of the gonococcal *fur* regulon. One of these Fur-regulated genes, *recN*, was found to be up-regulated 3-fold after exposure to H_2O_2. A *recN* mutant showed decreased resistance to both H_2O_2 and paraquat, a superoxide-generating agent. Two of the most highly up-regulated genes (70 and 19-fold up-regulation) were predicted to encode hypothetical proteins. We insertionally inactivated both of these genes to further investigate their role in survival to oxidative damage. Disruption of the gene up-regulated 19 fold resulted in up to 100-fold decreased survival to H_2O_2, whereas disruption of the gene up-regulated 70-fold had no effect on H_2O_2 survival; however, both mutants showed decreased survival to paraquat. These results suggest that one protein may provide general protection against oxidative damage, whereas the other may offer only protection against a specific type of damage. Further investigation of the bases of this decreased resistance is ongoing.

Isolation of an *ihf* mutant in *Neisseria meningitidis* and development of widely-applicable tools for complementation of mutations and overexpression of gene products in *Neisseria*

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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, to date the generation of non-lethal *ihf* mutants has not been reported in either *Neisseria gonorrhoeae* or *Neisseria meningitidis*. Here we describe for the first time, the isolation of an *ihf* mutant in *Neisseria meningitidis*. This mutant has a growth defect, but we are attempting to use microarray analysis to assess the extent of the role of IHF in *N. meningitidis* gene regulation. In addition, we describe the development and application of an integrative plasmid system for the complementation or overexpression of genes in *N. meningitidis*, a procedure that in the past has been difficult or unavailable. Based on the inducible *lac* system from the *N. gonorrhoeae* shuttle vector Hermes, this plasmid is designed to be easily adapted for similar applications in other *Neisserial* strains. We have used this system to overexpress intact IhfA in a variety of different backgrounds.

SELECTIVE ISOLATION OF ANOMALOUS SEQUENCES FROM UNSEQUENCED NEISSERIA

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Introduction

The impact of horizontal gene transfer on prokaryotic genome evolution has been well established. Currently, the identification of acquired or anomalous DNA (aDNA) in a sequenced genome is performed by computational methods, while no *in vitro* technique is available to isolate aDNA from an unsequenced genome. In a genome sequence aDNA can be recognised among others by its atypical dinucleotide content. We hypothesise that the hexanucleotide content in aDNA differs from the rest of the genome as well. Thus, certain palindromic hexanucleotides, being restriction enzyme (RE) recognition sites, may be overrepresented in regions containing aDNA, which would allow the selective isolation of anomalous sequences.

Aim

We aim to develop a tool for the selective isolation of aDNA from genomes without prior knowledge of their complete sequence, based on differences in RE recognition site clustering between the genome and anomalous regions.

Methods

The sequenced human pathogen *N. meningitidis* MC58 (MC58) and the related unsequenced commensal *N. lactamica* 892586 (Nl) were used in this study. Recognition sites of BglII, NheI, ScaI and SpeI were scored for atypical clustering within the genome of *N. meningitidis* MC58. The GC% and dinucleotide frequency of the restriction fragments <5 kbp were determined *in silico* and compared to the average genomic values. *In vitro*, chromosomal DNA digested with the appropriate RE serves as a template in an adaptor-linked PCR (ALP) reaction. Amplicons were cloned and sequenced. To test the *in vitro* tool with an unsequenced genome, Nl was used.

Results

In silico analyses of different prokaryotic genome sequences illustrated RE site clustering specifically in aDNA regions. Analysis of the genome of MC58 yielded 22 restriction fragments <5 kbp, of which 20 were anomalous in GC% and/or dinucleotide composition. These predicted fragments were subsequently identified *in vitro*, demonstrating the applicability of this strategy. In Nl we identified 8 different amplicons *in vitro*, of which 6 displayed anomalous composition. The two fragments which did not meet our criteria for aDNA did however show similarity with mobile genetic elements, in this case a 4 kbp cryptic plasmid and a fragment annotated as part of an island of horizontal gene transfer in MC58. The fragments with aDNA composition not only yielded meningococcal putative virulence-related genes such as hemolysin genes and Maf related sequences, but also previously unknown ORFs such as a subtilisin-like protease locus, displaying 70% sequence similarity with *S. pneumoniae* and a novel putative adhesin encoding ORF.

Conclusions

A new and easy strategy for the selective isolation of aDNA from diverse unsequenced bacterial genomes was devised. It allowed the identification of novel genes in Nl, expanding the neisserial gene pool.

Expression and Purification of MtrA: A Putative Transcriptional Activator of the *mtrCDE* Efflux System.

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The MtrA protein of *Neisseria gonorrhoeae* is a member of the AraC/XylS family of bacterial transcriptional activators. It is necessary for induction of the *mtrCDE* efflux system required for increased resistance of gonococci to hydrophobic agents (i.e. Triton-X 100). The predicted MtrA amino acid sequence contains a potential helix-turn-helix motif similar to that predicted for a number of DNA-binding proteins. Although MtrA is homologous to the carboxy terminal region of AraC-like regulatory proteins, the mechanism by which MtrA regulates the *mtrCDE* efflux system and/or other genes involved in antibiotic resistance is unknown. In order to define how MtrA regulates genes in gonococci, we sought to purify the protein so that DNA-binding studies could be pursued. The *mtrA* gene from strain FA19 was cloned into a His-tag expression vector (pET-15b), over-expressed in Rosetta-gami^{TM} B(DE3)pLysS cells, and partially purified by metal-affinity chromatography. The protein was further purified to homogeneity (>90%) by reverse phase. The protein was detected at 210 nm and two major peaks were collected. The major peaks were designated MtrA-I and MtrA-II. Based on SELDI-TOF MS the MtrA-I and -II have a molecular mass of 35,356 and 35,393 daltons, respectively. Because the N-terminal amino acid sequence of MtrA-I could not be determined, possibly due to blockage, we analyzed MtrA-II by internal protein digestion by trypsin and TOF/TOF-MS analysis. A confident match for MtrA-II peptides was made to the MtrA protein of *Neisseria gonorrhoeae*. Although there is slight difference in molecular mass, SDS-PAGE analysis showed that the bands have a relative molecular mass of 35 kDa, which suggests that MtrA-I and -II are the same protein but possibly isoforms.

Anaerobic Growth in the Presence of Nitrite Pre-adapts *Neisseria gonorrhoeae* for Murine Genital Tract Infection and Induces Virulence Gene Expression [1]WU H, [1]BEGUM AA, [2]AL-KHALDI SF, [3]ARVIDSON CG, [1]JERSE AE. [1]Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland, [2]Division of Microbiological Studies, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, and [3]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, U.S.A.

Pathogenic bacteria frequently utilize transcriptional regulation of genes in response to host stimuli to adapt to different microenvironments during infection. The role of oxygen limitation as a cue for gene expression in *N. gonorrhoeae* is supported by evidence that anaerobic growth promotes gonococcal evasion of certain innate defenses *in vitro* and the detection of convalescent serum antibodies to nitrite reductase, an anaerobically-induced gene. The ability of *N. gonorrhoeae* to grow anaerobically is coupled to nitrite reduction. Speculation regarding the role of oxygen in gonococcal adaptation to the host is limited by a lack of *in vivo* studies and the fact that anaerobically- and nitrite-induced phenotypes have not been fully assessed. Here we tested the hypothesis that oxygen limitation promotes survival of *N. gonorrhoeae* in the female genital tract using a mouse model of genital tract infection. We also performed DNA microarray analysis to identify oxygen and, or nitrite-regulated genes that may promote survival in this body site. Dose response studies in which we compared the infectivity of *N. gonorrhoeae* strain MS11 cultured anaerobically in the presence of nitrite versus aerobically in 5% CO_2 showed significantly more mice were colonized with anaerobically grown *N. gonorrhoeae* following intra-vaginal inoculation with 10^2 and 10^3 CFU. A similar rate of infection was observed at doses of 10^4 and 10^5 CFU. The ID_{50} as determined by a Probit analysis was $\log_{10}$ 3.2 (anaerobic) and $\log_{10}$ 3.9 (aerobic). To screen for genes that might be responsible for this pre-adaptation phenotype, labeled RNA from gonococci cultured under anaerobic or aerobic culture conditions was hybridized against DNA microarrays that represented >90% of the FA1090 genome and included the MS11 genetic island. Using a 1.75 fold increase or decrease in the mean intensity ratio from the reference standard as the criteria for differential expression, we identified 30 putative up-regulated genes and 20 genes that appear to be repressed during anaerobic growth in the presence of nitrite. Several genes predicted to encode periplasmic or membrane proteins, including sialyltransferase (*lst*) and three known anaerobically-induced genes (nitrite reductase (*aniA*), cytochrome c peroxidase (*ccp*), and *pil*C), were identified as up-regulated. Induction of *aniA*, *ccp*, and sialyltransferase (*lst*) gene expression under anaerobic culture conditions was verified by quantitative real-time PCR. A nonpolar mutant in *lst* was attenuated for murine infection; in contrast, mutation in *ani*A did not cause reproducible attenuation *in vivo*. Confirmation of other microarray results and further examination of the significance of these findings with regard to the pre-adaptation phenotype in mice will be discussed.

Poster Session II

Surface Structures

LPS mediated meningococcal serum resistance in the presence and absence of capsule.

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Introduction
The importance of LPS structure in colonization and virulence of pathogenic *Neisseria* has been established. LPS is known to contribute to resistance to serum mediated killing. It has also been shown that LPS structure can affect interactions with host cells and as such has the potential to affect adherence, invasion, and phagocytosis of the bacterium.
Of the four genes responsible for the biosynthesis of the terminal LPS structures in *N. meningitidis* strain MC58, two - *lgtG* and *lgtA* - are phase variable. Phase variation results from instability within homopolymeric tracts (HPT) which are present in these genes. This leads to the production of four distinct LPS types on the bacterial surface and since fully extended LPS phenotypes can also be sialylated, the LPS presented on the surface of the bacteria can vary considerably and play a variety of roles with regards to colonization and infection. To elucidate the contributions the different LPS structures make during the infection process, we have constructed a series of unmarked mutants, each with a defined, stable LPS phenotype.
Methods
We have established a new method to stabilize the phase variable genes involved in biosythesis of LPS in *Neisseria meningitidis*, using selection counter-selection (S-CS) methodology. Our S-CS method uses a kanamycin resistance cassette (selection) and the beta galactosidase gene (counter selection). Using this approach we have introduced unmarked alterations within the phase variable genes, resulting in strains which possess no HPT but have genes that are either in the ON or OFF configuration, as they would occur in their natural states. The introduced changes in the HPT are the only changes in the gene and therefore the alterations produce no polar effects. Furthermore, this facilitates the use of antibiotic cassettes for later mutagenesis. With serial transformation we have built up a collection of all possible naturally occurring LPS phenotypes in MC58. In addition we used the kanamycin resistance cassette to delete the *lst* gene, in strains with normally sialylated extensions, to generate strains that are sialylated and non-sialylated. Likewise an erythromycin cassette was employed to delete a region of the *siaD* gene resulting in capsulated and non capsulated variants.
Results
Consistent with previous reports, serum killing assays indicate that the fully extended LPS phenotypes are the most resistant in the presence of a capsule. However in the absence of a capsule, presence of non-extended LPS phenotype result in the greatest resistance to serum. This suggests that, in the absence of a capsule, the immune component of serum selects for the non extended LPS phenotypes.
In addition, a reciprocal relationship exists between LPS sialylation and capsule expression, possibly due to substrate availability.

A comparative analysis of pilin-encoding genes from ten species of *Neisseria*

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Although all *Neisseria* live in association with host cells, the role of pili has not been well characterized for most *Neisseria* species found as human commensals or for non-human animal isolates of *Neisseria*. Previously characterized neisserial pilin-encoding genes display features typical of type IV pilin expression loci and can be categorized into two structural groups; one containing *pilE* loci from *N. gonorrhoeae* and class I pilin-producing *N. meningitidis* strains, the other containing pilin-encoding genes from class II pilin-producing *N. meningitidis* strains, *N. lactamica*, and *N. cinerea*. In addition to displaying structural differences, the genomic location also differs for meningococcal pilin-encoding genes from these two groups. The genome of the class II pilin-producing meningococcal strain FAM18 displays evidence of a deletion in the region that contains the *pilE* locus in the class I pilin-producing meningococcal strains Z2491 and MC58. The FAM18 class II *pilE* gene is found on an insertion located at an alternate site (1).

We have isolated putative *pilE* loci from type strains of the human commensal species *N. elongata*, *N. mucosa*, *N. polysaccharea*, *N. sicca, N. subflava*, and from the guinea pig isolate *N. denitrificans*. Three of these species, *N. elongata*, *N. sicca*, and *N. subflava*, contain two complete pilin-like genes arranged in tandem and separated by a short intergenic region. This arrangement is also seen in *Eikenella* and *Kingella* species, while *Moraxella* species possess two inverted pilin genes. We have identified single putative pilin-encoding genes in *N. mucosa*, *N. polysaccharea*, and *N. denitrificans*. The predicted protein sequences derived from these neisserial genes display hallmark features typical of type IV pilin. The genes from all species except *N. elongata* also display the conserved SV2 and cys2 regions found in all previously studied neisserial pilin genes.

The neisserial pilin gene family is quite diverse with respect to overall sequence similarity. Pairwise predicted amino acid similarity amongst pilin genes from different *Neisseria* species ranges from 39-96 percent, with *N. elongata* possessing the most divergent loci. The putative pilin gene from the non-human animal isolate *N. denitrificans* shows a moderate degree of similarity to other neisserial *pilE* loci, with closest similarity to *N. gonorrhoeae*. Some commensal species display greatest pilin similarity to bacteria from genera other than *Neisseria*. These include *N. elongata*, *N. sicca*, and *N. subflava*, whose putitive pilin genes are most closely related to those found in *Moraxella*, *Eikenella*, and *Xanthomonas* species, respectively. These data suggest that neisserial pilin genes have a complex evolutionary history most likely involving horizontal gene transfer both within the genus *Neisseria* and with other type IV pilin-producing genera.

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Peptidoglycan of *Neisseria meningitidis* : structure and functional analysis

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Reduced susceptibility to penicillin G (PenI phenotype) in *Neisseria meningitidis* is directly correlated with alterations in the *penA* gene, which encodes the penicillin-binding protein 2 (PBP2). We used reverse-phase high pressure liquid chromatography (HPLC), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and post source decay analysis (MALDI-PSD) to determine the muropeptide composition of *N. meningitidis*. Our results indicate that muropeptide composition of penicillin-susceptible and penicillin-intermediate clinical strains of meningococci showed a positive correlation between the minimum inhibitory concentration (MIC) of penicillin G and the amount of muropeptides carrying an intact pentapeptide chain in the peptidoglycan. However, no role was suggested for *O*-acetylation or cross-linking in PenI phenotype.

This suggests that reduced susceptibility to penicillin G in *N. meningitidis* is associated with a decrease in D,D-carboxypeptidase activity and/or D,D-transpeptidase activity. These results suggest that the D,D-transpeptidase and/or D,D-carboxypeptidase activities of PBP2 are modified by the changes in *penA* gene.

Meningococcal peptidoglycan is a pathogen-associated molecular pattern (PAMP) that can be recognized by the intracellular nucleotide-binding oligomerization domain (NOD) proteins. These proteins therefore recognize components of meningococcal muropeptide. This recognition is followed by the activation of innate immunity signaling pathways

Structure and function analysis reveals that Neisseria NhhA is a new adhesin belonging to the OCA family

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NhhA (*Neisseria* hia/hsf homologue, also known as GNA0992) is an autotransporter protein homologues to Hia/Hsf adhesins of *Haemophilus influenzae.* It has been already reported that NhhA is widely expressed in different strains of *Neisseria meningitidis* and is able to induce bactericidal antibodies. NhhA has been recently included within the Oligomeric Coiled-coil Adhesin (OCA) family. With other members of this family, NhhA shares the ability to form stable high molecular weight oligomers likely associated to the presence of a well conserved C-terminal anchor.

In order to elucidate the mechanism responsible for oligomerization and export of NhhA, we have analyzed the amino acid sequence and identified different functional domains in silico. In particular we predicted the three-dimensional structure of the C-terminal region holding the putative translocation unit which consist of a trimeric beta-barrel preceded by an helix linker. We expressed in *Escherichia coli* full length NhhA and various deletions of the C-terminal region. Analyzing the surface localization and the tendency to form oligomers, we demonstrated that the region formed by the proximal portion of the linker region plus the beta-barrel is both necessary and sufficient for trimerization of NhhA and export of the passenger domain(s). We confirmed these results with chimeric proteins consisting of different portions of the NhhA C-terminal region fused to unrelated passenger domains.
Finally, we further extended the functional analysis by discovering that NhhA is able to mediate adhesion to epithelial cells as well as to extracellular matrix proteins.

Our results show that NhhA is a new member of the OCA family, an increasing class of autotransporters able to promote the export of the passenger domains through a trimeric beta-barrel pore. Moreover, we demonstrate that, as suggested by sequence similarity to Hia/Hsf, NhhA is involved in host-pathogen interaction.

A study of *Neisseria gonorrhoeae* pilin glycosylation and its pathogenic role

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Neisseria gonorrhoeae (gonococcus, GC) not only causes uncomplicated gonorrhoeae (UG) but also complications like Pelvic Inflammatory Disease (PID) and Disseminated Gonococcal Infection (DGI). These complications arise when bacteria from the UG sites of cervix and urethra further spread to the deeper parts of the body like the upper genitourinary areas (through mucosal extension) or various systemic sites (via blood-borne dissemination). Our studies showed that the phase-variation of pilin glycan likely promote the progression of UG to PID and DGI. In order to accomplish better understanding of the effect of pilin glycosylation variation on GC pathogenesis, we decided to further analyze the genetic loci involved with pilin glycosylation from a diverse collection of GC strains and clinical isolates, including bacteria from UG, PID and DGI. We have also performed in parallel the biochemical and structural characterization of the pilin glycans from some of these strains and isolates of GC. Our molecular-genetic analysis involves common techniques like PCR, restriction analysis, cloning, mutagenesis and DNA sequencing. The biochemical analysis includes various methodologies like SDS-PAGE, silver staining, Western blotting, mass spectrometry and High pH Anion Exchange Chromatography in conjunction with pulse amperometric detection (HPAE-PAD). Results of these experiments show that GC pilin glycosylation is much more complex than the previously proposed model and the nature of this glycosylation is considerably variable between different strains and isolates. In sum, these data confirm that the variation of pilin glycosylation promotes causation of DGI and PID by likely enhancing the general spread of bacteria from the UG sites. Also, overall, the results of the aforementioned experiments indicate important roles of pilin glycosylation in various processes related to GC pathogenesis.

Sequence variation in the vaccine candidate NspA

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Introduction

NspA is a promising vaccine candidate, as it elicits protective antibody responses in mice against *Neisseria meningitidis*. It is present in the outer membrane of all *N. meningitidis* strains tested so far, and was detected in *Neisseria gonorrhoeae* strains as well. Previous studies on limited numbers of isolates indicate that the NspA protein is conserved in the pathogenic Neisseria, currently 8 *nspA* alleles encoding 8 different polypeptides are present in the 15 available *nspA* Genbank database entries.

Aim

The variability of the neisserial NspA was assessed by PCR amplification and subsequent sequence analysis of the *nspA* gene for 45 *N. meningitidis* and 2 *Neisseria lactamica* isolates, spanning the period 1960-2001, present in the database of the Netherlands Reference Laboratory for Bacterial Meningitis (RIVM/AMC, Amsterdam)

Results

Six novel *nspA* alleles were identified, and deduced amino acid sequences yielded three novel NspA protein sequences. All novel polymorphisms were located in the transmembrane regions of the protein, except for one polymorphism in the surface exposed loop 4. The latter polymorphism was found in both *N. lactamica* strains tested and in one meningococcal isolate, suggesting a horizontal gene transfer event from *N. lactamica* to a meningococcus.

Conclusions

In conclusion, the sequences of the surface exposed loops of NspA are highly conserved. However, the presence of polymorphisms in these regions in commensal Neisseria warrants further investigation, as horizontal transfer of *nspA* from commensal Neisseria to *N. meningitidis* may contribute to antigenic variation, which could limit the effectiveness of NspA as a vaccine component.

The structure and function of meningococcal T-cell stimulating protein A (TspA)

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Introduction: TspA is a highly conserved, high molecular weight, T-cell and B-cell stimulating protein of *Neisseria meningitidis*. TspA has a highly positively charged N-terminus, which contains a putative peptidoglycan-binding domain. There is a hydrophobic putative trans-membrane region and a highly negatively charged C-terminus containing a multiple repeat region and a coiled coil domain. In this study the molecular features and function of TspA were investigated.
Results and Discussion: An isogenic deletion mutant and a bank of truncated *tspA* mutants were produced. Homology to *fimV*, the *Pseudomonas* gene involved in twitching motility, suggested a link between TspA and the type IV pilus. Since pili are important in the association of meningococci to human cells, the adherence of TspA mutant bacteria and their wild type counterparts were compared. The mutants were dramatically reduced in their ability to adhere to cell culture monolayers. However, twitching motility stab assays showed no differences between wild type and mutants. Pilus expression and assembly appeared unaffected in the *tspA* mutant, under electron microscopy.
Conclusion: TspA mutants are dramatically reduced in their ability to adhere to human epithelial cell monolayers.

The inner membrane protein MsbA is involved only in lipopolysaccharide and not in phospholipid transport in *Neisseria meningitidis*

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The ABC transporter MsbA is thought to be involved in the transport of both lipopolysaccharide (LPS) and phospholipids across the inner membrane and/or the transport of these compounds to the outer membrane, as experimentally demonstrated using a conditional *msbA* mutant of *E. coli* (1). However, *in vitro*, MsbA appeared non-essential for phospholipid flip-flop (2). To shed light on this discrepancy, we tested the possibility to generate an *msbA* mutant in *N. meningitidis*. Since this bacterium is viable without LPS, but not without phospholipids, a mutant is expected to be viable only if the function of MsbA is restricted to LPS transport. We inactivated the *msbA* gene in *N. meningitidis* strain H44/76 by replacing its 5' end with a kanamycin-resistance cassette by allelic exchange. Transformants were selected on kanamycin-containing plates and tested by PCR. Correct transformants were easily obtained, showing that MsbA is not essential in *N. meningitidis*, in contrast to *E. coli*. Thus, MsbA is not required for phospholipid transport in *N. meningitidis.*
The phenotype of the *msbA* mutant was very similar to that of an H44/76 *imp* mutant, a strain that lacks an outer membrane protein involved in the transport of LPS over the outer membrane (3). Both mutant colonies showed enhanced colony opacity, which did not correlate with increased Opa protein expression, they were both retarded in growth, and most strikingly, both produced only low amounts (i.e. less than 10% of wild-type levels) of LPS. Thus, both the *imp* and *msbA* mutant down-regulate LPS biosynthesis. This phenomenon is likely due to stalled LPS transport, which occurs at different levels in these mutants. Both mutants could be complemented by introduction of the appropriate gene in trans. Electron microscopy studies showed that both mutants still possessed double membranes. We conclude from the similar phenotypes of the *imp* and *msbA* mutants that MsbA is involved in LPS transport also in *N. meningitidis*.

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Molecular evolution of the Opa protein repertoire of *Neisseria meningitidis*: unifying biodiversity and function.

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Opa proteins are implicated in the pathogenesis of meningococcal disease due to their role in mediating adhesion to and invasion of host tissues *via* CEACAM proteins and heparan sulphate proteoglycans. The high level of molecular diversity in Opa has limited our understanding of their range of functionality. We characterised over 2300 *opa* genes in a large collection of genetically and epidemiological diverse meningococci. Despite a high overall level of variation, the Opa protein repertoire was structured on a number of levels and only a fraction of the total possible number of hypervariable region combinations (which define Opa protein functionality) were observed. The evolution of the Opa repertoire of the seven major hyperinvasive meningococcal lineages was investigated. Whereas no single Opa variant, or combination of variants was solely associated with hyperinvasiveness, the Opa repertoire was highly structured over 30 years of global epidemic spread of these lineages. This structuring was reflected in an asymptomatically carried meningococcal population. The distribution of a recently discovered CEACAM binding motif in the HV2 region of Opa proteins was also investigated in our isolate collection. A number of variants of this motif had persisted in the meningococcal population, including one that does not bind any CEACAM. Investigations into the association between the Opa protein repertoire and the severity or symptoms of meningococcal disease are continuing using isolates collected for a study supported by EU-MenNet.

Structuring of Opa diversity in the carried meningococcal population was consistent with a theoretical model of positive immunological selection. Maximum likelihood analysis was used to further investigate the selective pressures on *opa* genes. Although many sites in the putative extracellular loops of Opa proteins were under positive selection, including those comprising the CEACAM binding motif, a number of sites in the hypervariable regions were highly conserved. These sites may be involved in receptor interactions or in maintaining the tertiary structure of Opa proteins. While the diversifying influence of the human immune response is a probable cause of molecular heterogeneity in Opa proteins, the effect of functional niche adaptation cannot be excluded and a case/control study aimed at determining the effect of variation in the CEACAM proteins on the evolution of meningococcal Opa proteins is ongoing.

Analysis of the FetA outer membrane protein in meningococci collected in Scotland and Iceland between 1970 and 2004

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FetA is an iron-regulated 76-kDa outer membrane protein of *Neisseria meningitidis* which shows sequence homology to the TonB-dependent family of receptors. It is a potential vaccine candidate for both *Neisseria gonorrhoeae* and *N. meningitidis* and we therefore aimed to determine its variation amongst a collection of meningococci.

Our laboratories have unique collections of meningococci from invasive infection from the 1970's to date. We therefore selected strains from these collections to represent a range of serogroups and sequence types, including types that are prominent in causing meningococcal disease in Scotland and Iceland. The *fetA* gene in one hundred meningococci isolated in Scotland and Iceland between 1970 and 2004 was sequenced and the peptide sequence of FetA determined using the FetA variable region peptide sequences database (http://neisseria.org/nm/typing/feta/). We found that, in some cases, FetA peptide sequences can be used to further differentiate between strains that possess identical sequence types and *porA* variable regions. It may therefore be useful for the characterisation of hyperendemic stranis although its routine use may be limited as no further discrimination is provided for endemic strains. Moreover, for some strains, genosubtyping provided greater discrimination compared to FetA typing.

STRUCTURAL STUDIES OF THE INNER CORE REGION OF LIPOOLIGOSACCHARIDE (LOS) FROM *NEISSERIA MENINGITIDIS* STRAIN NMB: LOCATION AND DISTRIBUTION OF PHOSPHOETHANOLAMINE AND GLYCINE ON THE INNER CORE HEPII RESIDUE

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Meningococcal strain NMB expresses a mixed population of LOS structures consisting of L2, and L4 immunotypes. The HepII residue of the LOS in the L2 immunotype is substituted with Glc and PEA at O-3 and O-6, respectively; and in L4 HepII lacks Glc but retains the O-6 PEA group. Greater than 80% of the LOS in NMB is of the L2 structure. In addition, NMB contains L3 reactive LOS even though chemical analysis did not reveal a PEA group at O-3 of HepII; the presumed epitope for the L3 immunotype. However, even though HepII lacks a PEA at O-3, NMB does have an intact *lpt-3* gene. The gene encoding the O-6 PEA transferase, tentatively termed *lpt-6*, has not yet been identified. The putative *lpt-6* gene was insertionally inactivated in strain NMB and the LOS-derived oligosaccharides were structurally characterized by glycosyl composition and linkage analyses, ^{1}H and ^{31}P NMR spectroscopy, and matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS). The results showed that inactivation of the putative *lpt-6* gene in strain NMB resulted in the complete absence of PEA groups on the LOS inner core, and, in comparison to the parental NMB LOS, ^{1}H and ^{31}P NMR analyses of the oligosaccharides confirmed the loss of the O-6 PEA groups from inner core HepII residue. An NMB mutant, NMB*rfaK*, produces a truncated oligosaccharide consisting of a HepII→HepI→Kdo trisaccharide in which HepII contains PEA groups at both O-3 and O-6. This observation supports the conclusion that even though the LOS from the parent NMB lacks PEA at this position, the *lpt-3* gene product is active in the NMB*rfaK* mutant. Therefore, the function of the *lpt-3* and putative *lpt-6* genes were examined by characterizing the structures of the LOS oligosaccharides from the double mutants, NMB*rfaKlpt-3* and NMB*rfaKlpt-6*. The results showed that the NMB*rfaKlpt-3* double mutant produced an LOS containing the HepII→HepI→Kdo trisaccharide which completely lacked PEA at O-3 and contained PEA at O-6 of HepII. The NMB*rfaKlpt-6* double mutant contained an LOS with a HepII→HepI→Kdo trisaccharide that lacked PEA at O-6 but contained PEA at O-3 of Hept II. Also, during these investigations it was discovered that glycine is present on the LOS inner core of parental strain NMB, but absent in the NMB*rfak*, NMB*rfaKlpt-3*, and NMB*rfaKlpt-6* mutant LOSs.

THE *NEISSERIA MENINGITIDIS* SEROGROUP A CAPSULAR POLYSACCHARIDE O-3 and O-4 ACETYLTRANSFERASE.

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Neisseria meningitidis serogroup A capsular polysaccharide (CPS) is composed of a homopolymer of O-acetylated, ($\alpha 1 \rightarrow 6$) linked N-acetyl–D-mannosamine (ManNAc)-1-phosphate that is distinct from the capsule structures of the other meningococcal disease causing serogroups B, C, Y and W-135. The serogroup A capsule biosynthetic genetic cassette consists of four ORFs, *mynA-D (sacA-D*) that are specific to serogroup A. We found that *mynC* encoded an acetyltransferase that was responsible for the O-acetylation of the CPS of serogroup A. The wild type CPS as revealed by ^{1}H NMR had 60 to 70% O-acetylated ManNAc residues that contained acetyl groups at O-3, with some species acetylated at O-4 and O-3 and O-4. A nonpolar *mynC* mutant, generated by introducing an *aphA-3* kanamycin resistance cassette, produced CPS with no O-acetylation. A serogroup A capsule-specific monoclonal antibody was shown to recognize the wild type O-acetylated CPS but not the OAc- CPS of the *mynC* mutant. Competitive inhibition ELISAs using sera from vaccinated individuals showed that the mutant CPS competitively inhibited less antibody binding to the solid-phase CPS than the wild type CPS, indicating OAc- CPS lacks an important immunogenic epitope(s). MynC was C-terminally His-tagged and over expressed in *E. coli* to obtain the predicted ~26kDa protein. The acetyltransferase activity of purified MynC was demonstrated *in vitro* using ^{14}C labeled acetyl CoA. MynC, O-acetylated the OAc- CPS of the *mynC* mutant, and further acetylated the wild type CPS of serogroup A but not the CPS of serogroup B or serogroup C meningococci. Genetic complementation of the *mynC* mutant confirmed the function of MynC as the serogroup A CPS O-3 and O-4 acetyltransferase. MynC is tightly associated to inner membrane and exists presumably as a component of multienzyme CPS biosynthetic complexes. MynC belongs to a new subclass of O-acetyltransferases utilizing acetyl CoA to decorate the serogroup A capsule.

Expression of *Neisseria meningitides* PorA protein on the outer membrane of *Escherichia coli.*

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Introduction: The PorA porin protein on the outer membrane of *Neisseria meningitidis* is an important vaccine antigen. The protein is one of the main targets for bactericidal antibodies induced by two outer membrane vesicle (OMV) group B meningococcal vaccines developed in Norway based on strain 44/76, B:15:P1.7,16 (MenBvac), and strain NZ98/254, B:4:P1.7b,4 (MeNZB). The PorA protein express two immunodominant epitopes located to variable region 1 (serosubtype 7 and 7b) and variable region 2 (serosubtype 4 and 16) on the extracellular loops I and IV, respectively. We wanted to express meningococcal PorA on the outer membrane of *E. col* .in order to be able to discriminate the vaccine immuneresponse against PorA without interference with other meningococcal antigens.

Materials & Methods: The PorA protein gene was isolated from strains 44/76 (P1.7,16) and NZ98/254 (P1.7b,4), and the gene cloned into the pET3a vector. The construct included the use of *E. coli* lpp signal peptide in order to direct the PorA protein to the *E. coli* outer membrane. To verify the presentation of correctly folded PorA protein on the outer membrane, the bacteria was examined by dot-blot and flowcytometry techniques using monoclonal antibodies against P1.7, P1.4 and P1.16. As a control the DNA vector constructs were also expressed in cell-free *E. coli* extracts, and the resulting PorA protein products were detected.

Results and discussion: The PorA proteins from both strain 44/76 and NZ98/254 were detected on the outer membrane of *E. coli* by dot-blot and by flowcytometry. Interesting, we found that PorA from strain NZ98/254 present on the outer membrane of *E. coli,* did not expose the hidden epitope P1.7b similar as for meningococci. However, when the protein was expressed in cell-free extracts, the resulting PorA protein, having no structural restrains, readily presented the hidden P1.7b epitope, further indicating that the folding of PorA on the outer membrane of *E. coli*, is identical or close to the structure found on *Neisseria meningitides*.

Conclusion: We were able to express the PorA protein from the vaccine strains 44/76 and NZ98/254 on the outer membrane of *E. coli*, presumably in a correct folded structure expressing the serosubtype epitopes P1.7, P1.4 and P1.16. These bacteria can be used for multiple purposes to study the immuneresponse and protective activity of PorA antibodies in more discriminating test systems.

The phase variable allele of the pili glycosylation gene *pglA* (*pgtA*) is not strongly associated with strains of *Neisseria gonorrhoeae* isolated from patients with disseminated gonococcal infection.

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Neisseria gonorrheae infections may cause a spectrum of disease from asymptomatic carriage and uncomplicated gonorrhoeae (UG) and disseminated gonococcal infection (DGI). Pili are a major virulence factor of pathogenic Neisseria with a key role in adherence. Pili of pathogenic Neisseria are modified by glycosylation. In our previous studies, a number of genes have been identified that are involved in the glycosylation of pili. A feature found in the glycosyltransferases involved in the glycosylation of pili is phase variation (rapid on/off switching of gene expression). PglA was first described as a glycosyltransferase which catalyses the transfer of the first galactose to the trideoxyhexose sugar in the pilin-linked trisaccharide (Gal(β1-4)Gal(α1-3)2,4-diacetamido-2,4,6-trideoxyhexose) of *N. meningitidis* (Jennings *et al* 1998. Mol Micro 29:975-984). Banerjee *et al.* (J. Exp Med. 2002. 196:147-62) demonstrated that the same gene in *N. gonorrhoeae* (in that study called *pgtA*; 95% identical *pglA*) is responsible for an analogous function. The *N. gonorrhoeae pglA* gene has two alleles, one containing a homopolymeric tract with potential to phase vary, and another lacking the tract, which would not be expected to phase vary. A key finding in this study was an absolute correlation between strains that contained the phase-variable *pglA* allele and DGI. This was an exciting observation since it identified a virulence factor invariably associated with DGI and presented an important example of the role of phase variation and of pili glycosylation in pathogenesis.

In the current study we sought to confirm this observation and extend the study to include other phase variable genes involved in post-translational modification of pili. A large collection of *N. gonorrhoeae* strains isolated from patients with UG or DGI (n=81) was used to determine the phase variation potential of *pglA* (presence or absence of the homopolymeric tract). In this study only 36% of strains that were isolated from patients with DGI were found to contain a homopolymeric tract in the *pglA* gene, indicating no correlation with, or requirement for the phase variable *pglA* allele in DGI. Furthermore, the presence and length of phase-variable tracts in two other genes involved in the post-translational modification of pili, *pglE* and *pptA*, were also not linked with DGI.

PorA subtypes amongst invasive meningococcal isolates in Iceland between 1977 and 2003

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Neisseria meningitidis is an important cause of meningitis and bacteraemia worldwide. In Iceland, a relatively isolated community in the Mid-Atlantic, invasive meningococcal infections are hyperendemic. Meningococci have frequently caused epidemics in the country. As well as differentiation through multilocus sequence typing (MLST) it was of interest to discriminate among isolates using the sequence of three variable regions of PorA which determines the meningococcal serosubtype. PorA is a class-I outer membrane protein and plays an important role in human immunity and is therefore a vaccine candidate. A nationwide registry of all cases of meningococcal disease has been kept in Iceland since 1975. In this study our laboratory performed PorA sequencing on all viable strains from invasive infections which were collected during the period 1977-2003. This did not include imported cases. The nucleotide sequences of the three variable regions, VR1, VR2 and VR3, were determined and translated into an amino acid sequence. Variant names were assigned using the PorA variable region databases (http://neisseria.org/nm/typing/pora/ and http://www.show.scot.nhs.uk/smprl/)

A total of 41 different variable regions were found (14 VR1's, 29 VR2's, 7 VR3's). The most common families amongst VR1 were 7 (74/236 or 31.4%) and 5 (61/236 or 25.8%), and amongst VR2 were 16 (100/236 or 42.4%) and 2 (48/236 or 20.3%). VR3 results were equally split between the families 35, 36 and 37 and appear to show no specific relationships with the other variable regions. Interestingly, while the PorA profile with VR1 as 5 was almost exclusively associated with serogroup C isolates, those isolates with VR1 as 5-1 were almost solely serogroup B. The most common VR2 family, 16, would appear to be non-specific to any sergroup while all serogroup A isolates, except one, had the PorA profile 5-2, 10, 37-1. This study illustrates a substantial heterogeneity of meningococcal isolates, even in a relatively isolated population. It shows that by sequencing these three PorA variable regions that more discrimination or linking between isolates is possible and is extremely valuable for determining the epidemiology of the disease and influencing future vaccine policy.

Ng-MIP, a novel surface-exposed PPIase of *Neisseria gonorrhoeae* involved in persistence into macrophages.

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Infection of *Neisseria gonorrhoeae* provokes a massive inflammatory response in genitourinary mucosae and a consequent infiltration of mononuclear phagocytes in the subepithelial tissues. While the primary interaction of *N. gonorrhoeae* with human phagocytes is mediated by pili and opacity outer membrane protein (Opa), very little is known on the fate of gonococci after the internalization, although it is likely that entry and survival of gonococci into resident macrophages play an important role in the persistent phases of inflammation as well as in the spread of microorganisms.
By genome analysis we identified in *N. gonorrhoeae* a protein with high homology to Macrophage-Infectivity-Potentiator (MIP) of *Legionella pneumophila*, a virulence factor with peptidyl-prolyl-*cis/trans* isomerase (PPIase) activity, contributing to the initiation of macrophage infection.
We showed that *N. gonorrhoeae* MIP, that we named Ng-MIP, is a lipoprotein and exhibits a PPIase activity. The inhibition of the activity by rapamicyn and the predicted structural features place the protein in the family of prokaryotic and eukaryotic FK506-binding proteins (FKBP). Ng-MIP is expressed in *N. gonorrhoeae* F62 strain as well as in a panel of 21 clinical isolates; in particular, the protein is detected by Western blot analysis in the total cell extracts and in the OMV preparation and is secreted in the culture supernatant.
To test the hypothesis that Ng-MIP protein is involved in the interaction of *N. gonorrhoeae* with macrophages, the events of phagocytic process, including the adherence to the surface of macrophages, the internalization and the intracellular killing, were investigated. For this purpose we studied the interaction of F62 strain and the Ng-MIP knock-out mutant with RAW 264.7 mouse macrophages cell line and human macrophages differentiated from peripheral human blood. We observed that Ng-MIP does not affect the binding and internalization into macrophages, suggesting that the protein is not involved in the primary interaction with phagocytes. On the contrary we demonstrated that Ng-MIP plays an important role in the phases sub-sequent to macrophage-mediated internalization since we observed a marked decrease of intracellular survival of Ng-MIP-negative strain compared to the wild-type. These results strongly suggest that Ng-MIP is involved in promoting the survival of *N. gonorrhoeae* into macrophages with an important impact on the persistence of the infectivity.

The importance of the trimeric structure of neisserial porins for their biological effects.

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The outer membrane protein, PorB, of *Neisseria meningitidis* is a bacterial pore-forming protein which has various effects on eukaryotic cells. It has been shown to form pores in bacteria and in eukaryotic cells, to act as a potent immune adjuvant by up-regulating the surface expression of the co-stimulatory molecule CD86 (B7-2) and class II MHC (an event mediated by TLR2 and MyD88), and is involved in apoptosis by modulating the mitochondrial membrane potential. In this study, we have investigated the connection between native PorB structure and the above mentioned properties. Purified PorB, when in its native trimeric form, maintained its immune stimulating and anti-apoptotic properties, as well as its pore function. Upon treatment at low temperatures, the trimeric structure of PorB was dissociated and its pore-forming activity was lost. Moreover, it become unable to stimulate up-regulation of CD86 on the surface of B cells and to protect mitochondria from depolarization. Therefore, pore-forming ability, B cell activation and anti-apoptotic activity of PorB depend on its native trimeric conformation, which appears to be disrupted when PorB is incubated at low temperature. The pore forming ability of purified meningococcal PorB, along with its ability to interact with eukaryotic cells either in the context of the immune response or in the apoptotic cascade, are effects that are dependent on an intact, functional trimeric structure. This structure can be disrupted by low temperatures, probably due to a mechanical effect of trimer dissociation, which does not re-associate even upon returning to room temperature. In contrast, when porin preparations are boiled and then allowed to cool back to room temperature, the native trimeric structure is reformed. The mechanism of the irreversible disruption of the Por trimer by cooling is unknown but is important in regards to its use as a vaccine and immune adjuvant.

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A COMPARISON OF THE OLIGOSACCHARIDE BINDING SPECIFICITIES OF THE OpcA, OpaB AND OpaD NEISSERIAL OUTER MEMBRANE PROTEINS

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Many pathogens use binding to carbohydrate ligands on the surface of host cells as a means of attachment. Both the OpcA and the opacity (Opa) proteins from *Neisseria* are known to bind to proteoglycan ligands but a detailed biochemical characterization of their individual ligand binding specificities has not been been attempted to date. Here we compared the binding specificities of the adhesin OpcA with two different opacity proteins, OpaD from *N. meningitidis* MC58 and OpaB from *N. meningitidis* Z2491, for a range of different oligosaccharide ligands. All three proteins were expressed as inclusion bodies in *E. coli*, refolded and purified to homogeneity. We have developed a sensitive spectrofluorometric assay which allows us to measure accurately the stoichiometry and equilibrium binding constants (K_ds) for the binding of oligosaccharide ligands to these purified proteins. It is based on the sensitivity of the fluorescence of tyrosine residues to the change in environment caused by the binding of a ligand. The results show a broad range of oligosaccharide ligands for these proteins, primarily based around a 6-membered pyranose ring, with individual proteins showing distinct preferences for different ligands. For example, OpcA has a sub-micromolar K_d value for sialic acid (0.67microM): using sialyl [2-3] lactose and sialyl [2-6] lactose as model compounds, we were able to show that the specificity for this ligand is independent of the adjoining saccharide linkage conformation. Together with data from a range of sialic acid polysaccharides, we have concluded that OpcA shows a preference for sialic acid over other saccharides when it occupies the terminal position of an oligosaccharide chain. Mutagenesis experiments implicated Lys 27, Tyr 169 and Tyr 218 as being involved in ligand binding. In contrast to OpcA, both Opa proteins failed to bind monosaccharides but showed high affinity for lactose-containing polysaccharides, including lactose itself (K_d=4.0microM). Interestingly, OpaD bound the sialyl Lewis a and sialyl Lewis x blood group antigens with high affinity (K_ds of 0.7 and 0.8microM respectively). OpaB showed a similar specificity profile to OpcA, whereas OpaD showed less affinity for sialic acid-containing saccharides. The K_d for sialyl [2-3] lactose for OpaB was very low (1.0microM), compared with that for OpaD (51microM). The results show that the binding of OpcA or Opa proteins to complex oligosaccharides can be attributed to the recognition of much smaller mono- and disaccharide units. For all three proteins studied, a broad-based affinity for saccharides containing a pyranose ring appears to be supplemented with subtle variations in specificity for different ligands.

Meningococcal PilC2 proteins regulate pilus retraction but do not competitively inhibit adhesion to human cells.

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Pathogenic *Neisseria* express type IV pili (tfp), which have been shown to play a central role in the interactions of bacteria with their environment. The PilC proteins are outer membrane-associated proteins that have a key-role in tfp biogenesis since PilC-null mutants appear poorly piliated. The PilC proteins are also involved in tfp-mediated adhesion to human cells, and have been described in gonococcus as pilus-tip located adhesins. The two PilC variants that can be concomitantly expressed by *N. gonorrhoeae* mediate identical adhesion phenotypes. However, in *N. meningitidis*, only the PilC1 variant is functionally identical to the gonococcal variants since the PilC2 protein is unable to promote adhesion to cells.

Fibre assembly and extrusion to the bacterial surface take place independently from PilC, and recent results have shown that the meningococcal PilC1 proteins have a regulatory role in tfp biogenesis, by controlling fibre expression on the bacterial surface through the regulation of PilT-driven fibre retraction.

In this work, we first analysed the role of PilC proteins in the regulation of fibre retraction, depending on their ability to promote adhesion. Our results show that fibre expression and clump formation correlate with the level of PilC expression in a non-adhesive $PilC2^{+}/PilC1^{-}$ strain, similarly to the adhesive $PilC1^{+}/PilC2^{-}$ strain as control.

Furthermore, we purified a meningococcal PilC2 variant, expressed in gonococcus. Although purified adhesion-promoting PilC variants used as control would competitively inhibit adhesion of pathogenic *Neisseria* to human cells, the purified meningococcal PilC2 protein did not.

Taken together, these results support the model that all neisserial PilC variants share identical functions in the control of fibre expression. They also suggest that the meningococcal PilC2 variant fails to act as an adhesin, thus preventing tfp-mediated adhesion to cells.

The pilin-linked glycan of *Neisseria meningitidis* C311 is transferred to pilin by a process similar to wzy-dependent O-antigen biosynthesis in *E. coli.*

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The pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae* are glycosylated by the addition of O-linked sugars. The genetics of the glycosylation of pilin in *N. meningitidis* has been explored in a number of papers from our laboratory. Recent work in our laboratory has shown an additional gene, *pglF*, is required for the biosynthesis of the pilin-linked glycan. PglF has sequence and transmembrane homology with proteins that have been identified as putative O-antigen 'flippases' suggesting that the addition of glycan to pilin may occur in a manner analogous to the addition of O-antigen to the core-LPS.

In the wzy-dependent model of LPS O-antigen biosynthesis in *E. coli* O-antigen 'flippases' (wzx) are required for the transport of the undecaprenol-linked O-antigen unit across the cytoplasmic membrane to its periplasmic face where the polymerisation of the O-antigen units, and subsequent ligation of the polymerised O-antigen from the undecaprenol carrier onto the core-LPS occurs and requires the O-antigen ligase WaaL. *N. meningitidis* strain MC58 serogroup B genome was examined for O-antigen ligase homologs using a PFAM hidden Markov model and a candidate gene was identified and termed *pglL* (pilin glycosylation ligase). The transmembrane profile of this protein is similar to the profile to those of O-antigen ligases. Homologues were identified in the Z2491, FAM18 and FA1090 genomes.

A mutant of *pglL* was made in *N. meningitidis* strain C311#3. Silver stain gels for the detection of LPS revealed that the C311*pglF* and C311*pglL* mutants showed no alteration of LPS expression, ruling out a role in LPS biosynthesis. Western blots of pilin, detected with the pilin-specific antibody SM1, revealed a change in the molecular weight of pilin. Pilin from the C311*pglL* mutant also lost reactivity with anti- trisaccharide sera (specific for the C311 pilin linked trisaccharide), indicating that the altered migration was due to loss of the pilin-linked trisaccharide. These results suggest that the glycosylation of pilin occurs in a manner analogous to the wzy-dependent biosynthesis of LPS in *E. coli* - specifically that the pilin-linked trisacharide is first synthesised on an undecaprenol carrier (the first sugar transferred to the carrier by PglB) and then transported across the membrane by PglF and then ligated to the pilin by PglL. The presence of these genes in all strains of *N. meningitidis* and *N. gonorrhoeae* tested suggests that this mechanism is a common pathway for pilin glycosylation.

Analysis of NadA oligomerization process

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NadA is a novel vaccine candidate of *Neisseria meningitidis* and a new adhesin identified by genome analysis of Neisseria meningitidis serogroup B strain (MC58). The *nadA* gene is present in approximately 50% of the disease-associate meningococcal isolates and in three out of the four hypervirulent lineages ET37, ET5 and cluster A4. It is unusually well conserved in sequence, and present as only three alleles (1, 2 and 3). Very recently, a new allele has been identified in a subset of carrier strains (allele 4). The *nadA* gene encodes for a protein of 362 residues in the case of allele 1 and of 398 and 405 in the case of allele 2 and 3 respectively. The protein can be divided in three domains: an N-terminal globular head, an internal region with high coiled-coil propensity and a leuzine zipper motif, and a C-terminal membrane anchoring region. The carboxy-terminal region predicted to form the membrane anchor of the protein is formed by four amphipatic β-strands.

The protein is surface-exposed in meningococcus and migrates as high molecular weight oligomers, most probably trimers. The oligomers are very stable to heat and reducing agents

On the basis of the secondary structure prediction and of its oligomeric organization on the bacterial membrane is possible to classify NadA in a novel class of nonfimbrial adhesins named “Oca” family.

The aim of this study is to investigate the NadA oligomerization process. The NadA gene has been expressed as full-length or as amino and carboxy-terminal deletion mutants in E.*coli.* The expression, localization and oligomerization of the full-length NadA and of the deletion mutants have been investigated. Preliminary analysis show that the mutant devoid of the membrane anchor domain is still able to oligomerize. A biochemical characterization of all NadA form expressed in E.*coli* is ongoing.

Neisseria gonorrhoeae OmpA Protein Interacts with Human Epithelial Cells and Mediates Serum Resistance by Binding to C4bp

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Neisseria gonorrhoeae, the causative agent of gonorrhoea, is one of the most commonly reported sexually-transmitted disease. The interaction of gonococcus with epithelial cell surfaces has been extensively studied using different cell culture systems. Several bacterial cell surface components, such as type IV pili, Opa proteins and lipooligosaccharide, play a significant role in host-pathogen interaction. After the release of the complete genome sequence of *N. gonorrhoeae* FA1090, a number of surface-exposed antigens were identified by genome analysis. On the basis of homology with known bacterial virulence factors, proteins potentially involved in promoting adhesion and invasion of gonococcus into human host cells has been selected for further characterization. In particular, we identified a protein with high similarity to the outer membrane protein A (OmpA) of *E. coli* and other Gram-negative bacteria. OmpA has been extensively studied in *E. coli* and many functions have been attributed to this 35 kDa, heat-modifiable protein. OmpA is thought to contribute to the structural integrity of the outer membrane in combination with the outer membrane lipoprotein Lpp. The expression of OmpA appears to confer a significant selective advantage during the pathogenesis in vivo, since an *ompA* mutant showed attenuated virulence in two different models of *E. coli* K1 infection and increased sensitivity to serum bactericidal activity.

In our study we showed that the gonococcal OmpA protein plays a significant role in the adhesion and invasion process into human cervical carcinoma and endometrial cells. We observed a marked decrease in both adhesion and invasion of an *ompA* knockout mutant compared to the wild type *N. gonorrhoeae* F62 strain. In addition, a similar phenomenon was shown using a macrophage cell line, suggesting that OmpA is also required for entry and intracellular survival into macrophages. In addition, in *E. coli* OmpA contributes to the serum resistance by binding to the complement regulatory protein C4b binding protein (C4bp), leading to a decrease in serum killing, a mechanism which is used by many pathogens to avoid complement attack. *N. gonorrhoeae* OmpA is also able to bind C4bp and we are further investigating the serum resistance of the strain. Finally, in order to evaluate the conservation of this protein in different strains, we analysed a panel of clinical isolates, obtained from different geographical areas and observed the expression of OmpA in all strains and at comparable levels. Taken together, our observations support the hypothesis that OmpA could represent an important factor involved in the interaction between *N. gonorrhoeae* and the human host in the establishment of the infection process.

N. meningitidis produces three non-redundant DsbA proteins

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The thiol-disulphide oxidoreductase DsbA ensures the correct folding of many exported bacterial proteins by catalysing the formation of intramolecular disulphide bonds. DsbA has been implicated in the virulence of many bacterial pathogens including *N. meningitidis*, through its function in the folding of virulence factors.
The *N. meningitidis* MC58 genome contains three genes (*nmb0278, nmb0294* and *nmb0407*) with homology to *E. coli dsbA*. Only one of these, *nmb0407*, encodes a product with the N-terminal motif typical of a periplasmic protein (the location of DsbA in other organisms). NMB0278 and NMB0294, in contrast, bear N-terminal signal motifs typical of membrane lipoproteins. From inspection of sequence, NMB0278 and NMB0294 are very similar to each other, while differing significantly from NMB0407. The GC content of their genes, identical at 56.47 %, is also different from that of *nmb0407* (49.22 %).
We have investigated the DsbA activity of the products of these genes by studying their ability to complement a *dsbA*-deficient *E. coli* strain. All three genes successfully complemented the mutation in *dsbA* as indicated by DTT sensitivity assays. However, the meningococcal proteins differed in their capacity to complement *E. coli* DsbA in various functional assays. NMB0278 was the most active in complementing defects in motility and alkaline phosphatase activity, while NMB0294 protein was the most active in folding periplasmic MalF. NMB0407 showed the weakest activity in all assays.
We have constructed knockout mutants of individual and multiple *dsbA* genes in *N. meningitidis*. These strains, with wild-type, have been used to study the role of the different DsbAs in meningococcal biology and virulence.
DNA uptake in Neisseria is dependent on type IV pilus assembly, a process dependent on functional DsbA activity. Single knockouts in *nmb0278* or *nmb0294* were as transformable as wild-type, but a double mutant containing insertions in both of these genes was significantly impaired in DNA uptake / binding, as indicated by a 1-2 log lower transformation frequency.
We conclude that *N. meningitidis* contains three functional DsbA proteins which have the same enzymatic activity but different target specificities in *E. coli*. In *N. meningitidis*, we speculate that NMB0407 may be the native homologue of *E. coli* DsbA, while NMB0278 and NMB0294 could have been horizontally acquired and their protein-folding function directed at a restricted range of targets, perhaps involved specifically in host-pathogen interactive biology.

Meningococcal serine protease A (MspA) – an immunogenic autotransporter protein with bactericidal activity

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Introduction: Autotransporter proteins are well recognised as virulence determinants in Gram-negative bacteria, and have been used as vaccine antigens. We have previously demonstrated the surface-exposure, conservation and immunogenicity of the meningococcal autotransporter proteins App and AspA(NalP), which are potential virulence determinants and serogroup B vaccine candidates. Here we describe the characterisation of another meningococcal autotransporter protein designated Meningococcal serine protease A (MspA; NMB1998).

Methods and Results: MspA was identified via an *in silico* screen of the available genomic sequence of MC58. The *mspA* gene is absent from the genomic sequence of Z2491 and the gonococcal strain FA1090. An orthologue is present in FAM18 (serogroup C; ET-37), but the coding sequence is split into two regions by a *c*. 180 bp insertion, suggesting that the protein may not be expressed in this strain. MspA is predicted to be a 157-kDa protein with a low cysteine content (5), and exhibits 33% identity to App. A 26-amino-acid N-terminal signal peptide is predicted and an extreme C-terminal motif, typical of autotransporter proteins, is present. Search of the Pfam database, predicts the presence of IgA1 protease (residues 18-842) and autotransporter beta-barrel (residues 1169-1431) domains. A search of the Prosite database demonstrated the presence of a single RGD motif (residues 799-801) and a trypsin domain (residues 87-287). The autotransporter intramolecular chaperone (PD002475) domain is also predicted (834-964). MspA was cloned and a recombinant protein of the expected size was expressed, affinity-purified used to raise rabbit polyclonal monospecific antiserum. Western blot studies have shown that a *c*. 125-kDa fragment of MspA is secreted in meningococcal strain MC58, which is absent from Z2491. A strain survey showed that MspA is expressed by ET-5 and lineage 3 hypervirulent clones, and anti-MspA antibodies were found in patients' convalescent-phase sera. In bactericidal assays, anti-MspA serum was shown to kill the homologous strain (MC58) and another ET-5 strain (Z4664).

Conclusion: MspA is a novel, immunogenic meningococcal autotransporter protein, which is expressed in ET-5 and lineage 3 clonal groups. A *c*. 125 kDa fragment of MspA is secreted in the external milieu. Antibodies raised against recombinant MspA exhibit bactericidal activity.

The *porA* pseudogene of *Neisseria gonorrhoeae* - genetic polymorphism and inactivating mutations

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Neisseria gonorrhoeae (GC) and *Neisseria meningitidis* (MC) are genetically and morphologically similar. However, MC is the only Neisseria species known to express two outer membrane porins, PorA and PorB, whereas GC only expresses PorB. Still, a *porA* pseudogene, inactivated by mutations, in the genome of six GC strains has been identified (1).
Aims: To investigate the genetic polymorphism and identify different silencing mutations in the *porA* pseudogene of *N. gonorrhoeae* (GC).
Materials & methods: GC reference strains (n=28) and clinical isolates (n=24), comprising 21 different serovars and isolated in different geographic localities 1973-2001, were included. The entire *porA* pseudogene, including an upstream segment with the promoter region, was sequenced and compared with equivalent sequences of the previously examined six GC strains and two MC strains (1). Phylogenetic trees were constructed with Neighbor-joining method.
Results: The *porA* pseudogene was identified in all examined GC isolates. In the upstream segment containing the promoter region, 6% (12/216) of the nucleotide sites were polymorphic, resulting in 11 sequence variants (seven not previously described). In comparison with one of the MC strains, 27% of the nucleotide sites varied and all the GC isolates comprised a conserved start codon and ribosome binding site (rbs), an eight bp deletion between the transcription start and the rbs, an inactivating deletion of the first nucleotides (TA) in the MC TATAAT-box, and between the proposed -10 to -35 consensus sequence regions a variable poly T region, a deletion of a G, and a variable poly G stretch.
In the "coding sequence" of the GC *porA* pseudogene, six polymorphisms (0.5%) were identified, resulting in 11 different alleles. Three of the polymorphisms and seven of the alleles have not been previously described. In comparison with one of the MC strains, 14% of the nucleotide sites varied and all the GC isolates comprised an inactivating frameshift mutation at the same nucleotide position. A phylogenetic tree of the entire GC *porA* pseudogene (upstream segment with the promoter region and "coding sequence") identified 26 different alleles, of which 22 have not been previously described. Overall, the results were concordant with the previous study (1), however, many new alleles of the pseudogene were also found.
Discussion: The *porA* pseudogene and its in many cases identical silencing mutations are widespread in the GC population and the substantial homology with the MC *porA* gene certainly reflects their common origin. In contrast to this gene, the GC *porA* pseudogene is highly conserved most likely because the PorA protein is not expressed. Consequently, there exists no strong diversifying selection, as for the MC *porA* gene, due to immune response of the host. Thus, the GC *porA* pseudogene may reflect a molecular clock in comparison with the MC *porA* gene.
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pptA: an ORF involved in the addition of ChoP to pilin of *Neisseria meningitidis*.

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Pili of pathogenic *Neisseria* are major virulence factors associated with adhesion, cytotoxicity, twitching motility, autoaggregation and DNA transformation. Pili are important colonisation factors, playing a major role in adhesion and contributing to host specificity. Pili primarily consist of the repeating subunit pilin. Pilin of *Neisseria meningitidis* is post-translationally modified by several factors including the addition of phosphorylcholine (ChoP) and by glycosylation.

ChoP is found on the surface structures of many pathogenic and commensal organisms of the respiratory tract. In *Streptococcus pneumoniae*, *Haemophilus influenzae*, and commensal *Neisseria*, ChoP is attached to sugar moieties (teichoic acid, lipoteichoic acid, and LPS). In these organisms, ChoP is important for the colonisation of the nasopharynx and invasion of the epithelium. Expression of ChoP is often subject to phase-variation (high frequency on-off switching). The absence of ChoP is important for survival of the bacteria in the blood. In pathogenic *Neisseria* ChoP is covalently-linked to the pilus subunit protein, pilin. Prior to this study, no genes involved in either the biosynthesis or the transfer of ChoP in pathogenic *Neisseria* had been identified.

Several ORFs homologous to genes involved in the biosynthesis or attachment of ChoP and similar compounds were insertionally inactivated in *N. meningitidis* strain C311#3. Western immunoblots of pilin with the ChoP specific mAb TEPC-15 showed that the ORF NMB0415, now defined as *pptA* (Pilin Phosphorylcholine Transferase A), is involved in the addition of ChoP to pilin in *N. meningitidis*. In *N. meningitidis pptA* contains a homopolymeric guanosine repeat, sequencing of this repeat from previously isolated ChoP phase-variants showed that the loss of ChoP in these phase-variants correlated with changes to the length of this repeat. These changes to the repeat resulted in a frame-shift in *pptA* and thus its premature termination. The variation in ChoP presence is possibly important in the disease process. Therefore, the variation of ChoP accessibility was analysed using the mAb TEPC-15 with native pili, by colony immunoblots, and denatured pilin, by western immunoblots. These immunoblots showed that ChoP is surface exposed and accessibility to the mAb is variable dependent on other pilin modifications. Current investigations are focusing on the importance of this variation in host interactions.

Characterisation of Meningococcal Outer Membranes by Two-Dimensional Gel Electrophoresis and Mass Spectrophotometry.

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Serogroup B meningococci account for >50% of meningococcal infections in the US and many European countries including the UK. In contrast to the decline in the number of serogroup C infections following introduction of the serogroup C conjugate vaccine into the UK routine immunisation schedule, the number of reported cases of confirmed serogroup B infection has increased. Existing serogroup B vaccines based on outer membrane proteins (OMPs) are immunogenic, but heterogeneous and the identity of protection is not entirely known. The aim of this study is to identify the proteins in the outer membrane and in particular immunogenic proteins recognised by human antibodies.

The recent sequencing of the meningococcal serogroup B strain, MC58 (Tettelin *et al*., 2000) has facilitated the proteomic analysis of this organism. In the current study, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used to separate MC58 membrane proteins on the basis of differences in isoelectric point (p*I*) in the first dimension and molecular weight in the second dimension. All the separated proteins were excised, digested and then subjected to MALDI-ToF (matrix-assisted laser desorption-ionisation time-of-flight) mass spectrometry. The identity of proteins was established by comparison of mass spectrometry data with the MC58 genomic sequence database and proteins not characterised by MALDI-ToF were further analysed with MS/MS.

High quality 2-D gels of membrane preparations were achieved with the optimal combination of detergents prior to the first dimension separation. Circa 130 resolved protein spots could be detected on each 2-D gel. Initial peptide mass data led to the identification of 28 proteins by MALDI-ToF MS. One protein (4%) was identified as an outer membrane protein in the MC58 database and a further 32% (n = 9) proteins were identified as cytosolic proteins. For the majority of identified gene products (64%, n = 18) no annotation existed in the MC58 database concerning their subcellular location. Additional proteins are being identified by MS-MS.

In conclusion, this project has generated a 2-D map of the meningococcal outer membrane proteins. The results show that OM protein preparations contain both cytosolic proteins and other proteins whose subcellular location is yet to be identified. A significant percentage of the non-annotated proteins may be membrane proteins. 2-D immunoblotting studies are underway to determine the immunogenicity of these proteins in humans. The finding of new immunogenic proteins in outer membrane preparations will have consequences for vaccine design.

LC-MS–Analysis of the Outer Membrane Composition of a Lipopolysaccharide-Deficient *Neisseria meningitidis* Mutant and a Deoxycholate Treated Wild Strain

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Serogroup B vaccines based on outer membrane (OM) proteins are under development. The wild-type meningococcal OM contains lipopolysaccharide (LPS) which is toxic and therefore must be removed from OM preparations by sodium deoxycholate treatment prior to incorporation into a vaccine. This step could be avoided by using the OM of an LPS negative mutant strain, however the lack of LPS might affect the membrane protein composition of the mutant strain. In addition the effect of deoxycholate treatment of the wild OM preparation on the protein content of OMV vaccines is unknown.

Sequencing of the meningococcal serogroup B strain, MC58 (Tettelin *et al*., 2000) together with improvements in proteomic techniques have facilitated studies of *N. meningitidis*. Proteome analyses are usually carried out using two-dimensional electrophoresis followed by mass spectrometric protein identification. Although 2-D gels are able to resolve complex protein mixtures, low abundance proteins are difficult to detect. In addition, a major limitation of the technique is the poor solubility of membrane proteins, therefore membrane proteins are under-represented on 2D gels. An alternative approach involves 1-D electrophoretic separation of proteins followed by LC-MS on a nano-LC system coupled to an ESI ion trap using the MS-MS mode. The latter technology was applied to the comprehensive characterisation of OM preparations of the MC58 strain and an LPS-deficient mutant. In addition, the effect of sodium deoxycholate treatment on the protein composition of OMV vaccines was examined. Peptide mass fingerprints generated by LC-MS were matched to the experimentally derived masses of peptides calculated for MC58 meningococcal proteins and the subcellular locations of the proteins were then obtained from the MC58 database.

These results show that mass spectrophotometry coupled to liquid chromotography is particularly suited to catalogueing the complex mixture of hydrophobic proteins found in the meningococcal outer membrane. Specifically, this study compares the protein content of outer membrane preparations of the wild-type MC58, OMV vaccines and the LPS-negative mutant. A total of 263 different proteins were identified in mass spectrophotometry results of all three outer membrane preparations. Each membrane preparation contained circa 10% membrane proteins, 30% cytosolic proteins and 10% periplasmic proteins. The cellular location of >50% of proteins were not annotated in the MC58 database and some of these may prove to be membrane proteins.

Comparison of the protein profiles of the three OM preparations provides important information on the protein content of putative vaccine preparations.

Evaluating the virulence of meningococcal LOS mutants in a new mouse model of invasive meningococcal infection.

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Introduction. Epidemiological data support a strong association between the incidence of influenza A virus outbreaks and the subsequent increase in the incidence of meningococcal diseases and other bacterial infections. We used this epidemiological association to develop a mouse model of sequential influenza A virus (IAV)-*Neisseria meningitidis* (Nm) infection in BALB/c mice. In this study we evaluated the virulence of lipooligosaccharide (LOS) *lpxA* and *rfaD* mutants.

Methods. Five-week-old female BALB/c mice were first infected intranasally with a sublethal dose of IAV and then superinfected by intranasal challenge with a serogroup C Nm or its respective isogenic mutants for the capsule or LOS. Bacterial colonization of pulmonary tissues and bacteraemia were followed by bacterial counts in the lung and blood, respectively. The Th1- and Th2-type cytokine responses after IAV infection and after Nm superinfection were tested by ELISA

Results. Fatal meningococcal pneumonia and bacteremia were observed in IAV-infected mice superinfected with Nm on day 7, but not in those superinfected on day 10. The transient phase of susceptibility of mice to Nm superinfection correlated with the peak IFN-gamma production in the lungs and recovery from IAV previous infection. After Nm challenge, both IAV-infected and uninfected control mice produced the inflammatory cytokines IL-1 and IL-6. However, IL-10 was only detected in susceptible mice superinfected wih Nm on day 7 after IAV infection, but not in resistant mice. As expected, the capsule-defective isogenic mutant of serogroup C Nm was rapidly cleared from the lungs and was unable to induce bacteremia. Both *rfaD* and *lpxA* LOS mutants were affected in their capacity to persist in the lung and the *lpxA* mutant was unable to invade and/or persist in blood.

Conclusions. We demonstrated that mild IAV infection might transiently modify innate immune defenses in the respiratory tract, rendering the host susceptible to pulmonary superinfection by serogroup C Nm. This experimental approach of dual IAV-Nm infection allowed us to evaluate the role of major bacterial virulence factors such as the capsule and LOS. Therefore, this model of meningococcal disease in adult mice reproduces the pathogenesis of human meningococcemia with fatal sepsis and is useful for analyzing known or putative genes involved in meningococcal pathogenesis.

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IPNC2004
Poster Abstract
Addendum

The following posters were omitted from the abstract book but are presented in this Addendum. All of these posters will be presented during Poster Session II on Thursday September 9.

Epidemiology

Gray: Phenotypic and genotypic trends observed amongst invasive serogroup B meningococcal disease isolates (1987 – 2003) in England and Wales. (SII–117)
Harrison: Distribution of transferrin binding protein B (*tbpB*) gene isotypes in the *Neisseriaceae* family (SII-118)
Kriz: Epidemiology of invasive meningococcal disease and vaccination strategy in the Czech Republic (SII-119)

Vaccinology

MacKenzie: Immunomodulatory Effects of Meningococcal Outer Membrane Vesicles (SII-120)

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Folster: Regulation of *mtrF* expression and its role in high-level hydrophobic antimicrobial resistance in *Neisseria gonorrhoeae* (SII-114)(

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Brooks: The neisserial Opa protein adhesins: Conservation of CEACAM receptor binding in the context of sequence hypervariability (SII-113)

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Lee: IHF is required for FarR repression of the *farAB*-encoded efflux pump of *Neisseria gonorrhoeae* (SII-115)
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Phenotypic and genotypic trends observed amongst invasive serogroup B meningococcal disease isolates (1987 – 2003) in England and Wales.

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The Health Protection Agency (HPA) Meningococcal Reference Unit (MRU) for England and Wales has a database of all isolates received from invasive meningococcal disease in England and Wales since 1984 that includes phenotypic characterization (by the serological reagents available at the time of receipt). From 1987 meningococci have been characterized to serogroup, sero-type and sero-subtype.

Analysis of the predominant and selected serogroup B phenotypes by year has enabled phenotypic trends to be observed and depicted graphically. Observations suggest that there may be periodicity or cycles of antigen expression exhibited by the meningococcal population possibly under host immune-selective pressures.

Recently sequencing information (porA and MLST) enabled genetically related but antigenically distinct phenotypes to be considered as hyper invasive lineages defined by clonal complexes (CCs). Using clonal complex and phenotype associations it is possible to describe trends and changes in the meningococcal population causing disease in England and Wales.

The CC ST-32 (ET-5 MEE designation) meningococci represented by phenotypically B:15:P1.7,16 and B:15:P1.16 have declined from 20% to 2% of serogroup B case isolates during 1987-2003. Whereas CC ST-41/44 (lineage 3) meningococci represented by B:NT:P1.4 and B:4:P1.4 accounted for 11% in 1991, rose to 30% in 1998 but declined to 23% in 2003. CC ST-8 (cluster A4) represented by B:2b meningococci rose from 18% in 1988 to 22% in 1992 and have since declined 1% in 2003. A rise in CC ST-269 represented by phenotypes B:NT:P1.15, B:NT:P1.19,15, B:NT:P1.19, B:NT:P1.5,2 and B:NT:P1.9 from 17% in 1993 to 26% in 2003. Where the B:NT:P1.9 component rose from 1.5% to 11% over the same period. ST-213 represented by B:1:P1.14 has increased from 0.1% in 1991 to 5.5% in 2003.

Continued surveillance of invasive meningococcal isolates by sequence typing methods and retrospective studies on archived material will allow greater insight into the epidemiology and changing hyper-invasive lineages and the relationship between variable surface antigens (such as porA) and the host populations. This will be extremely important if approaches to serogroup B vaccine design and implementation include surface antigen components the duration of success of which may be dependent on their rate of change.

Distribution of transferrin binding protein B (*tbpB*) gene isotypes in the *Neisseriaceae* family.

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Background: Transferrin protein B (*tbpB*), a surface exposed lipoprotein, is required by meningococci for the acquisition of iron from human transferrin. The importance of *tbpB* for neisserial virulence together with its *in vivo* expression and the fact that it was a surface exposed antigen made it an attractive vaccine candidate (1). However, its subsequent heterogeneity, discovered amongst meningococci, made it difficult to envisage a vaccine capable of conferring protection against all meningococcal strains. Subsequently, two types of *tbpB* gene families were found amongst meningococci: an isotype I *tbpB* gene of 1.8 kb and a larger isotype II *tbpB* gene of 2.1 kb, the former harboured by clonal complexes ET-5 (ST-32), Lineage III (ST-41) and Cluster A4 (ST-8), the latter exclusively found in meningococci belonging to the ET-37 (ST-11) clonal complex (5). The heterogeneity of the *tbpB* genes is a consequence of horizontal genetic exchange resulting in the generation of a mosaic gene (2). Recombinational events are known to occur frequently, particularly in genes present on the surface membrane due to immune selective pressure (3), and this is clearly facilitated by the fact that many neisserial species co-exist in the same environmental niche thereby providing an abundance of genetic material with which to recombine and transform (4). Accordingly, for future vaccine research, it may be valuable to investigate the distribution of potential vaccine candidates amongst both commensal and pathogenic *Neisseria*. Thus, the aim of this study was to track the distribution of the *tbpB* gene amongst all non-pathogenic *Neisseria* whilst drawing particular attention to the smaller isotype I *tbpB* gene, which has been solely harboured by ET-37 (ST-11) complex meningococci. This would enable us to gain an insight into the evolution and origins of this gene and would provide a model with which to investigate future neisserial genes.
Methods: A collection of non-pathogenic *Neisseria* was gathered from varied geographical areas including Europe, the Middle East, and North America. These strains were screened for the *tbpB* gene by PCR and Southern Blot. Subsequent PCR fragments were sequenced and aligned from which phylogenetic analysis was undertaken.
Results: Three groups of *tbpB* genes could be deduced based on sequence alignments and phylogenetic analysis: Group 1 *tbpB* genes belonging to the diverse commensal *Neisseria* sp. harbouring an isotype I *tbpB* gene, Group 2 *tbpB* sequences belonging to the reference isotype I *tbpB* of B16B6, several *N. polysaccharea* strains along with 2 *N. lactamica* strains, and finally Group 3 the isotype II *tbpB* genes belonging to the reference Nm strain M982 and *N. lactamica* strains. In conclusion, this study demonstrates that the origin of the isotype I *tbpB* gene may be from commensal *Neisseria*.

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Epidemiology of invasive meningococcal disease and vaccination strategy in the Czech Republic

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Introduction: An emergency epidemiological situation of invasive meningococcal disease (IMD) started in the Czech Republic in 1993, when a clone, *N. meningitidis* C, ET-15/37, ST-11, occurred and caused an increased IMD morbidity and case fatality rate. Here, the epidemiological situation and vaccination strategy in the country is presented.
Methods: Epidemiological data came from passive notification since 1943 and from active surveillance of IMD since 1993. Strains isolated from IMD in the clinical microbiology laboratories country-wide were sent to the National Reference Laboratory (NRL) for Meningococcal Infections in Prague for further investigation by classical and molecular methods. Multilocus sequence typing (MLST) was performed according to the method described on the MLST website. A PCR method was implemented into routine use in the NRL and the active surveillance of IMD was improved by MLST performed directly from clinical material, introduced in 2001.
Results:
Long-term trends (since 1970) show that the morbidity caused by *N. meningitidis* B has been stable over more than 30 years, while the morbidity caused by serogroup C had an increasing trend similar to total morbidity. After the prevalence of serogroup C (ET-15/37, ST-11) in the period 1994-1999, serogroup B was found to be prevalent in the period 2000-2002. The serogroup B strains belonged to the hypervirulent complexes (ET-5/ST-32 complex and ST-18 complex) and the case fatality rate remained at a high level. Serogroup C morbidity started to increase after 2001 again and an equal proportion of serogroup B and C was found in 2003. The morbidity in the 15-19 years age group caused by serogroup C has had an increasing trend since 2001 (2.2 per 100 000 in 2001, 1.7 in 2002 and 2.7 in 2003). A strategy of targeted vaccination of parts of the population at highest risk has been adopted since 1993. The vaccination strategy was updated in 2004 and the vaccination of the 15-19 years age group with a meningococcal conjugated C vaccine was recommended. Mass vaccination is not planned in the current epidemiological situation.
Conclusions: Age specific morbidity of 15-19 year olds caused by serogroup C has increased in the Czech Republic in recent years. The vaccination of the 15-19 years age group with a meningococcal conjugated C vaccine was recommended. **Acknowledgement:** This study was supported by research grants NI/7109-3 and NJ/7458-3 of the Internal Grant Agency of Ministry of Health of the Czech Republic, by EU project no. QLK2-CT-2001-01436 and made use of the Multi Locus Sequence Typing website (http://neisseria.mlst.net) sited at the University of Oxford and funded by the Wellcome Trust and European Union. We thank Dr. K. Jolley (University of Oxford, UK) for kind editing of the text.

Immunomodulatory Effects of Meningococcal Outer Membrane Vesicles

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Introduction: During the growth of *Neisseria meningitidis* (Nm) in vitro and in vivo, natural outer membrane vesicles (NOMVs) are released from the cell surface. NOMVs are highly representative of the meningococcal outer membrane, containing a high proportion of lipopolysaccharide (LPS) and outer membrane proteins (OMPs). Our previous studies have demonstrated that NOMVs have inherent adjuvant properties enhancing local, nasal associated lymphoid tissue, and systemic antibody responses toward intranasally administered NOMV proteins and LPS. Intranasal administration of NOMVs with inactivated respiratory syncytial virus resulted in the protection of mice against viral challenge in a mouse model. The aim of this study is to investigate the immunomodulatory effects of NOMVs and their components.

Methods: NOMVs prepared from Mut-4 Nm strain 44/76 and cell lysate of a LPS-deficient, but non-OMV producing mutant ($lpxA^-$), were fractionated using preparative SDS-PAGE. The fractions were assayed, either individually or in pools according to molecular weights, for their ability to stimulate maturation and/or cytokine production from human U937 monocyte-derived macrophages (MDMs), murine bone-marrow derived dendritic cells (BMDCs) and/or macrophages (BMDMs) from C3H/HeN, C3H/HeJ, Toll-like receptor 2 (TLR2) and TLR4 knockout mice. Cytokine levels were analysed by ELISA and cytometric bead array kits and surface expression of markers on BMDCs were examined by FACS. Proliferation of murine splenocytes by NOMV fractions was assessed by thymidine incorporation.

Results: Our findings show that many of the fractions stimulated U937 MDMs and BMDCs to produce high levels of TNF-alpha and IL-10. Stimulated BMDCs also produced high levels of IL-6. Surprisingly none or very little IL-12p70 was induced, despite the presence of LPS in NOMVs. The contribution of LPS to the immunomodulatory effects was investigated using the $lpxA^-$ isogenic mutant and LPS-hyporesponsive mice. As with NOMV fractions, $lpxA^-$ fractions also produced TNF-alpha and IL-10, but no IL-12p70. Pooled fractions from NOMVs were also able to induce splenocyte proliferation and upregulate cell surface markers such as MHC II, CD40, CD80 and CD86 on BMDCs. Interestingly, production of nitric oxide (NO) and TNF-alpha was abrogated only when BMDCs and BMDMs from TLR2 and TLR4 double knockout mice were used. Stimulated cells from TLR2 or TLR4 knockout mice had similar or slightly lower levels as the treated controls.

Conclusions: Our results demonstrated that the immunomodulatory effects of NOMVs are not wholly dependent on LPS. TLR2 signalling is sufficient to induce maturation of and cytokine production by BMDCs and BMDMs. Studies into the mechanisms responsible for the NOMVs' immunomodulatory effects in vivo are now in progress.

Regulation of *mtrF* expression and its role in high-level hydrophobic antimicrobial resistance in *Neisseria gonorrhoeae*.

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Neisseria gonorrhoeae has the ability to colonize and infect numerous sites within the human host, suggesting that it possesses mechanisms to resist diverse antimicrobial agents, which bathe these mucosal surfaces. One of these mechanisms is the expression of the *mtr*-encoded efflux pump, which mediates the resistance of gonococci to structurally diverse hydrophobic agents (HAs). HAs include antibiotics, nonionic detergents, bile salts, hormones, and certain antimicrobial peptides. Negative regulation of the operon encoding the main proteins (MtrC, MtrD, and MtrE) of the pump is mediated by MtrR, a transcriptional repressor. Mutations which disrupt the function or expression of *mtrR* result in de-repression of the efflux pump operon and high-level HA resistance. Previously, mutations in a gene encoding an accessory factor to the pump, MtrF, were found to suppress mutations in *mtrR*. Utilizing a translational *lacZ* fusion system, inactivation of *mtrF* did not effect the expression of *mtrCDE* in a strain lacking MtrR. However, we found that HA resistance was significantly reduced in this genetic derivative. High-level HA resistance can also be mediated via an MtrR-independent mechanism through an induction process that requires enhanced transcription of *mtrCDE* when MtrR+ gonococci are grown in the presence of a sub-lethal concentration of TX-100. We obtained similar results for the role of MtrF on TX-100 induction of resistance. We found that insertional-inactivation of *mtrF* had no effect on the induction of *mtrCDE* expression. However, HA resistance was significantly reduced in an MtrF-negative strain. The regulation of *mtrF* was studied so as to learn if its expression is coupled to the regulatory processes that modulate *mtrCDE* expression. Like *mtrCDE*, the expression of *mtrF* was inducible by sub-lethal concentrations of TX-100 and was enhanced in an MtrR-negative strain. However, while MtrR is known to repress *mtrCDE* expression by specific binding to the *mtrCDE* promoter, specific binding of MtrR to the promoter region of *mtrF* could not be detected. This suggests that MtrR- regulation of *mtrF* is by an indirect process. We have identified an additional transcriptional regulator of *mtrF*, which we term MafR. Expression of *mtrF* increased upon inactivation of *mafR*, suggesting that *mafR* encodes a repressor of *mtrF* expression. MafR had no effect on *mtrCDE* expression. Repression of *mtrF* by MtrR and MafR was additive, demonstrating that these repressive effects were independent. These results show that MtrF plays an important role of the activity of the *mtr*-encoded efflux pump and demonstrates both similarities and differences in the regulation of expression of *mtrF* and *mtrCDE*.

The neisserial Opa protein adhesins: Conservation of CEACAM receptor binding in the context of sequence hypervariability.

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The neisserial colony opacity-associated (Opa) outer membrane proteins mediate intimate attachment and penetration into a variety of different human cell types. A single bacterium possesses up to 11 different *opa* alleles that each typically encode antigenically distinct proteins. The antigenic variability is based on the presence of one semi-variable and two hypervariable sequences that are predicted to be exposed at the bacterial surfaces. However, in the context of this sequence hypervariability Opa proteins display remarkable functional conservation: 10 of 11 Opa proteins of *N. gonorrhoeae* MS11 specifically bind one or more receptors of the carcinoembryonic-related cellular adhesion molecules (CEACAMs). Because of this, tissue tropism and the host cellular response depend upon both Opa variant expressed and the CEACAM expression pattern of target cell. With the goal of understanding this conservation of binding function in the context of sequence hypervariability, we have employed a phage display-based approach to isolate regions of Opa that are sufficient to mediate binding to CEACAM family receptors. Opa-derived sequences were fused to the M13 phage pVIII coat protein, and these were panned over CEACAM receptors and CEACAM-expressing cells. Our results indicate that peptides derived from hypervariable region 2 are sufficient to mediate binding to CEACAM5. This information, along with bioinformatic and computational sequences analyses have been employed to guide mutagenesis-based study of this region to reveal residues that correspond to the Opa proteins' binding function.

IHF is required for FarR repression of the *farAB*-encoded efflux pump of *Neisseria gonorrhoeae.*

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Introduction: The *farAB* operon encodes an efflux pump that confers resistance to antibacterial fatty acids (FA) in gonococci. This operon is negatively regulated by FarR, a member of the MarR family of transcriptional repressors. FarR binds to three sites of the upstream sequence of *farA*. Promoter deletion studies indicated that FarR-binding site III (-80 to –116 relative to the transcription start point) plays a predominant role in repression of the *farAB* operon. However, the repression of *farAB* due to FarR binding that occurred at site III was not completely reversed by the loss of FarR. Therefore, we asked whether another regulatory protein was required for this repression.

Materials and Methods: We used the techniques of polymerase chain reaction (PCR), gene cloning, site directed mutagenesis, DNA sequencing, *farAB-lacZ* fusions, electrophoretic mobility shift assay (EMSA), DNase I-foot printing analysis, and a DNA bending assay to conduct this study.

Results and Discussion: Through the analysis of the upstream sequence of *farA*, five binding consensus sequences of the *E. coli* Integration Host Factor (IHF), a histone-like DNA bending protein, were identified. Using an *E. coli* IHF-negative mutant and its IHF-positive parental strain, we observed that IHF was involved in repression of the *farAB* operon. The results from EMSA and DNase I foot printing assays with purified gonococcal IHF indicated that it binds to two sites upstream of *farA*; -21 through-54 on the sense strand and -44 to -77 on the antisense strand. Interestingly, these IHF-binding sites are located between FarR-binding site I and III. Furthermore, site-directed mutagenesis and DNA bending analysis results demonstrated that IHF-binding to these sites induces bending of the *farAB* promoter. The IHF-binding domain II (-35 to -47) is especially important for the bending of the *farAB* promoter and full repression of the *farAB* operon.

Conclusion: Our results demonstrate that IHF is required for the full repression of the *farAB* operon and that IHF binding to the sequence, located between FarR binding site I and III, introduces bending of the *farAB* upstream sequence. Therefore, we propose a model in which the DNA bending induced by IHF enables the simultaneous binding of FarR to both site I and III, which results in stable repression of the *farAB* operon.

Modulation of the *mtrCDE*-encoded efflux pump gene complex of *Neisseria meningitidis* due to a Correia Element insertion sequence

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The *mtr* (*m*ultiple *t*ransferable *r*esistance) gene complex in *Neisseria gonorrhoeae* encodes an energy-dependent efflux pump system that is responsible for export of antibacterial hydrophobic agents. Expression of the *mtrCDE* operon in gonococci is negatively regulated by the MtrR protein. Hydrophobic agent-resistance mediated by the *mtr* system is also inducible, which is due to an AraC-like protein termed MtrA. In this work, we identified and characterized a pump similar to the gonococcal *mtr* system in various strains of *Neisseria meningitidis*. Unlike the situation with gonococci, the *mtr* system in meningococci is not subject to the MtrR or MtrA regulatory schemes. An analysis of the promoter region of the *mtrCDE* operon in a panel of meningococcal strains revealed the presence of one or two classes of insertion sequence elements. A 155-159 bp insertion sequence element known as the Correia Element, previously identified elsewhere in the gonococcal and meningococcal genomes, was present in the *mtrCDE* promoter region of all meningococcal strains tested. In addition to the Correia element, a minority of strains had a tandemly-linked, intact copy of IS*1301*. As described previously (Buisine *et al*., 2002), a binding site for the Integration Host Factor (IHF) was present at the center of the Correia element upstream of *mtrCDE* genes. IHF was found to bind specifically to this site and deletion of the IHF-binding site enhanced *mtrC* transcription. We also identified a post-transcriptional positive regulation of the *mtrCDE* transcript by cleavage in the inverted repeat of the Correia element, as previously described by Mazzone *et al*. (2001) and De Gregorio *et al*. (2002) for other Correia elements. We conclude that the *mtr* efflux system in meningococci is subject to negative transcriptional regulation by IHF and positive post-transcriptional regulation by cleavage in the inverted repeat of the Correia element.