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The Tenth International Pathogenic Neisseria Conference was held in Baltimore, Maryland, September 8-13, 1996. This major international conference was attended by 400 scientists and public health officials from over 30 countries. The meeting featured both oral and poster presentations. Over 200 posters were presented. Poster breakout sessions were moderated by preselected participants. "Meet the Professor" talks focused on the clinical aspects of gonococcal and meningococcal infection. The goal was to introduce the real world problems of these infections to young and "new" investigators to the field. These talks were very well attended and generated a great deal of enthusiasm.The conference supported the travel of approximately 30 young investigators. This was made possible by a combination of government grants and corporate contributions, all of which were acknowledged at the meeting. In summary, the public health impact in both developed and developing countries is still significant. A goal of this conference was to focus discussion on how basic and applied research are contributing to diagnosis, treatment, and prevention. The organizers feel that this goal was met. Assembling scientists from so many countries fostered international discussions and potential collaborations which will hopefully lead to the control of Neisserial infections worldwide.

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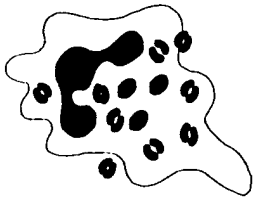
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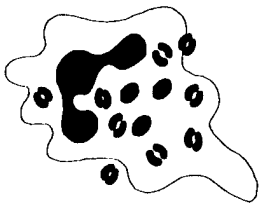
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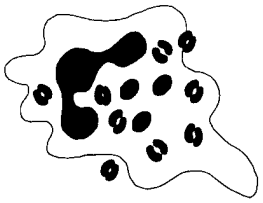
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of the
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The Tenth International Pathogenic Neisseria Conference

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Preface

The emphasis of the Tenth International Pathogenic Neisseria Conference is the public health impact of the pathogenic *Neisseria* in both developed and developing countries, and how basic and applied research are contributing to the diagnosis, treatment, and prevention of diseases caused by these bacteria. This is the major international conference in the field attracting over 300 scientists and public health officials from over 30 countries. The first conference in the series was held in San Francisco in 1978, and dealt primarily with *Neisseria gonorrhoeae*. The conferences have been held every two years since then, alternating between Europe and North America, and have resulted in the fruitful interaction of physicians, basic scientists, public health officers, and epidemiologists. The ninth conference was held in September 1994 in Winchester, England, and over 320 individuals attended.

A goal of these conferences has been to bring together investigators, from universities, governments and the biologics industry, who are involved with or are interested in *N. gonorrhoeae* and *N. meningitidis* and the diseases they cause. Over two million cases of gonococcal disease occur in the United States and Europe each year. Pelvic inflammatory disease in women exceeded $5 billion in health care costs in the U.S. in 1993 and is the leading cause of ectopic pregnancy and tubal infertility in much of Africa. *N. meningitidis* causes outbreaks or epidemics in many countries causing major problems within the public health system. It is therefore of major importance to understand the mechanisms by which these pathogenic *Neisseria* are able to invade and evade the host immune system. These conferences have resulted in dissemination of important new findings concerning the pathogenic mechanisms and epidemiological surveillance of these organisms, and will provide the information to improve control through immunization. Furthermore, developments in our understanding of how the pathogenic *Neisseria* express, through up-regulation, an array of new surface proteins for acquisition of iron from human iron binding proteins, and for *in vivo* survival has contributed greatly to our understanding of other invasive bacterial pathogens.

These conferences have clearly demonstrated the application of molecular biology and immunology to the analysis of gonococcal and meningococcal physiology, structure, pathogenicity and epidemiology, and have presented new developments in vaccines against gonococcal and meningococcal disease. The Tenth International Pathogenic Neisseria Conference extends these observations and provides the latest information about studies now in progress to control and prevent the morbidity and mortality associated with these organisms. Lectures and posters were submitted in nine broad areas, including over 40 oral presentations and 220 posters.

Carl E. Frasch Ph.D.
Chairman,
Conference Organizing Committee

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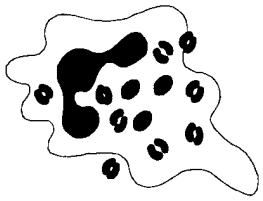
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Gonococcal Infection, Immunity, and Resistance

Is there protective immunity to gonococcal disease?

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Few data exist that clearly implicate acquisition of protective immunity from a previous gonococcal infection(s). Although a quantitative failure of the local immune response may explain this, a more likely explanation may lie in the qualitative response to gonococcal infection that fails to result in protective immunity against future incursions. Three reports suggest the possibility that a partially protective immune response may result from infection. A fourth suggests that, in some cases, the immune status of the host may actually be permissive for infection.

In 1946 Mahoney et al. (1) showed that only 26 of 108 male volunteers with a prior history of urethritis developed disease in response to intraurethral inoculation of gonococci, in contrast to 56 of 131 men without an antecedent history of urethritis. Buchanan et al. (2) showed that gonococcal pelvic inflammatory disease (PID) may produce some protective immunity to repeated episodes of salpingitis with gonococci that possess the same protein 1 serotype, but not to localized mucosal reinfections. In a study reported in 1989 (3), 227 prostitutes in Nairobi were followed at 2 week intervals for a 15-month period beginning in March 1985. Women previously infected with serovar-defined gonococci (except serovar 1B-1) were at 2- to 10-fold reduced risk of re-infection with the same serovar. Although protection was serovar specific, it was incomplete. Using this same population, Plummer et al (4) have also shown that serum antibody directed against reduction modifiable protein (Rmp) is associated with enhanced likelihood of re-infection with *N. gonorrhoeae*. Rmp antibody, previously has been shown to block the effect of bactericidal antibodies that are directed against porin (Por) protein and lipooligosaccharide (LOS) antigens (5).

To clarify the role of Rmp antibody in facilitating acquisition of gonorrhea, antibody concentrations to gonococcal Rmp were measured in serum samples of fifty-seven women who were the only sexual contacts of men with gonococcal urethritis. Antibody levels in infected patients were normalized for the influence of acute infection. In the group of women who became infected, the mean level of antibody (IgG) to Rmp protein at the time of exposure was approximately three-fold higher than that of exposed patients who did not acquire infection. A significant association between Rmp antibody level and transmission ($p<0.01$) was shown to be independent of the number of sexual exposures to the infected male partners (6).

Effective vaccination against gonococcal infection has been an elusive goal. Until host mechanisms that might protect against infection are better characterized and understood,

an effective vaccine that targets the genital tract for protection may continue to be out of reach. Recent evidence suggests that mucosal immune responses particularly local (eg vaginal) IgG antibody levels may mirror those measured serum (7). Therefore, a consideration of data available on serum based immune mechanisms may shed light on protection against mucosal pathogens such as *N. gonorrhoeae*. In particular, efficacy of most vaccines, targeted to prevent infections acquired by the mucosal route, have shown a correlation between efficacy and levels of IgG antibody in serum that result from vaccination (8).

A successful vaccine candidate against gonorrhea may require one or more of the following elements: (a) generation of serum or mucosal antibodies that either facilitate complement-mediated killing of the organism, and/or enhance phagocytosis and microbial killing by polymorphonuclear leukocytes; (b) ability to stimulate an immune response that blocks the attachment of *N. gonorrhoeae* to host tissues; and (c) evocation of cell-mediated defenses that prevent infection or modify fallopian tube damage (9). With the intent of satisfying one or more of these criteria, at least two gonococcal vaccine candidates have been tested in humans over the past 20 years (10,11). *N. gonorrhoeae* are obligate human pathogens, and therefore the most relevant testing of vaccine candidates can only be performed in humans. Nevertheless, certain useful predictions have been gleaned using animal models of infection. The chimpanzee model first developed by Lucas et al. (12) mimics uncomplicated gonococcal infection in man with respect to incubation period, certain clinical manifestations, sexual transmission, local cellular response in the exudate, *in vitro* culture characteristics of the infected material, and systemic immune responses. Arko et al. (13) inoculated male chimpanzees parenterally with a formalinized whole cell vaccine, and showed strain-related resistance to gonococcal challenge. Buchanan and Arko (14) used purified outer membranes, (prepared from the same strains used in the chimpanzees noted above) to immunize guinea pigs, and again showed strain-specific immunity in a chamber model. The use of animals to assess immunologic responses to vaccines may be particularly useful to elicit immune responses that involve antibody mediated complement dependent killing and opsonophagocytosis, because these may be shared with their human counterpart. One such approach has been taken in the assessment of LOS as a recent vaccine candidate.

Antibody against LOS has been shown to have several important functions: complement activation and bactericidal activity (15,16) and opsonic activity (17,18). Although these properties make LOS an excellent candidate vaccine antigen, considerable LOS heterogeneity is displayed by gonococci *in vivo* (19,20). Certain other limitations preclude the use of LOS as a vaccine antigen. First, the toxicity of the lipid A moiety of LOS limits its potential use as a vaccine immunogen. Second, purification of oligosaccharide (OS) from LOS may modify its antigenicity (21) and may result in a T-cell independent saccharide antigen that may be poorly immunogenic (22, 23).

Alternative strategies to the use of pure saccharide vaccines may include conjugation to a protein carrier and production of anti-idiotope monoclonal antibodies (Mabs) that may act as functional "molecular mimics". An anti-idiotope monoclonal antibody (Mab), called CA1 (Ab2), has been produced in mice against Mab 2C7, which recognizes a

widely *in vivo* expressed gonococcal lipooligosaccharide (LOS) epitope (24). Mice immunized with MAb CA1 initially had a 2.5-fold increase in IgG (12-fold after a booster), but no increase in IgM, anti-LOS (Ab1') antibody. Control mice immunized with LOS had a 4.5-fold rise in IgG and a 4-fold rise in IgM anti-LOS antibody. In rabbits, Mab CA1 elicited a 9-fold rise in IgG and a 3.3-fold rise in IgM anti-LOS (Ab1') antibody. Bactericidal activity of Ab1' antibody was one to two orders of magnitude greater than that resulting from immunization with LOS. Ab1' mediated complete human polymorphonuclear leukocyte (PMNL) phagocytosis of 2C7 epitope positive (but not 2C7 epitope negative) gonococci. Mab CA1 acts as a molecular surrogate (Ab2β) for the nominal LOS antigen and suggests a promising vaccine candidate for human immunization.

Soon after gonococcal pili were first described in 1971 (25), Buchanan et al. (26) showed that infected patients made antibodies against purified pili and Tramont et al. (27) showed that antibody generated from vaccination of volunteers with a gonococcal pilus could block the attachment of gonococci to human epithelial cells *in vitro*. In a small trial, Brinton et al (28) reported that a parenteral gonococcal pilus vaccine was protective in a human challenge model when the homologous strain was used as the challenge organism. A large randomized, placebo-controlled, double-blind efficacy trial of a purified gonococcal pilus vaccine composed of a single pilus type was tested in 3,250 volunteers (US military personnel stationed in the Republic of South Korea) (10). Vaccinees developed a sustained ELISA antibody response to homologous and heterologous pili, but the titers against heterologous antigen were only 40% as high as the homologous titers. Local antibodies measured from semen were also seen against homologous and heterologous pili. However, there were no increases in antibody titers that inhibited gonococcal attachment, *in vitro*, and this vaccine failed to protect men against gonococcal urethritis in an open field trial. In designing this or a subsequent vaccine trial (see below), a graded risk of acquiring gonorrhea was not considered in study participants, and all enrollees were considered equal with respect to their *a priori* susceptibility to gonorrhea. Although pre-vaccination serum antibody (IgG and IgA) levels against gonococcal pili initially were low in the military personnel, failure to consider antibody status against other common cross-reacting antigens may have undermined the effect of the vaccine. A placebo/control trial using human challenge might have facilitated this analysis.

In the most recent American trial that took place in 1985 (11), a placebo/control, human challenge trial was performed. Sixty-three male volunteers either were immunized with a vaccine prepared from the outer membranes of a single strain of *N. gonorrhoeae*, or were given a placebo. These men were challenged intraurethrally with viable organisms 2-4 weeks after completing the vaccination course. No significant difference in infection after challenge was observed in the two groups, but resistance to infection was high: 49% in vaccinees and 32% in placebo recipients. The goal for the outer membrane derived vaccine preparation was enrichment for the Por protein. The proposed mechanism of protection, had it occurred, was to generate complement fixing antibodies directed against Por that were directly bactericidal (and perhaps opsonophagocytic) to gonococci (29) in the urethra. Methods for preparing pure Por were not totally reliable at that time,

and preparations were contaminated with other outer membrane constitutents, particularly LOS and Rmp, which together with Por, also stimulated antibody responses in the vaccinees. Not completely appreciated in 1985, were the complex interactions of antibodies directed against these antigens that resulted in a net effect upon complement dependent bactericidal activity.

A graded risk of acquiring gonorrhea in both vaccine and placebo recipients was not considered prospectively in choosing the cohorts, because at that time, natural protective immunity against gonorrhea, while in some cases recognized (reviewed above), was not defined in specific enough terms that would have permitted immunologic stratification of volunteers into different risk categories. In a "look back" at that vaccine trial, volunteers were retrospectively stratified for immunologic risk, and the question asked whether susceptibility to infection after intraurethral inoculation was influenced by the vaccine. The ratio of the concentration of Por and LOS antibodies, summed, to Rmp antibody concentration was positively correlated with protection both in vaccine and placebo recipients, but none of these alone correlated with protection against challenge. Furthermore, changes in bactericidal activity, both positive and negative (blocking), elicited by the vaccine, correlated with enhanced protection or increased susceptibility respectively, to infectious challenge, when this variable was considered independently. This study emphasizes that the use of a placebo group and stratification for pre-existing immunity will be important considerations in the future design of gonococcal vaccine trials that involve vaccine candidates to which there may already be partial immunity.

Experimental challenge of humans to evaluate vaccine candidates offers several advantages. These are: (a) the ability, in some cases, to use genetically constructed deletional mutants, deficient in the vaccine targeted epitope(s), to establish the connection between virulence and the epitope; (b) the ability to assess naturally acquired immunity (placebo recipients), defined in terms of the vaccine candidate in protecting against disease; and (c) the ability to stratify participants on the basis of pre-existing immunity (placebo recipients) and post-immunization responses (vaccine recipients), while challenging participants with different sized inocula to assess the quantitative role of immunity.

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Absence of protective immunity from repeat infections by gonococci expressing the same Por protein

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Neisseria gonorrhoeae (GC) is a human pathogen which causes recurrent mucosal infections. Although several of the components of the outer-membrane have been shown to be immunogenic, significant inter- and intra-strain variation limits their capacity to serve as vaccines. Several vaccine development projects have focused on Porin protein I (Por) which is required by the bacteria and is antigenically stable.

Several characteristics of Por make it an attractive vaccine target; i) anti-Por antibodies are produced by complicated and uncomplicated infections, ii) some anti-Por antibodies are opsonic and bactericidal, iii) anti-Por antibodies have been produced in rabbits immunized with either whole gonococci or peptides corresponding to portions of the Por protein, and iv) Plummer *et al.* reported serovar-specific immunity in a study of commercial sex workers in Africa (1). In that study, the serovar distribution of gonococcal isolates from commercial sex workers repeatedly exposed to gonorrhea was analyzed. The authors reported that the study subjects were less likely to be re-infected by gonococcal strains with the same serovar suggesting that there was a serovar-specific -(i.e. Por-based) protective immunity.

In the current study we have re-examined the role of Por in a protective immune response. Subjects who presented to an STD clinic during a 17-month period were enrolled (n=2896). Each subject was cultured for GC, and isolates were serotyped as previously described (2,3). A total of 618 subjects had positive GC cultures and 91 had one or more repeat infections. 30.2% of subjects with repeat infections were re-infected with the same serovar and 69.9% with at different serovar. There was a shift in the predominant serovars in the community over time. Initially PIB-2 was the predominant serovar. By the 11th month of the study, PIB-3 and PIA-6 were the predominant serovars. The result of this serovar shift was that the risk of any one subject's exposure to a particular serovar changed over time. Taking the serovar shift into account, we found no serovar-specific protective immunity. The odds ratio of repeat infections with the same serovar versus a different serovar was 1.54 (i.e. subjects were more likely to be re-infected by the same serovar of GC than expected based on the serovar distribution in the community at the time of infection).

It is known that multiple GC strains can have the same serovar, and there is significant protein sequence variability among Pors of the same serovar. Having identified a group of subjects with multiple infections by GC with the same serovar, we examined the

possibility that these infections were due to different GC strains with the same serovar or that the Por of isolates from these subjects differed in primary sequence. 37 isolates from 17 subjects with multiple infections by GC of the same serovar (15 from the current study and 2 from a previous study) were examined by arbitrarily-primed PCR (AP-PCR) and *por* sequence analysis. AP-PCR uses a single PCR primer and a low-annealing temperature thermocycle. The primer anneals to sites on the PCR template where it matches or almost matches. When the primer anneals to two sites on opposite strands of the template close enough for PCR amplification a product is generated. Typically AP-PCR generates a series of amplified products (the AP-PCR pattern) which are specific to the given template and is therefore indicative of the genotype of the bacteria. One primer (1290) previously used to genotype other pathogenic bacteria was used for AP-PCR. 10 of 17 subjects were found to be re-infected by bacteria with the same 1290 AP-PCR pattern.

The Por sequence of the isolates was determined by the direct sequencing of a PCR amplified product corresponding to the *por* gene. Complete sequence was obtained from sequence corresponding to the start of the mature protein to the start of the 8th proposed surface exposed loop of the protein. Isolates with the same AP-PCR pattern were found to have Por sequences that were 98.9-100% identical (0-4 amino acid differences). In contrast, isolates with different AP-PCR patterns had Por sequences which were < 96% identical. These data demonstrate that patients can have multiple infections by gonococci expressing the identical Por protein and which are likely the same gonococcal strain. These results are not consistent with Por-based immunity following natural infection in this population.

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Infectivity of gonococcal mutants in the human challenge model

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Previous studies with the human challenge model have used only naturally occurring variants of wild type gonococcal strains. We have constructed mutants of strain FA1090 differing from the wild type parent in expression of individual components believed to be important in virulence. In our previous studies, an inoculum of 10^6 cfu of strain FA1090 variant A21 or A22 (Opa^-, P^+) resulted in infection in over 90% of volunteers, defined as the presence of a urethral discharge containing gram-negative diplococci, accompanied by positive urine or urethral swab cultures (1).

Pili promote attachment of gonococci to human cells or cell lines *in* vitro, and previous studies involving human challenge with wild type gonococci have suggested that pilus production may be a critical virulence factor (2-4). We constructed a non-reverting pilin-negative mutant of strain FA1090 with a deletion of 260 bp encompassing the promoter and the 5' end of the *pilE* gene. No new antibiotic resistance markers were introduced into the chromosome of the final strain. The FA1090 Pil^- variant used in human challenge was matched to wild type variant A22 in Opa and LOS expression. 6 subjects were inoculated with 10^6 cfu of the Pil^- mutant. 2 subjects developed a watery discharge; the remaining 4 subjects showed little or no sign of infection and were asymptomatic. Abundant nonpiliated gonococci were cultured from urine specimens from all 6 subjects throughout the five day trial. In one asymptomatic subject, colony counts from urine specimens rose rapidly for 2 days after inoculation, but then decreased equally rapidly to few or no colonies on days 4 and 5. These results suggest that pilin expression is not absolutely essential for colonization of the male urethra; alternative adhesins may facilitate pilus-independent colonization. However, the clinical manifestations of infection with the Pil^- mutant differed from those occurring with wild type FA1090, with little or no inflammatory response to the presence of the organisms.

Gonococcal lipooligosaccharide (LOS) is also believed to be important in pathogenesis of gonorrhea. The enzyme phosphoglucomutase (Pgm), which catalyzes interconversion of glucose-1-phosphate and glucose-6-phosphate, is necessary for synthesis of the sugar precursors that are assembled into LOS (5,6). We cloned the *pgm* gene from strain FA1090 using PCR amplimers based on the sequence of *pgm* from strain 1291 (5), and then constructed a deep rough *pgm* mutant of FA1090, using a two-step strategy involving an insertional inactivation cassette containing both a selectable and counterselectable marker (described in more detail in the abstract by Johnston et al). The

final mutant contained a linker insertion mutation in *pgm,* and expressed no new antibiotic resistance markers relative to the parent strain. Strain FA1090 *pgm* produced a single truncated LOS species that did not bind Mab 3F11, and had the same growth rate, serum resistance, and outer membrane protein profile as the parent strain. The mutant was matched to the wild type parent in Opa and pilin type (assessed by determining DNA sequence of *pilE.*). None of 9 subjects inoculated with 10^6 cfu or 3 subjects inoculated with 10^7 cfu of FA1090 *pgm* developed urethritis within a 5 day trial. All but two daily urine specimens from the subjects were culture-negative; the two positive specimens yielded only a few colonies. In contrast, FA1090 A22 infected 8 of 8 subjects receiving 10^6 cfu. These results emphasize the importance of intact LOS, which may influence key properties of the organisms and/or affect their interaction with host cells. The results with both mutants also demonstrate the ability of the human challenge model to discriminate among gonococci that are compromised to different extents in ability to cause urethral infection.

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Does an experimental gonococcal infection protect human volunteers from subsequent reinfection?

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We have previously reported that as few as 250 to 1600 MS11mkC gonococci can infect from 30% to 40% of inoculated volunteers, while 36,000 infects 100% (1). These experiments showed that expression of a terminal paragloboside, lacto-N- neotetraose, on its lipooligosaccharide (LOS) was a gonococcal virulence factor. We further showed that in vitro sialylation of this LOS lessened the infectivity of mkC gonococci (2). We sought to test the hypothesis that an experimental mkC gonococcal infection would prevent reinfection with homologous mkC gonococci two weeks after treatment of the initial infection. In the first experiment we inoculated 15 volunteers with a large number, 57,000 mkC gonococci; 14 (93%) became infected (median incubation time=54 hr; range-43 hr to 91 hr). These 14 and a control group of 10 "naive" volunteers were challenged with 8-fold fewer,7,000 mkC gonococci 2 weeks after the initial infection were treated Six (43%) previously infected volunteers became infected (median incubation time = 52 hr; range = 45 hr to 91 hr) and 5 of 10 (50%) of the naive volunteers became infected (median incubation time = 93 hr; range-69 hr to 139 hr), indicating that the first infection did not protect against homologous reinfection. We used recovery of gonococci from urinary sediments to monitor the course of each infection. There appeared to be no relationship between dose and infectious course. We found considerable variation among the subjects infected by either the high or low dose in the patterns of increase in the numbers of gonococci shed during the incubation period and the relationship of these numbers to the onset of dysuria and clinical urethritis. The course of our experimental infections is unpredictable, and does not appear to be dose related.. The unpredictability in the course of these experimental infections, both in incubation time and presentation of symptoms, mirrors the variation seen in naturally acquired infections, and appears more subject to individual variation than to dose of gonococci.

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Neisserial porins activate naive resting B lymphocytes, inducing proliferation and immunoglobulin secretion

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The Neisserial porins are immunogenic without the addition of adjuvants and have immunopotentiating activity. They have been demonstrated to augment the immune response to poorly immunogenic substances, like capsular polysaccharides and peptides (1). The mechanism of this action is unclear, but we postulate that their adjuvant ability is related to the porins' effect on lymphocytes and T-B cell interactions. We have previously reported that the porins can induce expression of the costimulatory ligand B7-2 on B lymphocytes and that these porin treated B cells can stimulate T cells (2). Porins, alone, have not been shown to directly stimulate T cells (2). Moreover, the porins induced B cell proliferation and increased expression of class II MHC. B7-2 expression is essential for B lymphocyte dependent T lymphocyte stimulation (3). The induction of B7-2 expression is one possible mechanism of the porins' adjuvant activity. However, other effects of the porins on B lymphocytes, as evidenced by proliferation and expression of activation markers, might also be involved in this mechanism.

To further characterize the porins effect on B lymphocytes, the following experiments were performed. Naive splenic B cells were obtained from murine strain C3H/HeJ (LPS non-responsive) by centrifugation of single cell suspensions of purified splenic B cells over a discontinuous percoll gradient, to insure that the cells obtained were small resting B cells (2,4). B cells (10^5/ml) were incubated various neisserial porins at increasing concentrations (0.1-10 μg/ml [0.003-0.3 μM]). The porins used were protein IA from gonococcal strain UU1, protein IB from gonococcal strain Pgh 3-2, and class 1 and 3 proteins from meningococcal strain 44/76 (1,2). After two days of incubation, proliferation was measured by ^{3}H-thymidine incorporation (2). All species of porins tested induced significantly greater B cell proliferation at concentrations as low as 0.1 μg/ml, when compared to control incubations of B cells with media alone.

When the B cell receptor (BCR), containing the surface immunoglobulin, is crosslinked, small resting B cells do not proliferate unless another stimulus is added, e.g. IL4, bacterial lipopetides, etc. (4). In this experimental model, BCR crosslinking was induced by incubation of B cells with dextran conjugated with anti-murine IgD (anti-IgD-dex). When porin treated B cells were co-incubated with anti-IgD-dex at concentrations of 0.03-3 ng/ml, a synergistic increase in B cell proliferation was found when 3 or 0.3 ng/ml of anti-IgD-dex was used. No significant proliferation was observed when B cells were incubated with anti-IgD-dex at 0.03 ng/ml or if anti-IgD-dex was used alone. These results demonstrate that neisserial porins, unlike BCR crosslinking, cytokines or

lipopeptides, can induce B cell proliferation without the addition of other stimuli. In addition, BCR crosslinking will synergistically increase the porin induced proliferation of B cells.

Another measurable read-out of the porins effect on B cells is the induction of secretion of immunoglobulin. Resting B cells were incubated with the neisserial porins as described. After two days, the supernatant of the cell cultures were obtained and the level of IgM was determined by ELISA (4). When incubated with porins at concentrations between 0.1-10 μg/ml (0.3-0.003 μM), B cells were induced to secrete IgM. When porin treated B cells were co-incubated with anti-IgD-dex, synergistic increases in IgM secretion were noted. Interestingly, at the highest concentration of anti-IgD-dex used in these experiments, 3 ng/ml, IgM secretion either was similar to or below the levels measured in wells containing B cells and porins alone. This finding is in contrast to the induction of proliferation when B cells were incubated with porins and anti-IgD-dex at 3 ng/ml. Similar to the proliferation experiments, the lowest concentration of anti-IgD-dex, 0.03 ng/ml, did not increase IgM secretion by porin treated B cells. This study implies that high BCR occupancy and crosslinking, as seen with high antigen concentrations, could have a negative influence on B cell function and immunoglobulin production. Bacterial capsular polysaccharides, with repeating epitopes, may act in a similar manner and crosslink BCR. There is, likely, an ideal concentration of polysaccharide or other antigens that are to be used in vaccines utilizing porins as adjuvants, as too high a concentration of the antigen could "over" crosslink the BCR and inhibit, rather than increase, antibody production.

Finally, CD40 engagement by soluble CD40-L synergistically increased proliferation and IgM secretion of porin treated B cells. CD40L is present on T cells and therefore, the porins might be able to increase B cell activity induced by T-B cell interaction directly as described and also increase T cell activation indirectly by inducing increased B cell expression of the costimulatory molecule B7-2 (5). Purified meningococcal or enterobacterial LPS had no effect on these B cells, demonstrating that this phenomena is not related to possible LPS contamination of the porin preparations

Crosslinking of the specific BCRs is expected to occur when antigens with repeating epitopes encounter B cells, i.e. polysaccharide. The effect of porins on B cell proliferation and antibody secretion, which are synergistically increased by BCR crosslinking, could be another possible mechanism of the porins' adjuvant activity which could improve the immune response to these classic T cell independent antigens.

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Selection for Opa$^+$ phenotypes of *Neisseria gonorrhoeae* in normal human serum

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The specificity of *Neisseria gonorrhoeae* for its human host and the lack of a suitable animal model has led to the use of a human male intraurethral challenge model to study gonococcal pathogenesis. One consistent observation made using this model, was that the organisms shed by infected volunteers showed a transition from an Opa$^-$ (transparent) phenotype of the inoculum to an Opa$^+$ (opaque) phenotype of the recovered organisms (1,2). Multiple members of the Opa protein family are expressed on gonococci (GC) shed during this early phase of infection. Another consistent result from the male volunteer studies was that not many viable input Opa$^-$ GC could be recovered from the first urine samples, suggesting that most of the input organisms were killed. The reappearance in urine of Opa$^+$ GC indicates there is a survival advantage for GC expressing an Opa protein.

A powerful bacterial killing mechanism exerted by the human host is antibody and complement mediated killing in normal human serum (NHS). Although the presence of NHS components on the male urethral mucosal surface is still a matter of debate, we believe NHS mediated killing may play a role in the urethra, since the presence of complement has been shown on other genital mucosal surfaces. Moreover, it was recently shown in the human volunteer model, that a rise in cytokine levels in urine, and therewith attraction of neutrophils and possibly serum factors to the site of infection, occurs within hours after instillation (3).

The goal of this study was to see if serum killing of GC could be a mechanism leading to selection of Opa$^+$ phenotypes, as a possible explanation for the in vivo observations. We therefore studied serum killing of Opa$^-$/Opa$^+$ mixtures of strain MS11, by measuring dose-response relationships, since minor differences in serum sensitivity could be important when serum availability is limited.

Expression of an Opa protein conferred a survival advantage upon the organism; i.e. the Opa$^+$ GC were always more serum resistant than their isogenic Opa$^-$ counter-parts, measured both in mixtures and in separate populations. This resulted in selection for the Opa$^+$ phenotype during serum killing of a mixture of Opa$^-$ and Opa$^+$ GC.

Since the type of lipooligosaccharide (LOS) expressed is a major determinant of serum sensitivity, we studied the selection phenomenon in different LOS backgrounds. Selection for Opa$^+$ occurred in all three LOS phenotypes examined, even in a LOS background that confers total serum resistance to the GC. In this case a monoclonal anti-

LOS antibody was added to NHS. These data indicate that the Opa related survival advantage is not due to a difference in LOS expression between the Opa^- and Opa^+ phenotype.

The micro-environment in the urethra may have a profound effect on the killing of GC. We therefore studied the effect of normal human urine (NHU) on the selection phenomenon. The bactericidal action of NHS was drastically reduced by the presence of NHU in the killing assay. This was probably due to complement inhibitors present in urine, since urine inhibited complement in an antibody-coated sheep erythrocyte lysis assay. However, also in the presence of urine, we observed a distinct selection for Opa^+ organisms during serum killing of an Opa^-/Opa^+ GC mixture.

Since in vivo most GC appear to be sialylated, we also studied selection of sialylated GC mixtures. We used a phenotype that expresses two major LOS bands on a silver-stained SDS-PAGE gel, with one being the main acceptor for sialic acid. Sialylation of these organisms led to an enhanced serum resistance, but with enough serum present, they were still killed. In Opa^-/Opa^+ mixtures of these sialylated GC, we also found a survival advantage for Opa^+ GC.

Thus, the expression of an Opa protein confers an enhanced serum resistance upon GC; this leads to selective survival of Opa^+ GC when limited amounts of NHS are present; this phenomenon may contribute to the enhanced appearance of Opa^+ GC, as observed in the human volunteer studies.

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In-vitro effects of contraceptive microbicides on *Neisseria gonorrhoeae* infection

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The objective of this study was to test a number of new agents with contraceptive activity for their potential as microbicides for STD pathogens. Our focus was on their antigonococcal activity (1-3). These compounds were originally formulated for use as vaginal topicals which has the advantage of the minimization of systemic exposure to pharmacologically active agents as well as increased availability of active agents at the target site where protection is most needed. Contraceptive compounds which include anionic quinonyl aldehyde copolymer (TC5-08PS500), anionic bioflavonoids (TC4-01PH000 and TC5-08SH00), anionic b-1,4-polysaccharide (TC5-12CS000) and arylalkenyl homopolymer M_r 500 Kd (TC6-02-PSS01), anionic aryl alpha-hydroxyl carboxylic acid (TC6-02MAS00) , macrocylic r-substituted phenol:formaldehyde condensation products (TC6-02C4A00 and TC6-02C6A00) , sulfated hydroxyalkyl homopolymer (TC6-02VAS01) were tested in varying concentrations (0.1-1000 mg/ml) against *N. gonorrhoeae.* The effect on infectivity was measured quantitatively by incubation of the gonococci on microbicide containing gc agar plates and by infection of human fallopian tube organ culture explants (4,5). Determinations were made for tissue cytotoxicity as well as quantification of the compound's ability to reduce or eliminate infectivity by *N. gonorrhoeae.*

The types of contraceptive compounds showing antigonococcal activity were anionic b-1,4-polysaccharide, arylalkenyl homopolymer M_r 500 Kd, anionic quinonyl aldehyde copolymer, anionic bioflavonoids, macrocylic r-substituted phenol:formaldehyde condensation products and sulfated hydroxyalkyl homopolymer when assayed using agar plates containing the microbicide. When the anionic b-1,4-polysaccharide was used in concentrations of 1000, 500 and 100 mg/ml kills of 100, 96 and 94 % respectively were obtained. Arylalkenyl homopolymer in concentrations of 1000, 500, 100 mg/ml all achieved 100% kill while 10 mg/ml resulted in 97% kill. The anionic quinonyl aldehyde copolymer was only effective at 1000 mg/ml with a 97% kill. The anionic bioflavonoids were less effective microbicides with TC4-01PH000 being an ineffective compound (<15% kill) while TC5-08SH00 exhibited 76% killing. At concentrations of 100 and 1000 mg/ml the anionic aryl alpha-hydroxy carboxylic acid was effective with 100% killing of the gonococci. However, at concentrations below 100 mg/ml the compound was less effective (~30% kill). Both macrocyclic r-substituted phenol:formalydehyde condensation products (TC6-02C4A00 and TC6-02C6A00) were effective at 1000 mg/ml but only partially effective at concentration of 100 mg/ml or less

(<30% kill). The sulfated hydroxyalkyl homopolymer was highly effective at concentrations of 1000 and 100 mg/ml with kills of 100 and 93% respectively.

The arylalkenyl homopolymer, anionic b-1,4-polysaccharide and anionic quinonyl aldehyde copolymer were also effective as antigonococcal microbicides in fallopian tube explants. The arylalkenyl homopolymer was effective with 100% kills at 100 and 1000 mg/ml. The anionic b-1,4-polysaccharide exhibited kills of 100 and 97% at 1000 and 100 mg/ml respectively. The anionic quinonyl aldehyde copolymer produced 100% kill at 1000 mg/ml.

In addition to a compound's ability to function as an effective topical antimicrobial agent it is important that they are not cytotoxic to local mucosal surfaces. These compounds were tested in both a cell and organ culture system for their ability to disrupt cell membranes and mucosal surfaces. None of the compounds, when tested at concentrations which were microbicidal, demonstrated cytotoxicity in either cell or organ culture assays.

A number of the compounds tested demonstrated promise as potential candidates for inclusion as a topically administered microbicide because of their effectiveness as antigonococcal agents. The compounds were effective in vitro at concentrations which are clinically achievable and do not demonstrate in vitro cytotoxicity, which are essential to be a useful candidate. Of the compounds tested the anionic b-1,4-polysaccharide and the arylalkenyl homopolymer were the most promising antigonococcal compounds because of their high degree of bactericidal activity over a wide range of concentrations. These data indicate that there are several compounds which have both contraceptive and microbicide effects against *N. gonorrhoeae* and have potential for further studies to determine their efficacy as topical microbicides.

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Epidemiological survey of plasmid mediated tetracycl[illegible] resistance gonococci in Argentina (April 1993 - April 1996).

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Neisseria gonorrhoeae (NG) with plasmid mediated tetracycline resistance was unknown in our country before 1993. The first strain was detected in April 1993 (1, 2). Three years after, the number of tetM-containing strains increased to 18 isolates, submitted from different areas of the country: Buenos Aires (DF), Jujuy, Tandil and Mendoza.

The strains were sent to the National Reference Center of STD (NRC) from periferic laboratories belonging to the National Network for Gonococcal Surveillance for characterization by sensitivity test (MIC), auxotyping, serogrouping and molecular studies.

The isolates could be divided into two groups by antimicrobial susceptibility test (3): 15 strains with plasmidic resistance to tetracycline (MIC 16 mg/ml) and penicillin (MIC 32 mg/ml) (PP-TRNG) and 3 strains with only plasmidic tetracycline resistance (MIC 32 mg/ml) (TRNG). All the strains were susceptible to cefuroxime, ceftriaxone, ciprofloxacin and spectinomycin.

The plasmid profiles by agarose gel electrophoresis (4) demonstrated the same pattern in all PP-TRNG: 2.6, 3.2 and 25.2 MDa plasmids. The TRNG strains bore the 2.6 and 25.2 MDa plasmids. The presence of tetM-determinant responsible for the high-level of tetracycline resistance was examined by hybridization using Probe 3 (5). The restriction endonuclease analysis with BglI, SmaI and HincII of 25.2 MDa plasmid, demonstrated a Dutch type plasmid (6).

The studies using the relation auxotype/serogroup (7), identified four phenotypic classes of NG isolates. Fourteen strains were arginine requiring, serogroup WI (A-/WI), and was the prevalent class. Two strains were methionine and arginine requiring, serogroup WI (MA-/WI). These strains were the first isolates in Argentina. Only one strain was proline and arginine requiring, serogroup WI (PA-/WI). The strain recently isolated was non-requiring, serogroup WII/III (NR/WII/III); and it strongly differed from the previous three classes.

The class A-/WI involved nine strains of a microepidemic that took place in Tandil city between February and April, 1995 (8). The comparison of the strains of this outbreak with the pattern of one strain previously isolated in Buenos Aires city in 1994, showed similar phenotype and tetM-containing plasmid type (Dutch).

Conclusions: From April 1993 to April 1995 all strains were PP-TRNG MA-/WI or A-/WI. These two phenotypes had never been identified among NG strains of our country before 1993, suggesting they were of foreign origin. Since May 1995 we have identified a new class of PP-TRNG, PA-/WI, besides the prevalent A-/WI. This auxotype is less common between our NG. During the same year, we found for the first time TRNG strains; two of them belonged to the prevalent A-/WI but the latest isolate was classified as NR/WII/III, the most common phenotype among NG in Argentina. It seems that the 25.2 MDa plasmid which has the ability to move itself, has been introduced in our indigenous NG population strains. The treatment of genital infections with tetracycline enhances the danger of dissemination. These strains may then spread trough the population but may not necessarily be recognized. In fact, all the PP-TRNG strains confirmed in our laboratory were not initially submitted as TRNG

Taking in account these results, we suspect that the true frequency and geographic distribution of tetM-containing NG in Argentina are underestimated.

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Oral inoculation with live attenuated *Neisseria gonorrhoeae* induces a vaginal IgA response.

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The gonococcus evades the immune system of the host during infection in a number of different ways. These include both a high level of antigenic variation of outer membrane components such as pili, the Opa protein (1,2) and LPS (3,4) and host mimicry, particularly through the sialylation of LPS (5). Such characteristics have rendered the use of both dead whole gonococcus and individual bacterial components, as vaccines, ineffective (6,7). The development of an attenuated strain of *Neisseria gonorrhoeae,* MS11 JKD298 which harbors a mutation in the *aroA* gene (8), allows the potential for vaccination with live gonococcus, thus avoiding these limitations. This *aroA*$^-$ gonococcus has been shown to display decreased virulence *in vivo* whilst retaining its immunogenicity (8).

In vaccinating against mucosal pathogens, such as *N. gonorrhoeae,* it is essential to elicit a mucosal immune response. In order to do this it is therefore highly desirable to deliver such vaccines to a mucosal surface. In previous studies with MS11 *aroA*$^-$, despite the expected reduced virulence *in vivo*, a strong inflammatory response was observed during infection with this strain. The induction of such a response precludes the possibility of delivery to the genitourinary tract, in common with natural gonococcal infection. However, it is now well established that immunization at one mucosal site leads to the induction of immunity not only at that site but also at other, distant mucosal sites (9). Therefore, as an alternative to vaginal vaccine delivery, we have investigated the outcome of oral inoculation with live attenuated gonococcus.

Female C_3H/He mice (OLAC, UK) were given three oral doses of MS11 JKD298, of between $6x10^9$ and $1x10^{10}$ gonococci, by gavage on days 1, 6 and 9. This was followed by a similar boosting dose on day 27. To investigate the systemic antibody (Ab) response, serum samples were prepared from tail vein bleeds on day 21, after the initial inoculation and day 34, following boosting. Most importantly, in order to assess the local mucosal Ab response in the vagina, vaginal washes were carried out with 200 µl of phosphate buffered saline to which a cocktail of protease inhibitors was added immediately after washing. Washes were carried out on day 21, following the first inoculating doses and then on days 34, 36 and 38 after boosting. Levels of specific IgG and IgA were measured by ELISA. To assess the Ab response elicited against a potential infecting gonococcus, ELISA were carried out against a whole cell lysate of the parental wild type strain MS11 JKD288.

Specific vaginal IgA was present in all mice on day 21 after the initial immunizing doses. However, the levels of Ab detected in vaginal washes performed varied greatly between animals, ranging from 1.2 to 24.7 times that detectable in similar washes from unimmunized, control animals. This observed variation was possibly due to differences in the overall response of the different mice or, alternatively, was related to the position of each individual mouse within the estrus cycle. To assess which of these was the case, following boosting, vaginal washes were taken over a six day period to cover the complete estrus cycle, on days 34, 36 and 38. Once again specific IgA was detected in vaginal washes from all mice. However the levels in consecutive washes from individual mice were highly variable. For one representative animal, IgA levels ranged from 0.6 times to 24.7 times greater than the levels present in washes from control mice. This observed variation in Ab levels between the washes of one individual mouse would suggest that levels of IgA secreted are indeed dependent on the position of each mouse within the estrus cycle. We are currently conducting experiments to assess the position within the cycle of each animal at the time of each vaginal wash. It is hoped that this will reveal whether the production of particularly high levels of IgA correlates with a particular stage of estrus. In addition to IgA, the presence of IgG and IgM within vaginal secretions was also determined. No Ab of either isotype could be detected within the vaginal washes of any of the mice.

A comparison of the systemic Ab response with the mucosal Ab response was carried out by assessing the levels of specific serum IgG and IgA. In common with the mucosal response, IgA was detected within the serum of all mice both after the initial inoculation, on day 21 and following boosting, on day 34. The mean IgA Ab level of all mice was 12.3±6.6 times (day 21) and 10.8±4.5 times (day 34) that detected in sera collected from control animals. In further contrast to the mucosal Ab response, in addition to IgA, IgG was also present within the sera from all animals at both times (means levels of 14.9±3.3 and 10.6±2.4 times that of control sera on days 21 and 34 respectively).

In summary, the above results have shown that oral delivery of attenuated gonococcus does indeed stimulate an immune response whilst avoiding the induction of an inflammatory response in the genitourinary tract. This resulting Ab response is reactive against the parental wild type strain, and thus potentially infective gonococcus. We are currently extending these observations to determine the specificity of both systemic IgG and IgA and mucosal IgA Ab using SDS-PAGE and western blotting. In addition we are also comparing the responses obtained following inoculation at other mucosal surfaces.

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Production of inflammatory cytokines by human macrophages and polymorphonuclear cells in response to *in vitro* stimulation with *Neisseria gonorrhoeae.*

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Uncomplicated gonorrhea is characterized by an intense inflammatory infiltrate consisting predominantly of polymorphonuclear cells (PMN). In our hands, in the guinea-pig subcutaneous chamber model, even in prolonged infections lasting several weeks, the inflammatory response remains acute with a majority of PMN and much fewer numbers of either macrophages or lymphocytes (1,2). The signals elicited by *Neisseria gonorrhoeae* early during infection which stimulate this acute inflammatory response are still not fully characterized. Additionally, host processes which may prevent dissemination and are thus responsible for localization of infection are not known.

Previous work carried out in this laboratory has shown the presence of the inflammatory cytokine TNF-α in chamber fluid removed during infection with *N. gonorrhoeae*, strain MS11 JKD288 or LPS variants of strain GC40, in the guinea-pig (R Demarco de Hormaeche, elsewhere in this book). In addition, experimental infection in male human volunteers has been shown to lead to an increase in the levels of TNF-α, IL-6 and IL-8 detected in urine (3). Whilst these studies suggest that such inflammatory cytokines may be of central importance during gonococcal infection, they do not address what specific cells are responsible for their production or which bacterial components mediate the stimulation. In order to investigate these points we have assessed the production of a range of inflammatory cytokines by human cells following stimulation with a number of bacterial components *in vitro*.

Monocytes and PMN were isolated from the blood of normal volunteers using commercially available centrifugation gradients. Macrophages were further purified from the monocyte fraction by adherence. In addition, the human myeloid cell line, U937, was utilized following differentiation in the presence of phorbol 12-myristate 13-acetate. Cells were stimulated at concentrations between $0.5x10^6$ and $1.0x10^6$ cells/ml, with either LPS purified from gonococcal strains MS11 JKD288 and GC40 variant D1 or from *Salmonella typhimurium* (all used at 5 μg/ml) or whole cell lysates of MS11 JKD288. Levels of TNF-α, GM-CSF, IL-1β and IL-8, present in the culture supernatants, at various time points after stimulation, were assayed by sandwich ELISA

Stimulation of differentiated U937 cells with GC40 D1 LPS and *S. typhimurium* LPS resulted in the production of all cytokines analyzed. IL-8 and TNF-α were detected first, 3 hours after stimulation. Production of TNF-α in response to the two different LPS was comparable and could be detected throughout the whole cell culture period. However, whilst production of IL-8 in response to gonococcal LPS was maintained for over 40

hours, levels decreased after 6 hours in cultures containing *Salmonella* LPS. IL-1β and GM-CSF were detected later, from 6 hours post stimulation. In common with the production of TNF-α, levels of GM-CSF were similar in the presence of either LPS. To confirm and extend the above observations macrophages and PMN isolated from normal volunteers were stimulated in a similar fashion. In common with the cell line, stimulation of fresh macrophages resulted in the production of IL-8, TNF-α and IL-1β. However, in contrast GM-CSF could not be detected at any time. IL-8 and TNF-α were again detected first, although some differences between the two systems were observed. An early peak of IL-8 was apparent 1 hour after stimulation of fresh macrophages. Interestingly, this was greater in the presence of gonococcal LPS than *Salmonella* LPS. Levels of IL-8 increased from 6 hours post stimulation. TNF-α was first detected at 6 hours, at which point production was maximal. No differences were observed with the responses to the two LPS types. In contrast to the results obtained with the cell line, IL-1β was detected in greater quantities after stimulation with gonococcal LPS, levels being lower or undetectable following stimulation with *Salmonella* LPS. In addition, presence of IL-1β was not detected until later in the culture, at 22 hours.

Stimulation of PMN with either gonococcal or *Salmonella* LPS gave rise to the production of IL-8. This was detected early in the culture, 3 hours post stimulation and continued throughout.

To investigate the potential contribution of gonococcal components other than LPS in the stimulation of cytokine production by the different cell types, freshly isolated macrophages and PMN were cultured in the presence of either LPS or whole cell lysate *N. gonorrhoea*, strain MS11 JKD288. Macrophage production of both IL-8 and TNF-α and PMN production of IL-8 was increased at least two fold following stimulation with whole cell lysate as compared to LPS. Concentrations of LPS in the cell lysate are estimated to be considerably smaller than the 5 μg/ml of purified LPS utilized in these experiments. The observed increase in cytokine production cannot therefore be accounted to an increase in LPS concentration. Such an increase suggests, therefore, that other gonococcal factors, in addition to LPS, contribute to the stimulation of both of these cell types. We are currently analyzing the production of TNF-α and IL-8 by macrophages, following stimulation with whole cell lysate, in the presence of either polymyxin B or mAb against CD14 to block the effects of the LPS. The results so far also indicate that other factors contribute to cell stimulation. Our recent work has revealed that stimulation of PMN with whole cell lysate, pre treated with proteinase K, results in a two fold reduction in the production of IL-8 indicating that gonococcal proteins contribute strongly to induction of cytokine production.

In summary, therefore, we have developed a system which enables us to analyze the activation of the individual cell types that predominate at sites of infection during gonorrhoea infections, following stimulation of such cells with *N. gonorrhoeae* . In addition we are able to further characterize the individual bacterial components responsible for the different effects observed. The results described here indicate that both gonococcal and *Salmonella* LPS are similarly capable of stimulating the production of inflammatory cytokines TNF-α, IL-8, IL-1β and GM-CSF by human macrophages

and IL-8 by human PMN. Some differences in the stimulation of IL-8 production by macrophages by these two different LPS were, however, observed. Further, preliminary results suggest that both gonococcal proteins and LPS may be important in stimulating cytokine production by both macrophages and PMN.

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Infection with *Neisseria gonorrhoeae* induces acute systemic, but not local, immune and inflammatory responses: effects of concomitant infection with *Trichomonas vaginalis* and/or *Chlamydia trachomatis*.

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Antibodies to *Neisseria gonorrhoeae* have been detected in both serum and mucosal secretions (1, 2). Despite the apparent presence of such antibodies, re-infection with *N. gonorrhoeae* is common. How *N. gonorrhoeae* is capable of re-infecting a host in the presence of such antibodies is not well understood. It is believed, however, that this organism evades the host immune response by a variety of mechanisms including antigenic variation. In addition, *N. gonorrhoeae* produces IgA1 proteases that may cleave IgA1 *in vivo*, protecting the organism from the immune system in some (as yet) unknown fashion. In this report we have examined how the mucosal and systemic immune and inflammatory responses to *N. gonorrhoeae* are effected by concomitant infection with *Trichomonas vaginalis* and/or *Chlamydia trachomatis*.

Female patients attending the Jefferson County STD clinic (Birmingham, AL) were recruited into a study examining the role of IgA1 proteases in the pathogenesis of *N. gonorrhoeae* induced disease. Informed consent was obtained from each patient prior to enrollment in the study. Where possible, samples of saliva, cervical mucus, vaginal wash, and serum were obtained from the patients during three visits to the STD clinic spaced at two week intervals following their initial visit. In patients infected with *N. gonorrhoeae,* 3/20 were also infected with *T. vaginalis*, 2/20 with *C. trachomatis*, and 3/20 were infected with both of these organisms. In the control patients, (not infected with *N. gonorrhoeae*) 3/31 were infected with *T. vaginalis*, 1/31 with *C. trachomatis*, and 3/31 were infected with both organisms. All infected patients were appropriately treated during their first visit and were tested for infection at subsequent visits. No patient remained infected with *N. gonorrhoeae* after treatment. All samples were treated with an antiprotease containing buffer, and stored at -70° until analyzed.

The concentrations of total IgA1, IgA2, IgG, and IgM immunoglobulins were determined for each sample. Infection with *N. gonorrhoeae* did not effect the concentrations of total IgA1, IgA2, or IgM in any samples. Total IgG concentrations in the vaginal wash, but not other samples, from patients infected with *N. gonorrhoeae* were significantly lower compared to the controls. Whole, formaldehyde treated, *N. gonorrhoeae* were used as the antigen for the estimation of antibody levels reacting to *N. gonorrhoeae* MS11. In addition, the antibody levels reacting to the patients infecting strain were tested (where possible). Antibodies to MS11 were present in all secretions and serum. There were no differences in the local antibody levels comparing *N.*

gonorrhoeae infected versus non-infected women. The levels of serum IgA1, but not other immunoglobulin types, reacting to the MS11 strain were significantly higher in women infected with *N. gonorrhoeae* compared to the controls. Infection with either *T. vaginalis* and/or *C. trachomatis* did not effect the immunoglobulin concentrations or antibody levels at any site, nor did they alter the response (or lack of response) to *N. gonorrhoeae*.

Experimental infection of males with *N. gonorrhoeae* induced a rapid local and systemic cytokine response including IL-1, IL-6, and IL-8 (3). We examined whether these cytokines would be produced during a natural infection in women. The levels of IL-6, IL-8, and IL-1β were quantitated by ELISA. Monoinfection either *N. gonorrhoeae*, *T. vaginalis*, or *C. trachomatis* did not induce a significant local cytokine response compared to the controls. Serum IL-6, but not other serum cytokines, was significantly elevated in patients infected with *N. gonorrhoeae*. This response was not observed in patients infected with *T. vaginalis* or *C. trachomatis* alone. Concomitant infection with *T. vaginalis* and/or *C. trachomatis* did not modify the cytokine responses to *N. gonorrhoeae* (examined in total), however, there was a significant subgroup (3/8) of these patients with highly elevated levels of these cytokines in serum. This effect was not seen in any other group.

The results summarized in this report suggest that there appears to be little or no acute mucosal immune or inflammatory responses to *N. gonorrhoeae*. Instead, IgA1 and IL-6 responses to this organism occur in serum. Concomitant infection with *T. vaginalis* and/or *C. trachomatis* does not affect those responses. There appears to be a subgroup of patients infected with *N. gonorrhoeae* with concomitant *T. vaginalis* and/or *C. trachomatis* infections that have very high levels of circulating inflammatory cytokines. These infections induce periodic cycles of cell mediated immunity. It is possible, therefore, that the high levels of serum cytokines in these patients may reflect an interaction between *N. gonorrhoeae* and PMN or monocytes previously recruited to the site of infection.

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Effects of *Neisseria gonorrhoeae* uretheitis on the concentration of HIV-1 in seminal plasma

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Objectives. To determine 1) if *N. gonorrhoeae* increases the concentration of HIV-1 in semen, and 2) if therapy for *N. gonorrhoeae* decreases the concentration of HIV in the semen.

Rationale. Gonorrhea has been linked to increased acquisition of HIV in women, with the assumption that mucosal inflammation provides greater access for HIV than normal tissue (1). However, an additional role for gonorrhea, which could biologically explain these findings includes increased infectiousness of male partners with untreated gonorrhea.

Methods. In 1996, men presenting with a urethral discharge to the STD Clinic in Lilongwe, Malawi were studied. Urethral swabs were evaluated with a gram stain and culture for *N. gonorrhoeae.* Semen was collected before treatment to measure the concentration of HIV-1 RNA with a modified nucleic acid sequence based analysis (NASBA) assay. This assay had a detection limit of 1,000 copies/ml. All patients were treated for gonorrhea with Gentamicin 240 mg IM (95% cure rate). If gonorrhea was not found on gram stain, either doxycycline or azithromycin were added to the gentamicin. HIV serostatus was determined by repeat ELISA. HIV+ subjects returned at 1 and 2 weeks post-treatment for semen collection and re-evaluation. Subjects who continued to have *N. gonorrhoeae* were treated with ciprofloxacin. An HIV+ control group of dermatology clinic men without urethritis on gram stain was selected and evaluated with semen collection at 2 visits, 2 weeks apart.

Results. 206 subjects with urethritis and 125 control subjects were enrolled. 113/205 (55%) of urethritis subjects and 60/125 (48%) of control subjects were HIV+. 55 of the 83 urethritis men who successfully gave semen were infected with *N. gonorrhoeae* by gram stain or culture. HIV-1 RNA concentrations of seminal plasma from 83 HIV+ subjects with urethritis and 43 HIV+ controls were evaluated. Before treatment, the median seminal plasma concentration in the urethritis group was 129,000 copies/ml, and the sub-set of 55 gonorrhea subjects was 178,000 HIV copies /ml. These values were significantly different than the control value of 17,000 copies /ml found at baseline ($p = .003$ and $p =. 003$, respectively). At 2 weeks post-treatment, the median concentration in

the gonorrhea group was 44,000 HIV copies/ml compared to, 23,00 copies/ml 2 weeks post-baseline in the controls (NS). In the gonorrhea group, the median individual HIV log concentration change comparing pre- and post-treatment values decreased .53 log (3.4 fold) ($p < .0001$). The individual log change in the control group comparing values at baseline and 2 weeks follow-up was -.02 log. Median HIV RNA blood plasma concentrations were not significantly different comparing the pre and post-gonorrhea treatment groups and the controls (95,000, 105,000 and 115,000 HIV copies/ml, respectively).

Conclusion. HIV infected men with urethritis have a significantly higher concentration of HIV RNA in seminal plasma compared to controls without urethritis. Men with *N. gonorrhoeae* urethritis had the highest level of HIV in seminal plasma. Effective antibiotic treatment for gonorrhea results in a reduction of HIV in seminal plasma. Blood plasma HIV concentrations do not appear to be affected by gonorrhea. Therapy for gonorrhea did not change blood plasma HIV RNA concentrations. Heretofore there have been nine studies related to the concentration of HIV in semen (2) and none have examined the effects of coinfection with STDs. The significant amplification of HIV in semen caused by gonorrhea suggests at lease part of the way treatment of STDs reduces the incidence of HIV (3).

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***In vivo* induction of TNFa by *Neisseria gonorrhoeae*.**

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The main clinical manifestation of gonorrhoea is the appearance of an acute inflammatory exudate containing mainly polymorphonuclear cells (PMN) some of which harbor large numbers of gonococci. This exudate is so typical of gonorrhoea that its examination has been an important aid for diagnosis for many decades. Apart from its diagnostic value, the inflammatory infiltrate may play an important role in the pathogenesis of gonorrhoea, particularly in precluding dissemination. The interaction of gonococci and phagocytic cells is complex and is not clear yet whether the restricted capacity of gonococci for invasion is due to the lack of capacity to invade on the part of the bacteria or to the efficiency of local non specific defense mechanisms like inflammation. The acute inflammatory cell infiltration may play an important part in the localization of gonococcal infection.

During our work on experimental infection in guinea pigs using open ended subcutaneous chambers coated only by connective tissue at each end, we have observed that the inflammatory infiltrate collected in the chambers is similar to that seen in humans. Untreated infections in guinea pigs may last several weeks, but the infection remains localized and the cellular infiltrate retains its acute characteristic with high proportion of PMN (1,2). This is in contrast with other bacterial infections which will progressively attract mononuclear cells, and suggests that the gonococcus may direct the host towards the maintenance of an acute inflammatory response. This may include induction of the production of particular cytokines that would promote the formation of an acute exudate during prolonged infection.

We studied the production of TNFa in guinea pig subcutaneous chambers during infection with LPS variants D1 and D2 of strain Gc40 and strain MS11JKD288. These strains differ in virulence as measured in this model, D1 and D2 are the most and the least virulent respectively, while JKD288 shows intermediate virulence. D2 produces only mild short infections which resolve by self cure. Each animal had two chambers, only one of which was inoculated with $5x10^4$ gonococci. Samples of chamber fluid were taken from the two chambers in each animal before inoculation, at the time of inoculation and at times during infection. The samples were used to determine TNFa production, kinetics of infection, and the type of cells present in the chambers. TNFa was measured by the biological L929 cells assay using recombinant TNFa as control.

TNFa was first detected at one hour after inoculation. The amounts detected at one hour were small, about 20 U/ml but were consistently found in all infected chambers. At 6 hours after inoculation all infected chambers had high levels of TNF varying between

200 and 800 U/ml. TNF levels peaked in most chambers between 6 and 24 hours. The highest amount detected was 820 U/ml and the lowest was 78. The differences seen in the size of the response in different chambers may represent individual variations between the animals but may clearly be due to a lack of correspondence between sampling and TNF peak times. Surprisingly, TNF was detected later during infection. Several samples obtained at 4, 7, and 15 days of infection had high levels of TNF and in some cases the highest levels were seen at 7 and 15 days. TNF was not found in samples obtained before inoculation nor at any time point in any of the samples from the non inoculated chambers carried by the same animals.

Viable counts were made on samples obtained at 24 hours and from then on at every subsequent time point. A correspondence between the numbers of cfu and TNFa U/ml was found only at 24 hours of infection. On later samples the amount of viable bacteria and detectable TNF appeared to vary independently. The strain used for infection did not have any effect on the size or kinetics of the TNF response.

During prolonged infections with JKD288 a peculiar pattern of TNFa production was observed. Several days after the initial peak seen early during infection, TNF production increased again at various times during infection. Some chambers had further TNF peaks at 14 or even 20 days of infection. Again, the size and time of the TNF response did not match variations on the number of viable gonococci in the chambers. No direct correlation was found between variations of TNF values during prolonged infections and variations in numbers of viable gonococci or cells in the chambers. This was expected, as the amount of a given cytokine detected during infection is most likely to be the result of a multifactorial system of stimuli, responses, and feed back mechanisms, with more than one gonococcal factor and host cells operating simultaneously.

In an attempt to find out whether gonococcal components other than LPS could be involved in induction of TNF production, C3H/He (LPS responsive) and C3H/HeJ (LPS non responsive) mice were inoculated intravenously with gonococcal LPS (20 μg), live gonococci ($\log_{10}$ 8), or dead gonococci (1 mg). TNF was measured in serum from tail vein blood obtained at 1 hour after inoculation. Sera from a group of uninoculated mice were used as controls. C3H/He mice produced high levels of TNF in response to all stimuli. As expected C3H/HeJ mice did not produce TNF after stimulation with purified LPS. They produced only non significant trace amounts after stimulation with live gonococci. After stimulation with 1 mg of dead gonococci C3H/HeJ mice responded with low levels of TNF. This suggests that although gonococcal products other than LPS might induce TNFa production, LPS or their combination with LPS may be needed to initiate stimulation.

Local production of TNFa, IL-6, and IL-8 has been shown in experimental urethral infections by *N. gonorrhoeae* in human male volunteers (3). Our current work is focused on the analysis of the role these and other cytokines in the subcutaneous chamber model of gonococcal infection which allows the study of prolonged infections and the effect of modulation of cytokine production (4,5).

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A strategy for constructing mutant strains of *Neisseria gonorrhoeae* containing no new antibiotic resistance markers using a two gene cassette with selectable and counterselectable markers, and its use in constructing a *pgm* mutant for use in human challenge trials

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Neisseria gonorrhoeae is the etiological agent of the sexually transmitted disease gonorrhea and an obligate human pathogen. Our lab, in collaboration with the laboratory of Myron Cohen, is using an experimental infection model with human male volunteers to study the early events in gonococcal urethritis and the requirement for putative virulence factors *in vivo* (1). Constructing genetically defined mutants for use in human challenge experiments is complicated by several restrictions, first of which is our desire to avoid the introduction of new antibiotic resistance markers into the strains to be tested. To create isogenic gonococcal mutants defective in the expression of putative virulence factors, we have adapted a two step mutagenesis strategy that allows for gene replacement without the introduction of new selectable markers in the final strain. The strategy uses a two-gene cassette containing both a selectable marker (*ermC'*) and a counterselectable marker (*rpsL*) (2). The cassette is cloned into the gene of interest, and the inactivated gene is used to replace the wild type gene on the chromosome of a Str^R strain by transformation and allelic exchange, selecting for erythromycin resistance. The resulting transformant is a Str^R/Str^S merodiploid and is relatively Str^S, because of the dominance of Str sensitivity over resistance. To replace the cassette-containing gene, a second transformation is done using a cloned gene copy with a deletion or linker insertion mutation as the donor, and the Erm^R/Str^S transformant as the recipient. Selection for the regain of high level Str^R results in a final strain with the same resistance phenotype as the original parent strain, but with the gene of interest inactivated. Using this approach, we have successfully engineered several mutants and tested their infectivity in the human challenge model. This technique has proven successful in creating genetically defined mutants for human challenge studies, and is broadly applicable to neisserial mutagenesis.

We demonstrated the feasibility of this approach by constructing a strain producing deep rough lipooligosaccharide (LOS) as a consequence of mutational inactivation of the gene encoding phosphoglucomutase (*pgm*) (3). Gonococcal LOS is believed to play an important role in pathogenesis. LOS undergoes high frequency antigenic variation, demonstrates molecular mimicry of human glycosphingolipids, and contains binding sites for bactericidal antibodies. Sialylation of gonococcal LOS affects several properties of gonococci, including serum resistance and invasion of epithelial cells *in vitro*. To determine if a complete LOS structure is required in pathogenesis, we used the two step counterselection strategy to engineer an isogenic *pgm* mutant of strain FA1090

(FA1090*pgm*). We cloned the *pgm* gene from strain FA1090 using PCR amplimers based on the sequence of the gene from strain 1291 (4), and constructed a mutant of FA1090 with a linker insertion in *pgm*. The mutant strain expressed a single truncated LOS species that did not bind mAB 3F11, lacked the LNnT structure, and had identical growth rates and outer membrane profiles as the parent strain. Strain FA1090*pgm* was non-infectious in the human challenge model. The results of the human challenge trials are described in more detail by Cannon et al in an accompanying abstract.

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Isolation of the outer membrane of *Neisseria gonorrhoeae*

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The outer surface of *Neisseria gonorrhoeae* interacts with the host to establish and maintain infection. Conversely, host defenses focus on exposed components of the outer membrane. Our studies center on the identification and characterization of outer membrane components. The goal of these studies is to find unique, constitutively expressed, invariant exposed outer membrane proteins which could be used in improved diagnostic assays or immunoprophylaxis. The physical isolation of gonococcal outer membranes is instrumental in identifying and confirming the location of outer membrane proteins.

A variety of methods have been used to isolate gonococcal "outer membrane" proteins for use in structural, functional and Immunological studies. These include solubility of membrane components in N-laurylsarkosine (sarkosyl), where putative outer membrane proteins remain in the sarkosyl insoluble fraction (1,2), isolation of "serotype-specific" outer membrane vesicles (3) using high-salt and shearing followed by size exclusion chromatography and differential centrifugation to recover "naturally-elaborated membrane vesicles" (membrane vesicles released by gonococci during log-phase growth)(4). Isopycnic sucrose gradient isolation, which separates membranes from EDTA-lysozyme spheroplasts based on membrane density (outer membrane $\sigma = \sim 1.22$) has been used to define and characterize the components of many Gram negative bacteria. Definitive, density gradient purification of gonococcal outer membranes has not been reported, possibly due to the unusual o-acetylated peptidoglycan which is refractory to hydrolysis by most lysozyme preparations and the presence of molecules which cause isolated outer membranes to clump such as Opa and Pil.

This paper reports the density isolation of the outer membrane of *N. gonorrhoeae*. The gonococcal outer membrane appeared to be typical of Gram negative bacteria. The gonococcal outer membrane appeared to be typical of Gram negative bacteria. Known outer membrane components such as Por, Rmp, Lip and LOS were restricted to fractions having a density of ~1.22 while dehydrogenases, cytochrome oxidase and heme-proteins were restricted to "inner membrane" fractions in the density range of ~1.1. However, comparison of gradient-isolated outer membrane components with those obtained by several common techniques revealed both qualitative and quantitative differences. None of the techniques yielded results that were completely comparable with density-separated outer membranes.

Antiserum raised against the outer membrane fractions has provided a valuable tool to identify such proteins. This antiserum, used to screen genomic expression libraries,

identified thirteen clones expressing unique proteins. One of these, the gonococcal homolog of *Hemophilus influenzae* D15 protective surface antigen, has been characterized (see abstract by Reschke, *et al.*) and several others are being evaluated. Future studies are planned to evaluate outer membranes isolated from gonococci grown under conditions suggested to mimic *in vivo* growth including anaerobic growth, heat-stress, iron limitation and pH-stress.

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Generation of polyclonal and monoclonal antiserum to a specific epitope of the MtrC protein of *Neisseria gonorrhoeae*

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Generation of antibody reagents to defined protein epitopes for research and diagnostic purposes requires significant time and effort. Peptides must be chemically linked to carrier molecules such as KLH or BSA which are, themselves, immunogenic. Extensive purification or clonal screening is needed to obtain the desired antibody reagent.

We used recombinant techniques and a multiple antigenic peptide system (MAPS) to generate polyclonal and monoclonal antibodies to a putative surface epitope of the MtrC lipoprotein. MtrC is a central component of an efflux pump (1) that contributes to gonococcal resistance to hydrophobic reagents and antibiotics. Computer analysis identified the sequence ^{119}-ISKQEYDAAVTAK-^{131} (MtrC Antigenic Peptide 1 - MtrC AP1) as a highly antigenic, possibly exposed epitope of this important protein.

DNA encoding the MtrC AP1 sequence was genetically fused to maltose binding protein. The resultant fusion protein was used as the primary immunogen to stimulate MtrC AP1-specific antiserum in rabbits. Boosting immunizations were performed with a synthetic octomeric MAPS immunogen. The resultant antiserum recognized the MtrC protein on Western blots and appeared to bind native MtrC protein *in situ*, showing bactericidal activity. Multiple protocols were used to immunize mice with MtrC AP1. Several clones producing MtrC AP1-specific monoclonal IgG were isolated and expanded. The resultant monoclonal antibodies were characterized by ELISA and Western blotting. These techniques make it possible to readily generate epitope-specific polyclonal and monoclonal antibody to evaluate surface availability, antibacterial activity and to produce antigen capture and signal monoclonal antibodies for diagnostic studies.

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Replication origins of b-lactamase-producing plasmids of *Neisseria gonorrhoeae.*

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Plasmid replication in Gram-negative bacteria may be divided into two classes, depending on the absence or presence of a plasmid-encoded protein (Rep protein) for replication initiation (1). ColE1-type plasmids exhibit an inhibition-target mechanism, using a counterscript RNA molecule as the main inhibitor in the control of initiation of replication. The other group of replicons uses an essential Rep protein, a cluster of direct repeats (iterons), binding sites for the DnaA protein, and A-T-rich sequences. Unlike ColE1-type plasmids, this latter group does not require PolI for replication initiation.

In isolates of *Neisseria gonorrhoeae*, penicillinase production is plasmid mediated. These plasmids have been separated into a related family of six types, based on their geographical source of isolation and size. They include the prototype Asia-type plasmid (e.g. pJD4, 7426 bp), the Africa-type (e.g. pJD5, 5599 bp), the Toronto-type (e.g. pJD7, 5154 bp), the Rio-type (e.g. pGO4717, 5154 bp, possibly identical to the Toronto-type), the Nnmes-type (e.g. pGF1, 6798 bp), and New Zealand-type (e.g. pAS84/417, 9309 bp) (2) (3).

The origins of replication of pJD4 were localized using branch-point analysis in an *E. coli* background. The plasmid carried three origins of replication, designated ori1 ori2, and ori3. Plasmid pJD5 contains only one of the three origins found in pJD4 (ori1), and EM analysis demonstrated that this origin was the least one preferred in pJD4. The remaining two origins (ori2 and ori3) of pJD4 were confirmed by the construction of pJD9, a deletion derivative of pJD4. DNA sequencing and EM analysis confirmed that the origin of replication found in pJD5 was not present in pJD9. Plasmid replication in pJD4, pJD5, and pJD9 was unidirectional.

Some naturally occurring -lactamase-producing type plasmids of *N. gonorrhoeae* representing each type were tested in an *E. coli* background for their dependence for DNA polymerase I. The Asia-type plasmid, pJD4, is able to maintain itself in both wild-type and polA- hosts, as can its insertion (duplication) derivative pAS84/417 (New Zealand-type), and its deletion derivatives pJD7 (Toronto-type), and pG04717 (Rio-type). DNA sequencing studies supported the concept of a Rep protein dependent mechanism of initiation. These plasmids contained features similar to the *ori*C of E. coli (1,4), such as DnaA boxes, integration host factor (IHF) sites, multiple repeats, and A-T rich sites. These features indicate the capacity for an iteron-based mechanism of plasmid initiation (1).

We used classical incompatibility experiments (5) involving resident and incoming plasmids to test for establishment and maintenance of more than one plasmid in a cell to study the incompatibility of pJD9. The origins of replication in pJD9 were found to belong to the incompatibility group W(6). At the present time, we are unable to differentiate whether ori2 or ori3 was the dominating incompatibility determinant. The two origins of pJD9 are further being investigated for presence of another incompatibility determinant, consistent with the presence of a second origin. pJD9 was found to be incompatible with pJD4, as expected.

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Characterization of an 85 kDa outer membrane protein of *Neisseria gonorrhoeae* having homology with the D15 surface protective antigen of *Haemophilus influenzae*

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A gene expressing an 85 kDa outer membrane protein from *Neisseria gonorrhoeae* FA19 was cloned and sequenced. Western blot analysis showed that polyclonal rabbit antiserum directed against a purified gonococcal outer membrane fraction (1 and see Judd, *et al.*) bound to a similar molecular mass protein in gonococcal strains FA635, FA1090, JS1, and F62 and in *N. meningiditis* strains, MP3, MP78, MP81 and HH. Southern blot analysis demonstrated that the protein was encoded by a single copy gene in all of the above strains. Computer analysis of the sequence showed a typical signal peptide (2) indicating the protein was located in the outer membrane. A BLAST search (3) revealed significant homology with a surface antigen of *Haemophilus influenzae* (4), antibodies against which provide protection against challenge in mice (5). Homology was also observed with OMP1 in *Brucella abortis* (6). Both of these organisms are human pathogens which suggests that the 85kDa outer membrane protein described here is possibly virulence associated.

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Recombinant CTB-linked mucosal immunogens for inducing antibodies in secretions

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The generation of antibody responses at the mucosal surfaces, including those of the genital and upper respiratory tracts, is most effectively accomplished by administration of vaccine antigens at one of the inductive sites of the mucosal immune system. While the best known of these is represented by the gut-associated lymphoid tissue, recent attention has turned also to the lymphoepithelial structures of the upper respiratory tract, such as Waldeyer's ring in humans, or its functional equivalent, the nasal lymphoid tissue of rodents. Intranasal (i.n.) immunization of mice or rhesus monkeys with a bacterial protein antigen chemically conjugated to cholera toxin (CT) B subunit induces strong IgA antibody responses in saliva and secretions of the intestinal, respiratory, and genital tracts, as well as serum IgG antibodies (1,2). Furthermore, mice immunized i.n. with pneumococcal surface protein A (PspA) and CTB develop protective immunity against nasopharyngeal carriage of pneumococci as well as against lethal infection (3). Although the enteric adjuvant effect of CTB may require the synergistic action of intact CT, recombinant CTB completely lacking the toxic A subunit functions effectively as an adjuvant by the i.n. route (4).

A more convenient approach for the development of hybrid immunogens, however, is to construct genetically engineered chimeric proteins, but the direct fusion of large segments of protein to CTB usually disrupts the ability of CTB to assemble into G_{M1} ganglioside-binding pentamers which are important for immunogenicity. To overcome this problem, we have modified the pET20b(+) vector (Novagen) by ligating DNA specifying the A2 and B subunits of CT into the multiple cloning site to form $pCT^{deltaA1}$ (5). DNA encoding a large (42 kDa) segment of a model bacterial protein antigen, AgI/II *from Streptococcus mutans*, was then inserted upstream of and in frame with the CTA2 gene segment, and behind the *pel*B leader. When this plasmid was expressed in *E. coli* BL21 (DE3), an AgI/II-CTA2 fusion protein and CTB subunit were synthesized separately and assembled into a chimeric protein in which the toxic A1 subunit of CT was totally replaced by the AgI/II segment (5). G_{M1} ganglioside-binding activity of the CTB component and antigenicity of the AgI/II segment were demonstrated by G_{M1}-ELISA, and the chimeric protein was purified chromatographically and characterized by SDS-PAGE and western blotting. Intragastric or i.n. administration of small doses of this protein to mice generated high levels of salivary and other mucosal IgA and serum IgG antibodies to AgI/II that persisted for a prolonged period, at least 11 months (6). The responses to AgI/II compared favorably with those to CT itself, a well-known potent mucosal immunogen. Similar CTA2/B chimeric proteins have been constructed from

group A streptococcal M protein and PspA, and DNA specifying other protein antigens identified as potential protective immunogens can be readily inserted into the plasmid construct. Because CTB-coupled immunogens induce strong mucosal and circulating antibody responses, we believe they may have application in the development of vaccines against numerous mucosally acquired infections, including those of the respiratory and genital tracts.

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Opacity protein expression by organisms recovered from volunteers infected with transparent *Neisseria gonorrhoeae* MS11mkC

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MS11mkC harbors 12 *opa* loci; 11 loci appear to be fully functional (1-3). In experimental infections where 34 human male volunteers were inoculated intraurethrally with piliated (P^+) transparent (Opa^-) strains of *Neisseria gonorrhoeae* MS11mkC (4-6), onset of clinical urethritis was preceded or accompanied by a phase change to the opaque (Opa^+) phenotype (4). Fourteen volunteers were reinfected with the homologous strain three weeks after the initial infection. Outer membrane proteins were extracted from amplified single colonies, or whole plates of primary isolates from infected volunteers. Opa specific MAb 4B12 (provided by Milan Blake) was used to identify opa proteins on western immunoblots and size was estimated by comparison with standard molecular weight size markers (BioRad) using a 420oe scanning densitometer and QS30 software (pdi, New York). Opa protein nomenclature was based on previously described determinations (1, 7-9). Among the ten opa proteins identified, some patterns occurred repeatedly. OpaK, the most frequently detected protein, was identified in gonococci isolated from all but one volunteer, and persisted over days. OpaK adheres to and mediates invasion of epithelial cells, while other opa proteins interact with human leukocytes (8). OpaI and OpaH predominated in isolates from people with blood type O+, and OpaF in type B+ and AB+ individuals. The predominance of specific opa proteins in a given host suggests clonal selection by host-pathogen interactions, in which the organisms expressing proteins that are most efficient in binding to and invading neutrophils (8,9) will find conditions for multiplication more favorable . At the same time, opa phase variation allows constant low-level cycling of the entire opa repertoire during an infection. Thus, gonococci expressing opa proteins with optimal affinity for particular host receptors would become predominant, and persist against a varying background of the other opa proteins during the course of infections.

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Characterization of *Neisseria gonorrhoeae* strains isolated from a conjunctivitis outbreak.

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Penicillin was introduced for the first time in 1940 in the treatment of gonococcal infection, and in the following decade the use of a single dose was the effective standard treatment. A few years later some strains having a decreased sensitivity appeared, and to control the disease the use of 4.8 million units as therapeutic dose were effective for a while (1). In 1976 the first penicillinase-producing *Neisseria gonorrhoeae* (PPNG) strains brought in simultaneously in England and USA, it was demonstrated that the strains showing high penicillin-resistance level had two types of resistance plasmids (plasmids R) with a molecular size of 3.2 Mda (African type), and 4.4 - 4.7 MDa (Asian type) respectively. Four new types of plasmids R have been described from that time on, and this way of resistance is wide distributed globally (2).

Neisseria gonorrhoeae has shown a remarkable adaptation to the evolutionary pressure made by the treatments used in the last 50 years, that is why the epidemiological studies and the disease control are based on the analysis of the distribution and behavior of the antimicrobial-resistant strains (3). Few antimicrobial susceptibility studies by Minimal Inhibitory Concentration Method have been carried out in our country with *N. gonorrhoeae*, and remains unknown which types of plasmids are responsible for the high resistance levels to penicillin and tetracycline.

We aim at knowing the susceptibility to penicillin and to tetracycline, as well as the plasmid profile in a group of *Neisseria gonorrhoeae* strains isolated from patients with conjunctivitis, and in this patients the use of penicillin brought about complications and sequelae.

Methods. A total of six strains were sent to the National Reference Laboratory of Pathogen Neisseria at IPK from the Provincial Center of Hygiene and Epidemiology at Camaguey Province. Those strains were confirmed *Neisseria gonorrhoeae* because of their growth in a selective medium of modified Thayer and Martin, Gram staining, positive oxidase and superoxol, and utilization of carbohydrates (4).

The agar dilution base GC plus Isovilatex supplement at 1% was used to determine the minimal inhibitory concentration, following the suggestions of the National Committee for Clinical Laboratory Standards, and of CDC, Atlanta (5). The detection of betalactamase was carried out using chromogenic cephalosporine using disks impregnated with nytrocephin (Unipath).

Extraction of the plasmid was under taken accordingly to the method described in the Protocol Guide and Applications, Promega (6).

Results. The conjunctivitis produced by *Neisseria gonorrhoeae* is one of the less frequently clinical manifestations of gonococcal infection. Some authors from all over the world have described gonococcal conjunctivitis outbreaks affecting different age groups, but mainly < 5 years (7).

A total of 6 cases of this disease in young adults were notified in one province from May to July 1995. It was found neither relation between them nor the source of infection though the clinical and epidemiological analysis was carried out. Each case presented severity of the ophthalmic invasive clinical manifestations and slow response to the antimicrobial therapy.

A complete study of this phenomenon, will necessarily depend on the services of a National Reference Laboratory, and a characterization of each patient isolation would be possible.

When analyzing our results, it was found that all strains were penicillinase-producing, showed high resistance levels to penicillin (MIC > 16 μg/mL), were resistant to tetracycline (MIC > 2-8 μg/mL) and had identical plasmid profiles (2.6 MDa, 3.2 MDa and 24.5 MDa.)

In a study carried out by Ebong et al, 68% of the *Neisseria gonorrhoeae* isolations from a gonococcal conjunctivitis were penicillinase-producing, showed high level of resistance to penicillin, and had any kind of plasmid R (8).

It should be highlighted that plasmid 3.2 MDa together with the conjugative plasmid 24.5 MDa are found in our isolations. Most of the outbreaks caused by penicillinase-producing strains are associated with few phenotypes accordingly to the MIC values and to the plasmid profiles, and many of them have no conjugative plasmids. In 1981, Dilon and Pauzé reported 5 PPNG strains that showed 3.2 and 24.5 MDa plasmid combination (9).

Though this was an unusual fact in literature, now there are authors from Spain, Uruguay, Argentina and Hawaii that have reported high percentages of strains having both plasmid types. Of the total strains that had 3.2 MDa plasmid they reported 50%, 95%, 87% and 58,3% respectively (10).

The homogenicity of the results in the virulence and epidemiological markers used is suggestive, in spite of the fact that this study was carried with few strains. The results contribute to the knowledge of the invasive behavior of the disease, and at the same time, it is made clear how antimicrobial susceptibility and plasmid profile studies help to understand the dynamic of an outbreak, and to determine the transmission patterns of a gonococcal infection, if they are applied early.

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Antimicrobial susceptibility of 42 *Neisseria gonorrhoeae* strains isolated in 1995 in Cuba.

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Antimicrobial resistance in *Neisseria gonorrhoeae* is an increasing and costly public health problem in the world, which can contribute to increased morbidity and complications associated with gonorrhea, making its management more difficult (1). In 1995, there were 41,185 confirmed cases of this disease reported to the Public Ministry of Health in Cuba (2). In this country, there exists few studies about the susceptibility of gonococcal isolates to antibiotics, which generally deal only with the detection of the ß lactamase enzyme (3). Penicillin is the drug recommended for the treatment of uncomplicated gonorrhea in Cuba (4). This study was conducted to determine the susceptibility of 42 gonococcal strains isolated in 1995 in Cuba to the antibiotics recommended by the WHO for the treatment of uncomplicated gonorrhea.

Methods. Forty two gonococcal strains isolated from patients with genital infections from some Cuban provinces occurring during the first six months of 1995 were sent to the National Reference Laboratory for Pathogenic Neisseria at the Tropical Medicine Institute "Pedro Kourí", Havana. The strains were identified as gonococci by standard procedures (5) and were stored frozen at - 70 °C. in trypticase soy broth (Unipath) containing 20 % glycerol until tested. Antimicrobial susceptibilities to penicillin G, tetracycline, spectinomycin, ceftriaxone, cefotaxime, cefuroxime, and ciprofloxacin were determined on GC agar base medium (Unipath) supplemented with 1% Isovitalex (Becton Dickinson) as described previously (6).

Antibiotics were supplied by the manufacturer as pure powders and used according to their instructions. *N. gonorrhoeae* ATCC 49226 was used as a control strain. A control plate without antibiotics was also included. The susceptibility of a strain to an agent was defined as the MIC, i.e., the lowest concentration inhibiting growth to ≤ 1 CFU. The results were interpreted according to the recommendations of the NCCLS and the CDC (1,6). ß lactamase production was detected using the chromogenic cephalosporin, Nitrocefin (Unipath). As positive and negative control strains were used *N. gonorrhoeae* WHO E and WHO A, respectively.

Results: Susceptibility to penicillin: Of the strains evaluated, 57,1% and 28,6 % resulted resistant and less susceptible, respectively, and only 14,3 % were entirely susceptible. The proportion of penicillinase (ß lactamase) producing *N. gonorrhoeae* (PPNG) MIC ≥ 16,0 µg/mL to penicillin and ≤ 16,0 µg/mL to tetracycline was 45,2 % (20/42).

Susceptibility to tetracycline: The proportion of strains that resulted resistant, less susceptible and susceptible were 43%, 19 % and 38 %, respectively. A total of 4 strains were presumably identified as a plasmid-mediated tetracycline-resistant *N. gonorrhoeae* (TRNG) MIC ≥ 16,0 µg/mL to tetracycline/ß lactamase negative. A total of 35.7 % of the strains was multi-resistant to both penicillin and tetracycline.

Susceptibility to other antibiotics: All the strains were susceptible to spectinomycin, cefuroxime, cefotaxime, ceftriaxone and ciprofloxacin, and the MIC 90 for such drugs were 10 ug/mL, 0.125 ug/mL, 0.012 ug/mL, 0,008 ug/mL and 0.004 ug/mL, respectively.

Comments and Recommendations. In this study we found that the occurrence of less sensitive and resistant strains of *N. gonorrhoeae* to penicillin and tetracycline and also the resistance level encountered for both drugs were very high.

Delgado et al. in some gonococcal strains isolated in 1983-1985 in Cuba, found no resistance to penicillin, but 63 % of the strains presented a diminished susceptibility to this antibiotic (7). However, some studies sporadically carried out in the Havana population have shown a high proportion of ß lactamase-producing *N. gonorrhoeae* isolates (3). We can construct the hypothesis that in Cuba there is an endemic-high gonococcal resistance to both penicillin and tetracycline.

Similar results have been reported almost from some countries in the Far East and Africa (8,9). However a recent study developed in Kingston, Jamaica also revealed an extremely high gonococcal resistance to penicillin and/ or tetracycline (10).

Could the same phenomenon be occurring nowadays in other Caribbean islands? We consider it obvious that some investigation on this matter should be done in the future in that geographical area.

As in previous reports from other countries, the rest of the drugs tested were shown to be very effective against *N. gonorrhoeae* (1,8).

Although the data presented here may not be representative of the Cuban population, they represent an attempt to analyze antimicrobial susceptibility of gonococcal isolates in this country. According to our results, neither penicillin nor tetracycline can any longer be regarded for the treatment of uncomplicated gonococcal infections in Cuba. Other regimens which have been proven to be effective against *N. gonorrhoeae*, i.e., wide-spectrum cephalosporins, quinolones or spectinomycin which are recommended by the WHO and the CDC to combat those infections should be used in Cuba.

We recommend that this study be extended to the rest of the Cuban provinces as soon as possible. Thus, it is mandatory to continue monitoring antimicrobial susceptibility in gonococcal isolates in this country, which can provide the necessary information to support local gonorrhea program efforts and guide the selection of the most effective therapeutic agents for the treatment and control of that disease in Cuba.

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In vitro induction of inflammatory cytokines by *Neisseria gonorrhoeae* and by gonococcal antigens

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Neisseria gonorrhoeae infections cause a localized inflammatory response which is accompanied by elevated levels of inflammatory cytokines (Il-6, Il-8, TNF-α) in the urine of experimentally challenged males (1,2). An in vitro tissue culture monolayer stimulation assay (3) has been developed to examine the induction of cytokine production by virulent gonococci and by gonococcal antigen extracts. *N. gonorrhoeae* has previously been shown to invade the ME180 (ATCC HTB 33) cell line (4), which is derived from a human cervical epidermoid carcinoma. In the present studies, we stimulated ME180 monolayers, grown in 24-well tissue culture plates, with live *N. gonorrhoeae* MS11mkC or antigen extracts [lipooligosaccharide (LOS), pili or outer membrane complex (OMC)] derived from Pil^+Opa^+ MS11mkC. Kinetics of Il-6 and Il-8 production were measured in the culture supernatant by testing samples taken at 2, 5 or 24 h following addition of the stimulant. Pil^+Opa^+ and Pil^+Opa^- phenotypic variants of MS11mkC, added to 48 h monolayers at about 50:1 bacteria:cell ratio, both stimulated about equal levels of Il-6 and Il-8 production. Cytokine levels reached a peak at the 24 h time point. The OMC (Pil^+Opa^+) was also able to induce Il-6 and Il-8 production when added at a concentration as low as 1 μg/ml. At 5 μg/ml, Il-6 and Il-8 were detected as early as the 2 h time point. The most rapid rise was seen between 2 and 5 h; levels continued to rise up to 24 h. Pili alone (10 μg/ml) was also able to stimulate Il-6 and Il-8, although its activity was reduced compared to OMC (10 μg/ml). LOS (10 μg/ml) used alone was unable to stimulate any cytokine production. A low level activity was seen when LOS and pili were used together (both at 10 μg/ml), but the activity was not greater than that of pili alone.

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Differential antibody and cytokine responses in male volunteers experimentally infected with *Neisseria gonorrhoeae* MS11mkC

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The effect of challenge dose size and duration of gonococcal infection upon cytokine (1) and antibody responses was studied in three groups of male volunteers experimentally challenged intraurethrally (2) with *Neisseria gonorrhoeae* strain MS11mkC. The three groups were challenged with 7000 cfu, 5700 cfu and 57,000 cfu, respectively, and infection rates were 5/10, 5/10 and 14/15. Group 1 was treated as soon as infection was diagnosed, Group 2 at 48 hours post diagnosis and Group 3 at times varying from 24 to 99 hours post diagnosis. All infected volunteers developed Il-8 detectable in the urine while the rate of positive urine Il-6 responses rose with increased time of infection and size of challenge dose. Time of onset of urine cytokine increase (24-48 hours after challenge) did not vary among the three groups; responses waned within the same period, approximately 48 hours, after antibiotic treatment. Peripheral blood IgG, IgA and IgM antibody secreting cell (ASC) responses against MS11mkC LOS, pili and outer membrane complex (OMC) were measured in order to assess B cell responses following infection in the urethral mucosa. ASC responses were detected in only 2 of 5 infected volunteers in Group 1; in Groups 2 and 3, the longer duration of infection resulted in positive responses in 4 of 5 and 13 of 14 infected volunteers, respectively. In contrast to responses to infection at other mucosal sites in which ASC responses peak around 6-10 days (3), ASC responses in these studies did not peak until 10-12 days after challenge. The highest numbers of ASC were detected in Group 3, and the responses were most often detected with the OMC antigen, a native vesicular extract (4) incorporating both pili and LOS. Serum antibody responses against LOS, pili or OMC were detected at low rates(<50% in almost all cases) with IgG against LOS the only response consistently seen in all three groups.

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Cellular immune response to *Neisseria gonorrhoeae* proteins in patients with urogenital gonorrhea

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Urogenital infection with *Neisseria gonorrhoeae* is a mucosal disease . Little is known about the immune response to this sexually transmitted disease. The anti-gonococcal humoral immune response has been investigated in patients with gonorrhea and it has been demonstrated that serum antibodies to certain *N. gonorrhoeae* outer membrane proteins correlates with immune protection from reinfection (1). However, there is a paucity of data examining the cellular immune response to *N. gonorrhoeae*. Particularly, whether T cell anti-gonococcal reactivity is present in individuals infected with gonorrhea and is it associated with protection from disease. In this preliminary study, the cellular immune response to gonococcal infections has begun to be examined.

The proliferative response of peripheral blood mononuclear cells (PBMC), isolated from *N. gonorrhoeae* infected individuals was measured, as determined by ^{3}H-thymidine incorporation assay (2), to the gonococcal proteins protein IA (PIA), protein IB (PIB) or GroES, an 11 kDa heat shock protein (obtained from Dr. William Shafer, Emory University School of Medicine). In 44% (8/18) of infected individuals we observed antigen-specific proliferation to both PIA and PIB (Stimulation Index (SI) >2). However, no antigen-specific proliferative response could be detected when the PBMC were stimulated with either GroES (SI, 1.3±0.6) or a non-relevant control antigen GST (SI, 1.1±0.6).

We also examined the cytokine profile of PIB stimulated PBMC using intracellular staining and FACS analysis (3). A significant percentage of T cells from gonococcal infected individuals produced IL-4 upon 7 days of incubation with PIB. Eleven to 37% of $CD4^{+}$ T cells were IL-4^{+}. Interestingly, we also observed that 15 to 39% of PIB stimulated $CD8^{+}$ T cells were also IL-4^{+}. Much smaller percentages of T cells when incubated with media alone produced IL-4 ($CD4^{+}$ 7% and $CD8^{+}$ 6%). No significant staining for IL-2, IFNγ or TNFα was observed in either PIB activated $CD4^{+}$ or $CD8^{+}$ T cells.

These preliminary data indicate that in some individuals, a portion of the cellular immune response to *N. gonorrhoeae* is directed against Protein I (Por). Furthermore, our results suggest that infected individuals mount a Th_2 type response to *N. gonorrhoeae*. These findings correlate with previous data demonstrating that individuals with mucosal *N. gonorrhoeae* infection develop antibodies that recognize the major gonococcal outer membrane proteins, especially the porins. Even though anti-gonococcal T cell reactivity

alone might not be protective, these T cells could offer help to B cells producing the potentially protective anti-gonococcal antibodies.

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

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

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

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

To determine if Opa binds PK in vivo, rabbit antiserum was raised against recombinant human PK subtype M2 and used to determine if PK surrounds *N. gonorrhoeae* within host cells. Opa-expressing gonococci were allowed to invade ME-180 human cervical epithelial cells for 4 hours, the cells were fixed and probed with anti-PK antiserum followed by fluoresceinated anti-rabbit antibody, and visualized via confocal fluorescent

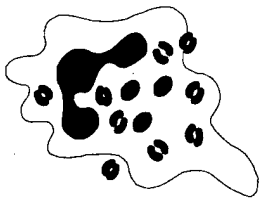
microscopy. These in vivo studies revealed that intracellular, but not extracellular, gonococci bind PK.

Gonococci are thought to use only three carbon sources - glucose, pyruvate, and lactate (7). Intracellularly, there is little available glucose, since glucose is present mainly as glucose-6-phosphate. Pyruvate, on the other hand, can be continuously produced intracellularly. It appears that intracellular gonococci bind active PK, and use it to supply themselves with pyruvate. Additionally, this interaction may enable intracellular gonococci to interact with the T3 metabolic pathway.

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

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Lipooligosaccharides

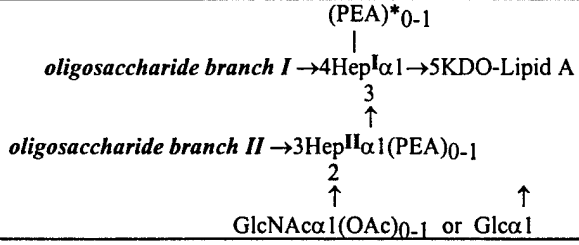
The lipooligosaccharides of the pathogenic *Neisseria.*

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The LOS of pathogenic *Neisseria* are distinct structures which have functions and features which have been appropriately adapted to allow the pathogenic *Neisseria* to be successful human pathogens (1).

Table 1: Major *Neisseria* LOS Structures



Oligosaccharide branch structures I and II	Comments
Galβ1→4Glcβ1-(I)	Lactose
Galβ1→4Glcβ1-(I), Galβ1→4Glcα1-(II)	Lactose(α, β)
Galα1→4Galβ1→4Glcβ1-(I)	P^k antigen
GlcNAcβ1→3Galβ1→4Glcβ1-(I)	L6 serotype
Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Glcβ1-(I), Glcα1-(II)	L5 serotype
Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-(I)	Lacto-*N*-neotetraose
Neu5Acα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-(I)	Sialyllactosamine
GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-(I)	GalNAc-capped

Abbreviation: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, L-*glycero*-D-*manno*-heptose; KDO, 2-keto-3-deoxy-*manno*-octulosonic acid, Neu5Ac, 5-N-acetylneuraminic acid or sialic acid.

Physicochemical analysis: A structural model has been proposed for the LOS of gram-negative pathogenic bacteria that colonize human mucosa, e.g. pathogenic *Neisseria* and *Haemophilus* (Table 1). This model shows that the deep core of *Neisseria* LOSs contain two KDO and two heptoses moieties. The proposed LOS model has several unique features that distinguish it from those developed for the lipopolysaccharides of enteric

bacteria. To simplify the nomenclature, the heptoses are numbered with the first heptose being the one directly linked to KDO. Oligosaccharide chains extending from the heptoses are designated "oligosaccharide branch I" if it extends from the first heptose and "oligosaccharide branch II" if the chain extends from the second heptose. The development of this model has involved analysis of a series of pyocin-resistant gonococcal mutants with altered LOS and other recent immunochemical and structural data. Analysis of this series of pyocin mutants revealed that the oligosaccharide attached to the first heptose, branch I, of the LOS structure was sequentially assembled to the structure Galβ1→4Glc (Table 1). After this point in assembly, divergence in assembly occurs. The Galβ1→4GlcNAcβ1→3Galβ1→4Glc structure is the predominant structure formed. The Galβ1→3Galβ1→4Glc structure can also be formed from the Galb1→4Glc.

The determinants responsible for the L3, L7, and L9 meningococcal lipopolysaccharide serotypes are situated in the oligosaccharide moiety of neisserial LOS . The striking feature of this structure is that the terminal tetrasaccharide at the non-reducing end of the oligosaccharide moiety is composed of the same terminal tetrasaccharide, (Galβ1→4GlcNAcβ1→3Galβ1→4Glc), as the carbohydrate portion of the mammalian glycosphingolipid paragloboside. Paragloboside is the precursor of the glycolipid ABH antigens of human erythrocytes. It has been shown that the non-reducing ends of an L2 and L5 meningococcal serotype LOS also contain lacto-N-neotetraose. Both meningococci and also most strains of gonococci express at least one LOS with a Galβ1→4GlcNAcβ1→3Gal trisaccharide indicating that this LOS structure is common among pathogenic *Neisseria*. Subsequent structural analysis of LOS from other gonococcal strains has confirmed that lacto-*N*-neotetraose is present in gonococcal LOS that bind the antilactosamine MAbs. It has been recently determined that the *N. gonorrhoeae,* strain 15253 has an unusual LOS structure and contains 2 lactosyl branches.

The structure of the lipid A from *N. meningitidis* and *N. gonorrhoeae* have been defined. The dominant forms of these lipid A's were hexacyl and pentacyl substituted species, with the major species being hexaacyl. *N. meningitidis* lipid A consists of a 1,4'-bisphosphorylated b(1-6)-linked glucosamine disaccharide backbone with the same fatty acid substitution pattern found in the gonococcal lipid A. However, differences were found in that the phosphate groups of the meningococcal lipid A are largely substituted with phosphoethanolamine.

Antigenic structure and molecular mimicry: It has been shown showed that anti-gonococcal LOS MAb's 3F11 and, a second MAb of a similar specificity, 06B4, could agglutinate human erythrocytes and that purified glycosphingolipids from human cells could bind the MAbs. Both MAbs could bind to a series of neutral glycosphingolipids that contain terminal Galb1→4GlcNAc (*N*-acetyllactosamine), but terminal sialic acid, galactose or fucose (Fuc) at the non-reducing end of the glycolipids blocked the binding of the MAbs.

It had been observed that gonococci in urethral exudates possess a virulence factor that is lost when the bacteria are subcultured onto an artificial medium. Attempts to purify the

inducing factor resulted in both a high and a low Mr factor being detected in extracts from human red blood cells. The low Mr activity was discovered to be the nucleotide sugar for sialic acid, cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA). Purified CMP-NANA in nanogram amounts duplicated all of the effects of the low Mr factor with gonococci including a major alteration in the strain's LOS. Immunoelectronmicroscopy of gonococcal infected male urethral exudates indicated in vivo sialylation of the 4.5 kDa paragloboside-like LOS component.

Previous studies had indicated that the 4.5 kDa lactoneoseries LOS is present in some strains of meningococcal serogroup B and C strains. However, the pattern of binding of MAb 3F11 to these meningococcal LOS was similar to the pattern of MAb binding to sialylated and nonsialylated gonococci. Since serogroup B and C meningococci synthesize a sialic acid capsule, and thus CMP-NANA, it seemed possible that endogenous CMP-NANA might also be used for sialylating LOS. Analysis of partially deacylated LOS of a serogroup B meningococcus by liquid secondary ion mass spectrometry revealed that a fragmentation pattern characteristic of a sialylated molecule was present and confirmed the terminal location of sialic acid on a tetrasaccharide consistent with lacto-*N*-neotetraose. Recent studies with defined mutations of capsule biosynthesis and transport operons of group B meningococci support these studies. They indicate that mutants defective in their ability to produce CMP-NANA are unable to endogenously sialylate their LOS. Sialylation was restored by the addition of exogenous CMP-NANA. Further study of these mutants demonstrated that LOS sialylation and (a2-8)-linked polysialic acid capsule production have a common biosynthetic pool and indicate that the endogenous LOS sialylation pathway requires CMP-NANA produced by the capsule biosynthetic system. Conversely, mutations which interfer with capsular expression following CMP-NANA biosynthesis (e.g. mutations in the polysialyltransferase [*synD*] or the capsular transport gene, *ctrA*, did not interfer with the ability of the organism to sialylate LOS. These studies also confirm that the (a2-8)-linked polysialyltransferase is not the LOS sialyltransferase and that CtrA is probably not involved in membrane transport of CMP-NANA. Most strains of meningococci that synthesize a sialic acid capsule (serogroups B, C, W and Y) and a 4 5 kDa LOS also sialylate their LOS endogenously. However, endogenously sialylated LOS are absent in meningococci that cannot synthesize sialic acid (e.g. serogroups A, 29E, X) and in non-pathogenic *Neisseria,* even those non-pathogenic strains that express the 4.5 kDa LOS acceptor for sialic acid.

Biosynthesis of *Neisserial* LOS: Attached to *Neisseria* lipid A through the conserved KDO-heptose core region (Table 1) is a variable oligosaccharide region consisting primarily of glucose, galactose, GlcNAc and GalNAc. This region is assembled through the action of glycosyltransferases which sequentially add sugar residues to a specific acceptor structure of the growing oligosaccharide chains making the oligosaccharide of an organism's LOS dependent on the glycosyltransferases that the organisms encodes. This situation is particularly well illustrated by the *lgt* gene cluster of pathogenic *Neisseria.* The *lgt* gene cluster of *N. gonorrhoeae* strain F62 consists of five genes, *lgtA-E* which act to assemble the lacto-*N*-neotetraose region of gonococcal LOS. Three of these genes, *lgtA*, *-C* and *-D* contain poly-G tracts within their coding sequences and are

potential sites of phase variation through slip-strand mispairing (see below) which is thought to contribute the LOS heterogeneity seen with *N. gonorrhoeae.*

An analogous *lgt* cluster has been characterised in *N. meningitidis* strain MC58. *N. meningitidis* can be divided into twelve different immunotypes based on the organism's LOS, although *N. meningitidis* LOS typically contains either a lacto-*N*-neotraose or digalactose terminal structure. A survey of all twelve meningococcal immunotypes using meningococcal *lgtA*, *-B*, *-C*, *-D* and *-E* genes as probes revealed that the restricted arrangement is typical of *N. meningitidis* strains and that meningococci fall into two groups, one able to express a terminal lacto-*N*-neotetraose structure, the other able to express a terminal digalactoside strucuture.

An operon responsible for the inner core biosynthesis of the LOS of *N. meningitidis* has been identified. Using *Tn916* mutagenesis, the a1,2 *N*-acetylglucosamine transferase gene (*rfaK*) was identified which, when inactivated, prevents the addition of GlcNAc to hepII of the meningococcal LOS inner core. This mutant was also deficient in the chain I oligosaccharide extension suggesting that the lack of extension of this chain was due to structural constraints imposed by the incomplete biosynthesis of the LOS inner core.

Many of the genes involved in *Neisserial* LOS biosynthesis have been cloned Each of these genes has had a function ascribed to it, through their ability to complement well-defined mutations in other systems, based on the properties of the LOS expressed when these genes are defective, or by biochemical characterization of the gene product. Most of the genes involved in the synthesis of the biosynthetic precursors are unlinked to the assembly genes in both the gonococcus and meningococcus (*pgm, galE, rfaD, rfaE*). The genes involved in the addition of heptose to KDO (*rfaC* and *rfaF*) are also unlinked. *rfaF is* part of a two gene operon, with the second gene having homology to the *smpB* gene of *E. coli.* Although mutations in the *smpB* homolog do not effect LOS biosynthesis, cell mutants in this gene are barely able to grow on agar plates, but grow normally in liquid cultured (DCS, unpublished observations).

Thus, the potential LOS repertoire of an organism is dependent on the glycosyltransferases it encodes although further complexities arise through mechanisms such as phase variation. Antigenic variation of the oligosaccharide I is due to changes in the number of guanines seen in a polyguanine tract of various glycosyl-transferase-encoding genes. Changes in these polyG tracts do not seem to explain a strains ability to express several LOS components on the surface of the same cell. It has been shown that this ability to simultaneously express multiple LOSs is due to transcriptional/translational frameshifting in this gene.

It is of note that genes whose predicted products share considerable amino-acid sequence homology with *S. typhimurium* Rfb proteins have been identified in *N. meningitidis* and *N. gonorrhoeae* . The role of the Rfb homologues is unclear, their mutation in *N. gonorrhoeae* failed to affect LOS biosynthesis, or indeed produce any detectable change in phenotype.

Many genes of central metabolism affect LOS biosynthesis through their effect on the availability of LOS components. Sugar and lipid metabolism that produces LOS components is fully integrated with central metabolism. *Neisseriae* differ in the arrangement of *gal* genes. *N. meningitidis* contains a duplication of the *galE* gene while the gonococcus genome contains only a single functional copy. *N. meningitidis* and *N. gonorrhoeae* does not appear to have other *gal* genes found in *E. coli*. This is consistent with and *N. gonorrhoeae* and *N. meningitidis galE* mutants failing to display galactose toxicity when grown in the presence of galactose.

Neisseriae phosphoglucomutase (PGM) mutants are unable to convert glucose-6-phosphate to glucose-1-phosphate and as a result are unable to synthesise UDP-glucose. *Neisseria* are unable to import galactose and thus these mutants are also unable to synthesise UDP-galactose which is formed from UDP-glucose by the action of the *galE* product. Thus, the LOS of *Neisseria* PGM mutants lacks glucose and is truncated. However, some compensatory mechanisms may exist as the LOS of *N. gonorrhoeae* PGM mutants contains some higher molecular weight structures and retains a small amount of reactivity with monoclonal antibodies which recognise terminal components of wild-type LOS.

The genetics of LOS sialylation is an area of intense research. Mutants unable to sialylate their LOS are potentially important to understanding the pathogenicity of the organism and represent high priority research goals. A sialyltransferase activity that catalyses the transfer of sialic acid from CMP-NeuNAc to the acceptor LOS has been detected in the outer membrane of *N. gonorrhoeae* and thus the enzyme is believed to be an outer membrane protein.

LOS in adherence and invasion. Recent studies have demonstrated that gonococci can invade urethral epithelial cells during urethral infection in males. As gonococci invade these cells, membrane fusion occurs suggesting that a very tight ligand-receptor based interaction is occurring between the gonococci and the host epithelial cell. Recent studies in experimental models suggest that the gonococcal LOS may be participating in the adhesion and invasion events as a ligand. Disruption of the *lsi-1* gene in gonococcal strain MS11 resulted in the production of LOS that migrated faster than that from an isogenic *galE* mutant, typical for a mutation that influences the core region. Infection experiments *in vitro* demonstrated that the *lsi-1* mutant could not invade human Chang epithelial cells despite expression of a genetically defined invasion-promoting gonococcal opacity protein. These data imply that the LOS phenotype is a critical factor for gonococcal invasion.

There is evidence that the lacto-*N*-neotetraose-containing LOS may be an important factor in these events. Studies in human experimental infection in two patients showed that the LOS phenotype of the inoculating strain shifted over 4 days from a 3.6 kDa species to the 4.5 kDa-lacto-*N*-neotetraose containing LOS species.

Recent studies in human volunteers demonstrated that inoculation of human volunteers with gonococci with *in vitro* sialylated LOS result in a marked reduction in infectivity.

Similarly, it has been shown that the lacto-*N*-neotetraose expressing meningococci were recovered systemically in the infant mouse model of meningococcal disease in animals initially infected intranasally with a L8-expressing inoculum. The importance of this finding was corroborated by analysis of meningococcal disease isolates from the Storehouse outbreak in England, which revealed systemic isolates were more likely to express lacto-*N*--neotretraose than did the nasopharyngeal isolates from the same patient.

Role of antibody to los in protection from neisserial disease. It has been shown by a number of investigators that gonococci isolated from patients with either local mucosal disease or disseminated gonococcal infection (DGI) differed in their sensitivity to the bactericidal action of normal serum - the former being susceptible and the latter being resistant. Subsequent studies showed that IgM naturally present in serum mediated both the direct complement dependent killing of the serum sensitive isolates as well as their opsonophagocytic uptake and killing by neutrophils . This IgM reacts with an epitope present on the LOS of these strains and its bactericidal potential can be inhibited by sialylation of the LOS prior to start of the experiment. Recent work by Densen and co-workers in which IgM binding to LOS purified from wild type gonococci was assessed on Western blots following absortion of IgM with the wild type strain or the 1291 gonococcal mutant series suggests that bactericidal IgM binds to a 3F11 MAb negative LOS species, that binding to this species is not affected by sialylation but that a terminal galactose may be part of the epitope recognition site. The observation that sialylation blocks killing but does not affect IgM binding to the putative bactericidal epitope on a different LOS species suggests that sialylation may interfere with the cross linking of the bactericidal epitopes that is required for IgM conformational change prior to complement activation (Abstract this meeting).

Gonococci causing DGI display a different array of LOS molecules than do isolates from patients with local disease. In particular, DGI isolates do not express the 4.5 kD LOS species and can not sialylate their LOS. Although these isolates are not killed by normal serum a given isolate is susceptible to killing when incubated in the serum from the individual in whom the isolate caused disease. It has been shown that this killing is mediated by an LOS specific IgG which can be blocked by the presence of antibody to P III, a protein present in the gonococcal outer membrane. The ratio of the concentration of these two antibodies appears to be an important determinant of the susceptibility of DGI isolates to complement dependent killing and the concentration of anti-P III itself may also be important in determining the susceptibility to disease caused by local isolates as well.

Studies of the human immune response *N. meningitidis* has demonstrated a striking inverse correlation between the presence of complement dependent bactericidal activity in serum and susceptibility to meningococcal disease. This activity was antibody (IgG) dependent and was acquired with increasing age beginning around the age of 6 months to a year. Anti-capsular antibodies were shown to be enormously effective in initiating complement dependent killing as well as opsonophagocytic killing of appropriate isolates. However, persons convalescing from meningococcal disease rarely develop a second infection despite the existence of multiple meningococcal serogroups. Those

individuals who do experience recurrent disease commonly have some immunologically identifiable defect - typically a defect in one of the terminal complement components that mediate complement dependent killing. In addition, quantitation of anti-capsular antibodies in the sera possessing bactericidal activity for a given strain demonstrates only weak correlation with the extent of that activity. In total, these observations suggest that antibodies, in addition to those directed at capsular polysaccharides, are important in mediating natural protection against meningococcal disease. This conclusion is of enormous impact for the prevention of serogroup B meningococcal disease, since the bulk of evidence suggests that immune exclusion in humans prevents them from making antibodies to the homopolymeric sialic acid which forms the capsule of this meningococcus.

A number of observations suggest that oropharyngeal colonization with relatively non-pathogenic Neisseria lactamica is responsible for the development of natural protection against meningococcal disease. Subsequent studies have provided evidence that meningococcal LOS was an important target of these antibodies. The relative protective potential of antibody to capsular antigen versus subcapsular antigens appears to differ in vitro, the former being more potent in assays of bactericidal and opsonophagocytic activity. This difference may be due to the fact that the capsular polysaccharide offers a series of repetitive epitopes which are sufficiently closely located in space to afford ample opportunity for cross linking of IgG by C1q and initiation of complement activation. Moreover binding of anti-capsular antibody to the organism surface makes it readily accessible to opsonophagocytic receptors on phagocytic cells. In contrast, the accessibility of antibody bound to subcapsular antigens to these receptors is hindered by the presence of the capsule. Nevertheless, evidence exists to support the notion that these antibodies do promote both bactericidal and opsonophagocytic activity, especially the former and that these cross reactive antibodies form the basis for a large part of the natural immunity against meningococcal disease. The epitopes on subcapsular antigens that give rise to these cross protective antibodies remain poorly defined.

Of interest are observations in patients with a late complement component deficiency (LCCD) who develop meningococcal infection and whose convalescent serum contains substantially greater amounts of antibody to subcapsular antigens than does the serum from normal individuals recovering from this infection. These IgG antibodies persist for extended periods of time, are primarily directed against meningococcal LOS, are broadly cross reactive with the LOS immunotypes associated with most cases of non-serogroup A disease, and are highly bactericidal in the presence of an intact complement system - especially against serogroup B meningococci. Assessment of the potential contribution of individual saccharide units to the IgG epitope recognition site on meningococcal LOS was carried out using the 1291 gonococccal mutant series and Western blotting techniques as described above. Absorption of the convalescent serum with either the wild type or the 1291A mutant, but not the 1291C mutant, removed the LOS reactive antibody. These findings suggest that the sub-terminal N-acetyl glucosamine may contribute to the bactericidal antibody recognition site on meningococcal LOS.

Conclusions. As we have learned more about the pathogenesis of *Neisseria* infections, the role of the LOS in instigating disease, promoting toxicity and the development of the immune response has been come clearer. The present ability to generate a wide range of LOS mutants will allow precise definition of the specific LOS components involved in these different aspects of *Neisseria* immunobiology.

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The *ice* (inner core extension) lipooligosaccharide biosynthesis operon of *Neisseria meningitidis* B.

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Lipooligosaccharide (LOS) is a critical virulence factor involved in many aspects of meningococcal pathogenesis. Gotschlich et al. (1) identified the *lgtA-E* operon required for the biosynthesis of the Galb1-4GlcNAcb1-3Galb1-4 epitope of the a-chain of LOS which is added to the substrate Glcb1-4Hep$_2$-R. We have used *Tn*916 mutagenesis to identify a second operon, termed *ice* (inner core extension), which is required for LOS inner core assembly and the addition of the a chain to this structure. We recently characterized a LOS mutant, 559, in which the a 1,2 *N*-acetylglucosamine transferase (*rfaK*) was inactivated by *Tn*916 (2). Inactivation of *rfaK* in the L2 serogroup B strain NMB results in a LOS structure without GlcNAc or glucose attached to HepII, as well as preventing the extension of a chain from HepI (ie. Hep$_2$-R). We concluded from these results that the addition of GlcNAc to HepII was a prerequisite for further LOS synthesis. During the study of *rfaK*, a second ORF (*lgtF*) of 720 bp was found upstream of *rfaK*. The amino terminus of LgtF had significant homology with a family of b-glucosyltransferases involved in the biosynthesis of polysaccharides and O-antigens of LPS (3). *LgtF* was inactivated by insertion of a non-polar *aphA-3* cassette, thereby minimizing potential polar effects on *rfaK*. Tricine SDS-PAGE and composition analysis of the LOS from the non-polar *lgtF* mutant showed that this strain produced a truncated LOS structure containing a complete LOS inner core, GlcNAc$_1$Hep$_2$-R, but without the a-chain attached to HepI or glucose to HepII. These results and the amino acid homology with b-glycosyltransferases indicate that *lgtF* is involved in LOS biosynthesis and suggest that it encodes the previously undefined UDP glucose:lipooligosaccharide b1,4 glucosyltransferase which attaches the first residue of the a chain to HepI to form Glcb1-4Hep$_2$-R which is the substrate for the *lgt* operon. RT-PCR and primer extension analysis indicate that both *lgtF* and *rfaK* are co-transcribed as a polycistronic message from a promoter upstream of *lgtF*. This arrangement suggests that the completion of the LOS inner core and the extension of the a-chain are coordinated in *N. meningitidis* through the *ice* operon.

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Lipooligosaccharides

Regulation of gonococcal sialyltransferase expression

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Strain F62 of *Neisseria gonorrhoeae* (GC) is sensitive to normal human serum (NHS) unless exogenous CMP-NANA is present. Sialic acid (NANA) is transferred primarily to a 4.5 kDa terminal galactose (gal) residue in the gal β-1,4 N-acetylglucosamine (gal-glcNac-R) lipooligosaccharide (LOS) structure by a GC sialyltransferase (Stase) (1,2). Sialylation results in an increase in LOS M_r to 4.9 kDa. Recently, lactic acid, utilized by GC from human neutrophils (3), was identified as a second serum resistance-inducing factor (4). In addition to lactate, GC use pyruvate and glucose as carbon sources. We asked the following question: Is Stase expression regulated by different environmental conditions?

We grew GC in pyruvate, lactate or glucose in plate vs broth conditions and analyzed LOS and Stase expression and serum resistance. GC grown in pyruvate broth expressed 2.4-fold more Stase activity than those grown in glucose broth (21,144 vs 8,759 cpm). When grown on pyruvate plates, GC expressed 5.6-fold more Stase activity than those grown on glucose plates (11,811 vs 2,106 cpm). Stase activity from lactate-grown GC was intermediate to pyruvate and glucose-grown GC for both growth conditions. There was also two to four-fold more Stase activity obtained from broth-grown GC than from plate-grown GC, irrespective of carbon source. Thus, Stase activity in GC is regulated both by variation in carbon source and in growth condition. We have been unable to completely repress Stase activity by growth in different environmental conditions, suggesting that basal production of Stase occurs.

LOS was analyzed from GC grown with pyruvate, lactate or glucose, under three different growth conditions: aerobic broth, aerobic plate, and anaerobic plate, and in the presence (25 μg/ml for plate, 50 μg/ml for broth) or absence of CMP-NANA. The LOS profiles of GC grown with pyruvate or lactate were identical to each other, irrespective of growth condition. Under these conditions, two additional lower M_r LOS species (4.1 kDa and 3.6 kDa) were detected, similar to those observed previously (5, 6). Additionally, GC grown in pyruvate or lactate constitutively express the sialylateable 4.5 kDa LOS species in greater quantity than the 4.9 kDa galNac-terminating species, under all growth conditions. Under aerobic conditions, glucose-grown GC express the 4.9 kDa galNac species in greater quantity than the 4.5 kDa species whereas under anaerobic conditions the 4.5 kDa species is equal or greater in quantity than the 4.9 kDa galNac species. Since there is both greater expression of Stase and the 4.5 kDa LOS when GC are grown in pyruvate or lactate than when grown in glucose, this suggests that expression of Stase and the 4.5 kDa LOS species may be co-regulated.

When GC are grown with 25 μg/ml of CMP-NANA, the NANA moiety is covalently transferred to the 4.5 kDa LOS species, causing a complete shift upward in migration to 4.9 kDa. This occurred under all growth conditions and carbon sources tested.

Since pyruvate-grown GC express more Stase activity than glucose-grown GC, we asked whether GC grown on pyruvate plates would incorporate more radiolabeled NANA onto the 4.5 kDa LOS species than GC grown on glucose plates. Pyruvate-grown GC incorporate 1.5 to 2.8-fold more radiolabeled sialic acid onto their LOS than do glucose-grown GC. There was no evidence for sialylation of other LOS species in these experiments. These results correlate with enhanced Stase activity observed for GC grown in pyruvate compared to glucose.

Since CMP-NANA concentrations may be limiting for GC in vivo, we grew GC on pyruvate or glucose plates plus 1.6 to 12.5 ug CMP-NANA per ml. At these concentrations, GC grown with pyruvate were 6.5 to 16.1-fold more resistant to 20% NHS than those grown with glucose. These results demonstrate that enhanced Stase activity in pyruvate-grown GC increases their serum resistance, and thus may have biological relevance. These data also strongly suggest that elevated Stase correlates with enhanced expression of the sialylateable 4.5 kDa LOS species and enhanced sialylation of LOS. Regardless of carbon source, at 25 μg/ml CMP-NANA GC were completely protected from killing by NHS (two to four log protection versus GC grown without CMP-NANA). No differences in serum resistance between pyruvate- and glucose-grown GC were observed when CMP-NANA was omitted.

CMP-NANA was the first heat labile compound in NHS that was found to induce serum resistance in GC. A second serum resistance-inducing factor is lactate (4), which we show increases expression of Stase activity in GC versus glucose under certain growth conditions. We speculate that GC grown in lactate would exhibit smaller differences in serum resistance and incorporation of radiolabeled NANA onto LOS than what we found between GC grown in pyruvate versus glucose, since smaller differences in Stase activity between lactate- and glucose-grown GC were observed. Indeed, pyruvate might also be a serum resistance-inducing factor in that it causes enhanced Stase expression and increased serum resistance in GC.

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The properties of a sialyltransferase-deficient mutant of *Neisseria gonorrhoeae* and studies on lactate enhancement of LPS sialylation.

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Previous studies indicate that sialylation of lipopolysaccharide (LPS) by host cytidine 5'-monophospho-N-acetyl neuraminic acid (CMP-NANA) catalysed by bacterial sialyltransferase rendered gonococci resistant to killing by phagocytes, to entry into epithelial cell-lines, to killing by immune serum and complement and to absorption of complement component C3. These results have been confirmed by comparing a sialyltransferase deficient mutant (strain JB1) (1) with its parent (strain F62) in appropriate tests (2). In contrast to F62, JB1 was very susceptible to killing by human polymorphonuclear phagocytes in opsonophagocytosis tests and incubation with CMP-NANA did not decrease the killing. The inherent resistance of F62 in these tests was probably due to LPS sialylation by CMP-NANA and lactate present in the phagocytes. A JB1 variant expressing the invasion- associated Opa protein was as able to enter Chang human conjunctiva epithelial cells as a similar variant of F62, suggesting that the sialyltransferase is not required for Opa-mediated entry. After incubation with CMP-NANA, entry of the F62 variant was drastically reduced but not that of the JB1 variant. Both JB1 and F62 were killed by incubation with rabbit antibody to gonococcal major outer membrane protein, Protein I and human complement, but only F62 was rendered resistant to the killing by incubation with CMP-NANA. Finally, both JB1 and F62 absorbed similar amounts of complement component C3 and the binding was decreased by incubation with CMP-NANA only for the wild type, F62.

The low Mr factor in blood cell extracts which enhances LPS sialylation and induction of serum resistance in gonococci by CMP-NANA has been identified as lactate (3). The mechanism of enhancement by lactate may have been due to a direct stimulation of sialyltransferase activity. To investigate this possibility an improved extraction of the enzyme and a reliable quantitative assay were devised (4). Gonococci (strain F62) were disrupted in a French pressure cell and the bacterial membranes were extracted for 1 h at 37°C with a detergent, NONIDET (1% v/v).

The assay involved sialylation of LPS by CMP-^{14}CNANA and scintillation counting of the labelled LPS after fixing it on filter paper strips by trichloracetic acid and washing it

free from unincorporated CMP-^{14}CNANA. It was rapid, reproducible and, although the enzyme preparations contained endogenous LPS, dependent on added LPS for maximum activity. Using these methods it was shown that a wide range of concentrations of lithium-L-lactate did not enhance the activity of the extracted sialyltransferase (4).

The process whereby lactate enhances the effect of CMP-NANA is separate from the action of CMP-NANA itself because pre-incubation of gonococci with lactate enhanced subsequent LPS sialylation and induction of serum resistance by CMP-NANA (4). Both processes were inhibited by a sublethal concentration of chloramphenicol, indicating that metabolic events were required (4).

Pyruvate as well as lactate enhances sialylation of gonococcal LPS by CMP-NANA (3). Since lactate and pyruvate can be interconverted by gonococci either could be the effector molecule. The possibility that lactate and pyruvate increase gonococcal contents of either the sialyltransferase or LPS receptors for sialyl groups is being investigated.

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Genetic basis for the production of multiple lipooligosaccharides by *Neisseria gonorrhoeae*

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LOS is an outer membrane component of *N. gonorrhoeae* that mediates many aspects of disease. It consists of a branched oligosaccharide structure which is anchored to the membrane via Lipid A. Variation, observed within as well as between strains, results in the production of LOSs that differ in the length and/or presence of any of three oligosaccharide chains, the number of LOS components expressed, and their relative concentrations (1-4). The implication that production of several epitopes may be necessary during some aspects of infection (5) underlies the need to understand the genetic mechanism which makes this possible.

Neisseria gonorrhoeae cells may express a single lipooligosaccharide (LOS) component on their cell surface, or they may simultaneously express multiple LOS structures. FA19 expresses 3 LOS components and each component binds one of the following MAbs: 2-1-L8, 1B2, and 17-1-L1. Gotschlich (6) identified an operon containing the genes needed for the biosynthesis of each of these structures (*lgtA-E)*. Danaher et al. (7) and Yang and Gotschlich (8) showed that changes in the number of guanines found in a polyguanine tract in three of these genes (*lsi-2 = lgtA, lgtC and lgtD)* effects which LOS component is made. From this work, one can explain how a cell makes an LOS that reacts with one of the MAbs described above by changes in the expression state of these three genes. If a strain is defective in *lgtA* and *lgtC*, it will express an LOS that reacts with MAb 2-1-L8. If the *lgtC* was functional in this background, it will express a single LOS that reacts with MAb 17-1-L1. When *lgtA* is functional and *lgtC* and *lgtD* are not, the strain will produce a single LOS component that will react with MAb 1B2. However, these data do not explain how a cell can simultaneously express multiple LOS's.

N. gonorrhoeae 1291 contains a functional *lsi-2 (lgtA)* gene and it expresses a single LOS component that reacts with MAb 1B2; when this gene is nonfunctional due to a +1 frame shift caused by the insertion of a guanine into a polyguanine tract within the gene, the gonococcus expresses a single LOS component that reacts with the MAb 2-1-L8 (3). We will present data that shows that *N. gonorrhoeae* strain FA19 simultaneously expresses in the same cell both of these LOS components. The genetic locus responsible for this phenotype in FA19 was identified by isolating a clone that is able to impart on strain 1291 the ability to simultaneously express both LOS molecules. This clone, pCLB1, was characterized by DNA sequence analysis. The data indicate that the gene responsible for the expression of both LOS components on the same cell is also *lsi-2*. DNA sequence analysis of $lsi\text{-}2_{FA19}$ indicated that there were several differences in the DNA sequence, relative to $lsi\text{-}2_{1291}$. The region responsible for the LOS-specific

phenotype change in *lsi-2*$_{FA19}$ was identified by deletion and transformation analysis. The region responsible for the phenotypic change mapped to the polyguanine tract within *lsi-2* where lsi-2$_{FA19}$ possessed a +2 frameshift within this polyguanine repeat, relative to *lsi-2*$_{1291}$. The polyguanine tract in *lsi-2*$_{FA19}$ was modified by site directed mutagenesis to change the sequence GGGGGGGGGGGG to GGGAGGTGGCGGA. This change in DNA sequence does not alter the protein sequence of the in- frame gene. This change in the DNA sequence will prevent the DNA sequence from changing during DNA replication. Transformants of 1291 generated with this clone only expressed a single LOS component that reacted with MAb 2-1-L8. From this data, we concluded that FA19, even though *lsi-2*$_{FA19}$ is out of frame with respect to *lsi-2*$_{1291}$, is able to generate a small amount of a functional LSI2 protein via transcriptional frame shifting, and this limited amount of protein allows for the expression of some "wild type" LOS molecules. This results in the production of cells that can express two different LOSs on its cell surface at the same time. These data indicate that a cell only needs to express a small amount of LSI2 protein to produce MAb 1B2 reactivity.

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Analysis of two loci involved in biosynthesis of the inner core and lipid A parts of Neisseria meningitidis lipopolysaccharide.

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Genes involved in biosynthesis of LPS from *Neisseria meningitidis* have been identified and studied with the following aims in mind: (i) mutant forms of LPS with a truncated oligosaccharide chain might be useful for vaccine purposes, if the immune system is directed to other, more conserved epitopes, and (ii) modification of lipid A biosynthesis could conceivably lead to less toxic forms of LPS, which could be more easily included in outer membrane vesicle vaccines.

By deletion mutagenesis in the entire meningococcal chromosome, we have previously identified the *ics*A gene, which encodes the glycosyltransferase required for adding GlcNac to Hep-II in the inner core of meningococcal LPS (1). This gene has homology to several LPS glycosyltransferases, notably to *rfa*K from *Salmonella typhimurium* and bplH from *Bordetella pertussis*, both of which encode GlcNac transferases. Directly upstream of *ics*A is an ORF showing significant homology to the hypothetical protein HI0653 from the *Haemophilus influenzae* genome sequence, and to a lesser degree to putative glycosyltransferases from *Streptococcus thermophilus* and *Yersinia enterocolitica*. Insertional inactivation of this ORF resulted in a meningococcal strain with truncated LPS. We have named this new LPS-involved gene *ics*B. Slight differences in binding of monoclonal antibodies and in mobility on Tricine-SDS-PAGE showed that LPS from *ics*A and *ics*B mutants is similar but not identical. On the basis of these results, we postulated that the new gene encodes the glycosyltransferase required for adding Glc to Hep-I. Structural analysis of purified mutant LPS by electrospray mass spectrometry was used to verify this hypothesis. The composition determined for *ics*A and *ics*B is lipid A - KDO2 - Hep2 - PEA and lipid A - KDO2 - Hep2 -PEA - GlcNac, respectively. The *ics*A and *ics*B genes thus form an operon encoding the glycosyltransferases required for chain elongation from the lipid A - KDO2 - Hep2 basal structure, with *Ics*A first adding GlcNAc to Hep-II and IcsB subsequently adding Glc to Hep-I. Only then is completion of the lacto-N-neotetraose structure possible through the action of the *lgt*A-E genes (2).

Up to date, no genes involved in Neisserial lipid A biosynthesis have been reported. By complementation of a temperature-sensitive *E.coli lpx*D mutant, we have cloned a meningococcal chromosomal fragment that carries the *lpx*D homologue. At the restrictive temperature of 420C, LPS biosynthesis was restored in this E. coli mutant when a plasmid carrying the meningococcal *lpx*D was present. Cloning and sequence analysis of chromosomal DNA downstream of *lpx*D revealed the presence of the *fabZ* and *lpx*A genes, followed by an inverted repeat that might function as transcriptional terminator. In contrast to *E. coli* and several other bacterial species, no *lpx*B gene was

found directly downstream of *lpx*A. The LpxA and LpxD proteins catalyze early steps in the lipid A biosynthesis pathway, adding the O- and N-linked 3-OH fatty acyl chains (3). In E. coli and N. meningitidis, the LpxD proteins have the same specificity, both adding 3-OH myristoyl chains; in contrast to *E. coli*, the meningococcal LpxA protein adds 3-OH lauroyl chains instead. We are currently trying to determine the molecular basis for this difference in specificity, in order to be able to modify lipid A biosynthesis. Both LpxD and LpxA contain an (I,V,L)GXXXX hexapeptide repeat motif; the recently published crystallographic structure of the E. coli LpxA protein has shown that this forms a beta-helix tubular domain, with the I, V and L side chains forming a hydrophobic interior (4). A unique feature of the meningococcal LpxA sequence is the replacement of two of these residues with F. In order to determine the role of this beta-helix domain in the fatty acid specificity, we are constructing both site-specific mutants and *E. coli - N. meningitidis* hybrid lpxA genes. A construct carrying a kanamycin-resistance cassette inserted in the intergenic region between fabz and lpxA is being used for the reintroduction of these modified lpxA genes into the meningococcal chromosome. This is expected to result in lipid A with C14 instead of C12 3-OH fatty acyl chains O-linked at the 3 and 3' positions.

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LPS sialylation studies with gonococcal strain F62 and a sialyltransferase-deficient mutant, JB1.

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A gonococcal mutant, strain JB1, has been isolated from strain F62 by transposon insertion mutagenesis using Tn1543-Δ3 (1). The mutant is defective in both conversion to serum resistance and LPS sialylation using either CMP-NANA or blood extracts as the sialyl donor. Similar LPS species including targets for sialylation are synthesized by both the mutant and the parental strain, albeit in different proportions (1).

The gonococcal sialyltransferase from strain F62 can be released quantitatively into a soluble, micellar fraction by breaking bacteria in a French pressure cell and extracting the membrane fraction sedimented by ultracentrifugation with 1 % (v/v) Nonidet. The specific activity of the resulting preparation, 4.8 nmol. of NANA transferred to LPS. Min^{-1}. (mg. protein)$^{-1}$, is far higher than that of the previously reported procedure for extracting the gonococcal sialyltransferase with Triton X100 (2). The specific activity can be increased a further three-fold by chromatography on a column of DEAE Sepharose CL6B. In contrast to the parental strain, no sialyltransferase activity was released from the mutant.

The extracted sialyltransferase catalyses the sialylation of purified lipopolysaccharides from both the mutant JB1 and the parental strain at similar rates. In both cases, only a single major LPS component with an apparent Mr of 4.5 kDa is sialylated rapidly. Similar data are also obtained for the sialylation of the same major component on the surface of live gonococci. These data confirm that strain JB1 is defective in the production of the sialyltransferase rather than in the LPS substrate for sialylation by exogenous CMP-NANA. They also indicate that both the sialyltransferase and the LPS substrate are sufficiently close to the bacterial surface to be accessible to exogenous CMP-NANA.

Sialyltransferase activity was also readily detected when membranes from a $GalE^-$ strain were extracted with Nonidet but, as expected, this activity was totally dependent upon the addition of exogenous LPS. The specific activity of the GalE extract, 9 nmol of NANA transferred to LPS. Min^{-1}. (mg. protein)$^{-1}$, was higher than that of the extract from strain F62. Studies of the effects of altering the major carbon source during growth and the kinetic and stability properties of the sialyltransferase preparation will be reported.

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Lipooligosaccharides, Poster 23

Inhibition of meningococcal induced inflammation by anti-CD14 monoclonal antibodies and bactericidal/permeability increasing protein *in vitro*.

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Meningococcal lipopolysaccharide (LPS) is thought to be a key mediator of meningococcal septicemia and plasma levels of LPS have been shown to correlate with the severity of illness and with outcome (1). The glycoprotein, CD14, present on the monocyte cell surface, functions as a receptor for LPS and is bound by a glycosyl-phosphatidylinositol anchor (2). Previously viewed as a useful marker molecule for monocytes and macrophages, there has been much interest in its role in binding to LPS complexes in recent years. LPS binds to a lipopolysaccharide binding protein (LBP) which is present in human plasma and the complex has an increased affinity for the CD14 receptor leading to cell activation. The mechanism of stimulation of the endothelium is unclear. However, current research suggests that a soluble form of CD14 (sCD14) present in serum produces a complex with LPS which is able to activate endothelial cells (3). Recognition that the CD14 receptor plays a key role in cellular activation by LPS has lead to the investigation of agents which can block the interaction of LPS and its receptor, thereby inhibiting inflammation. Bactericidal/permeability increasing protein (BPI) found in the neutrophil granules possesses both bactericidal and LPS-neutralizing properties. BPI binds with high affinity to the active region of LPS, lipid A, and can successfully neutralize LPS. The anti-LPS activity is contained in the 23KDa N-terminal region of BPI, and a recombinant fragment ($rBPI_{23}$) has been developed as a potential therapeutic agent.

We have evaluated the effect of monoclonal antibodies against the CD14 receptor (MAbs CD14), and also $rBPI_{23}$, in blocking the activation of monocytes and endothelial cells by meningococci or *E. coli* LPS *in vitro* in whole blood and in human umbilical vein endothelial cells (HUVECs).

Heat killed meningococci or LPS induced TNFa release when added to whole blood, and also tissue factor (TF) expression when incubated with HUVECs (4, 5). MAbs CD14 inhibited TNFa release induced by either LPS or meningococci when added prior to the inflammatory stimulus. The effect was critically dependent on timing, and no inhibition was observed when the MAbs CD14 were added after the LPS. $rBPI_{23}$ effectively reduced the LPS mediated TNFa release but had no effect on TNFa production induced by meningococci regardless of whether the $rBPI_{23}$ was added before or after the bacteria.

LPS induced TF expression in HUVECs was blocked by MAbs CD14 when either added before or 5 min after the LPS. However, MAbs CD14 were ineffective at blocking

meningococcal induced TF expression. Similarly, $rBPI_{23}$ blocked LPS induced TF expression if added before the LPS but had no effect on meningococcal induced TF expression.

Agents such as $rBPI_{23}$ and MAbs CD14 which block activation of inflammatory cells via the CD14 receptor may inhibit purified LPS yet be unable to inhibit the inflammatory effects of whole bacteria. Our results highlight the care needed when extrapolating data from in vitro studies with LPS to situations where whole organisms are involved.

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Sialylation of *Neisseria meningitidis* lipooligosaccharide (LOS) inhibits serum bactericidal activity by masking lacto-*N*-neotetraose.

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The majority of meningococcal disease in the United States is caused by serogroup B and C organisms. Most of these strains make LOS that contain a terminal polylactosamine structure, lacto-*N*-neotetraose (LNnT). This carbohydrate serves as the major site for sialylation of meningococcal LOS (1, 2). A monoclonal antibody (MAb) 1B2 binds to LNnT on the 4.5 kDa component of meningococcal LOS. Addition of sialic acid blocks the binding of 1B2. Groups B and C *N. meningitidis* can endogenously sialylate LOS (1) and do so in varying degrees. Some strains express LOS with LNnT that is not endogenously sialylated, while others are heavily sialylated (3). Groups B and C meningococci can also add additional sialic acid (exogenous sialylation) when grown in the presence of cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA).

Exogenous sialylation of *N. gonorrhoeae* LOS causes resistance to serum bactericidal activity (SBA) (4, 5, 6). To find out how sialylation affects the sensitivity of group C *N. meningitidis* to SBA, we assessed the relationship between the degree of sialylation and expression of LNnT of 9 strains and their sensitivity to a pool of 5 normal human sera (PHS). All strains were isolated from children during periods of endemic disease. Five were isolated from blood or cerebral spinal fluid, 3 were carrier isolates, and 1 was isolated from the middle ear fluid of a child with acute otitis media (3). SBA susceptibility was assessed by incubating organisms that had been grown to mid-log phase with or without 200 μg/ml CMP-NANA in serial 2-fold dilutions of PHS (maximum 50%). Chelation of PHS and use of depleted PHS was used to assess the classical (CP) and alternative (ACP) pathways of complement. Mid-log phase organisms that were used in the bactericidal assays were also washed and re-suspended in phosphate buffered saline for whole-cell ELISA. The degree of endogenous sialylation of LNnT was judged by the binding of MAb 1B2 before and after removal of sialic acid by neuraminidase to strains grown without exogenous CMP-NANA. The decrease in the binding of MAb 1B2 to LOS on strains grown with exogenous CMP-NANA was used to monitor exogenous LOS sialylation (3). The percentage survival of strains (grown with and without CMP-NANA) in serial 2-fold dilutions of serum was obtained. For each strain in each assay, a linear equation was used to calculate the $\log_2$ serum dilution where survival reached 100%. This was correlated with the binding of MAb 1B2 to LNnT for each strain in each assay and with the degree of endogenous and exogenous sialylation of LNnT.

All 9 strains made LNnT that was variously endogenously sialylated, as judged by the binding of MAb 1B2 before and after removal of sialic acid by neuraminidase. For endogenously sialylated meningococcal strains, (grown without CMP-NANA),

susceptibility to SBA of PHS correlated with the amount of unsialylated LNnT (r^2 = 0.83) above a threshold of LNnT expression; strains that expressed less than the threshold survived in 25% PHS. Non-disseminated isolates were not more sensitive to SBA than case isolates that expressed an equivalent amount of LNnT.

All strains added more sialic acid when grown with CMP-NANA. Exogenous sialylation reduced expression of free LNnT and significantly reduced susceptibility to SBA for strains that expressed more than the threshold of LNnT when grown without CMP-NANA ($p < 0.001$; two-tailed paired t-test). The amount of inhibition correlated directly with sensitivity to SBA. The 3 most resistant strains were heavily endogenously sialylated and did not become more resistant after growth in CMP-NANA. The strains that were most susceptible to SBA when not exogenously sialylated had the greatest inhibition of susceptibility to SBA when they were exogenously sialylated. Masking of LNnT expression by exogenous sialylation affected killing through both the CP and ACP.

We conclude that both endogenous and exogenous LOS sialylation are associated with increased serum resistance of some endemic group C *N. meningitidis* by masking LNnT. Susceptibility to serum bactericidal activity of PHS correlated with the amount of unsialylated LNnT as indicated by the binding of MAb 1B2 to this structure for both endogenously and exogenously sialylated strains.

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Non-opsonic phagocytosis of *Neisseria meningitidis* by human neutrophils.

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Recent work suggests that killing of *N. meningitidis* by neutrophils (PMN) might be a more important host defense than previously recognized (1-3). While phagocytosis of opsonized meningococci has been well studied, few data exist on non-opsonic phagocytosis, a well documented mechanism for gonococci. Most group B and C meningococci express lipooligosaccharide (LOS) molecules that contain the terminal structure, lacto-*N*-neotetraose (LNnT) that binds monoclonal antibody 1B2. LNnT is the major site of sialylation of LOS and addition of sialic acid blocks the binding of 1B2. We previously reported (1) that resistance of opsonized group C *N. meningitidis* strains to phagocytic killing by PMN correlated directly with the degree of sialylation of LNnT. We now report two group C endemic meningococcal strains (15029 and 8026) that are sensitive to non-opsonic phagocytosis by human PMN. Strain 8026 is a case isolate and 15029 is a carrier isolate. Both strains are encapsulated and express at least one Opa protein but neither expresses Opc protein. Both strains have little to no endogenous LOS sialylation but bind MAb 1B2 very well.

We used the assay described by us (1) to measure opsonic phagocytosis of *N. meningitidis*. C8 depleted serum (C8D) was used to allow complement activation through C3 (necessary for complement-dependent phagocytosis) but not through C9 (necessary for complement-mediated bacterial lysis). The opsonic phagocytosis assay consisted of a reaction mixture containing bacteria, neutrophils, and 10% C8D and a reaction mixture containing bacteria and 10% C8D. Gonococcal Buffer (GB) was added to each tube to bring the final volume to 250 µl. Survival was expressed as the percentage of organisms at time 0 that survived to 60 minutes. A modification of the above assay was used to measure non-opsonic phagocytosis. The assay consisted of a reaction mixture of bacteria and neutrophils in GB and a reaction mixture of just bacteria in GB. Because preliminary experiments showed that some strains survived less well than others in GB alone, 10% heat-inactivated agammaglobulinemic serum was added to all tubes in non-opsonic phagocytosis assays.

The mean survival at 60 min. for strain 15029 was 3 ± 3% with opsonization and 35 ± 16% without opsonins. Mean survival for strain 8026 was 1 ± 1% and 28 ± 8% with and without opsonization respectively. Cytospin and Wright stain analysis following the non-opsonic assays showed that organisms were internalized by PMN and were not merely adherent to the PMN surface. This was confirmed by washing and plating the PMN after the non-opsonic assays. Exogenous sialylation of LOS by growth in cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA), as confirmed by decreased 1B2 binding, increased the resistance of the strains to non-opsonic phagocytosis. Survival increased from 30% to 65% ($p = 0.02$) and 32% to 54% ($p = 0.008$) for the two strains.

This effect was lost when the sialic acid was removed from LOS by treatment with neuraminidase.

To examine the role of the LNnT LOS structure in non-opsonic phagocytosis, an isogenic mutant (8026-R6) was made that lacked this LOS structure. The Tn*916* mutants of meningococcal strain NMB that were generated by Stephens et al. (4) included a mutant (NMB-R6) that expressed only one LOS of 3.1-3.2 kDa while the parent NMB expressed a 4.5 kDa LOS that contained LNnT and bound MAb 1B2. The defect was identified as a deficiency of phosphoglucomutase (PGM) that converts glucose 6-phosphate to glucose 1-phosphate (5). Mutants were unable to add glucose to heptose. Genomic DNA from the tetracycline resistant NMB-R6 was used to transform strain 8026 to a *pgm* deficient mutant as described (4,5).The mutant was made and kindly provided by Dr. M. Apicella and Dr. D. Zhou. Whole-cell lysates and pk treated whole-cell lysates of strain 8026 and the PGM deficient mutant 8026-R6 were analyzed by SDS-PAGE and silver stain of the LOS and Coomassie stain of protein molecules. 8026 and 8026-R6 were identical except that 8026-R6 did not express the 4.5 kDa LOS that bears the LNnT structure but did express a new LOS molecule with apparent molecular weight of < 3.2 kDa. Immunoblot analysis confirmed that 8026-R6 did not bind MAb 1B2. Strains 8026 and 8026-R6 were analyzed together in the phagocytosis assays. The strains grew in broth at a similar rate and after washing were suspended in GB to an identical optical density. There were no significant differences in percentage survival of 8026 and 8026-R6 in the opsonic and non-opsonic phagocytosis assays. Whole-cell ELISA of the organisms used in the phagocytosis assays confirmed that 8026 bound MAb 1B2 strongly while 8026-R6 did not and thus did not make LOS containing LNnT. These data suggest that the loss of the LNnT structure did not interfere with opsonic and non-opsonic phagocytosis of this strain.

We conclude that some meningococcal strains, like gonococci, are highly susceptible to non-opsonic phagocytosis but resistance may be acquired *in vitro* by exogenous sialylation of LOS but not loss of the LNnT structure.

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Proposed specificity of the *Neisseria gonorrhoeae* lipooligosaccharide epitope identified by monoclonal antibody 2C7

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Monoclonal antibody (mAb) 2C7 identifies a conserved gonococcal lipooligosaccharide (LOS) epitope that is widely expressed *in vitro* and *in vivo*. The 2C7 epitope evokes a significant immune response after natural infection and vaccination that mediates both killing and opsonophagocytosis (1, 2). The current studies describe initial immunochemical characterization of the 2C7 epitope.

Several mAbs that bind to saccharide substitutions off heptose 1 of gonococcal LOS possess binding specificities that coincide with the lacto-*N*-neotetraose structure partially identified by mAb 3F11 (3). MAbs 1-1-M, neisserial-specific 4C4, and 9-2-L3,7,9 bound to strain 24-1 but did not inhibit binding of mAb 2C7 to whole gonococci or purified LOS in ELISA. MAb 2-1-L8 bound to the LOS of strain WR220, but did not inhibit binding of mAb 2C7 to WR220 LOS in ELISA. MAb 2C7 did not bind to LOS of strain 1291 or its pyocin mutants, which assemble sequentially increasing number of hexoses attached to heptose 1 (4). Thus the epitope identified by mAb 2C7 does not reside on the known lacto-*N*-neotetraose substitutions off heptose 1 of gonococcal LOS. An alternative structure substituted off heptose 1 of LOS is Galα1→4Gal (exemplified by pyocin mutant strain 1291b (4). Anti-P^k mAb 3D9 and *H. influenzae*-specific mAb 4C4 both bind to this epitope (5, 6). MAb 17-1-L1 binds Galα1→4Gal that is expressed by meningococci of the L1 serotype and some gonococci (3, 4). Neither mAb bound to certain gonococci or their purified LOSs (strains 24-1 and 15253) that otherwise expressed the 2C7 epitope. Three additional gonococcal isolates known to express this alternative digalactoside (strains 1291b, F62, and 4505 (3, 6) similarly did not bind mAb 2C7.

mAbs that recognize human GSL antigens previously shown to cross-react with gonococcal LOS epitopes (6, 7) were screened for binding to whole gonococci and purified LOS that express the 2C7 LOS epitope (strain 24-1). As expected, mAbs 3F11 (prefers branching over linear lactosamine structures) and O6B4 (prefers linear lactosamine) bound both to purified LOS and whole gonococci (strain 24-1), as did mAb 2D4 (anti-asialo-GM2 (6). MAb 2C7 did not inhibit binding of mAbs 3F11, O6B4, or 2D4 to either purified LOS or whole gonococci (strain 24-1). MAb SH-34 (anti-asialo-GM1, which exhibits variable binding to gonococci (6) bound to whole gonococci but not purified LOS (strain 24-1); mAb 2C7 did not inhibit binding of mAb SH-34. MAb 103HT30, a separate mAb that also binds asialo-GM1 antigen (6), did not bind to 2C7-containing LOS. The 2C7 epitope thus lacks antigenic similarity to known cross-reactive

human GSL (lactoneo series, asialo-GM1, -GM2) antigens and hence may not be expected to evoke an autoimmune response.

We examined mAb 2C7 binding to Western blots of LOS prepared from gonococcal strains. MAb 2C7 binds to strain 15253 LOS, which possesses lactosyl (Galβ1→4Glc) substitutions at both heptose 1 and 2 (8), but not to strain 1291c LOS, which differs from 15253 by the lack of this lactosyl substitution on heptose 2 (4). Collectively these data suggest that the 2C7 epitope may involve the lactosyl (Galβ1→4Glc) substitution of heptose 2.

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Lipopolysaccharide biosynthesis in *Neisseria meningitidis*: A genetic analysis of lgt loci in immunotype typing strains.

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Structural studies and the use of monoclonal antibodies have demonstrated the heterogeneity and complexity of meningococcal LPS (1) which can divided into 12 immunotypes (ITs; 2). A feature of meningococcal LPS is the reversible, high-frequency switching of expression (phase variation) of terminal LPS structures. A number of studies are strongly suggestive of a key role for these terminal structures and the phase variation of their expression in pathogenesis (3, 4, 5).

Recently a locus containing three genes, lgtABE, for the biosynthesis of the terminal LPS structure lacto-N-neotetraose (LNT) in *Neisseria meningitidis* (Nm) strain MC58 has been described (6). This study also describes the mechanism which controls the phase variable expression of this structure, which operates via slipped strand mispairing in a homopolymeric tract of 14 guanosine residues in the first gene of the locus. Structural studies of LPS from lgt mutant strains and enzyme assays have confirmed that these three genes encode glycosyl transferases for the biosynthesis of LNT (7). Prior to this work, Gotschlich (8) described a similar locus in *Neisseria gonorrhoeae* strain F62 (Ng) which contained 5 LPS biosynthetic genes, lgtABCDE. The Nm genes described above are present in the same orientation and order as those in the Ng locus, so that the major difference is the absence of lgtC and lgtD in the Nm locus. The lgtC and lgtD genes are involved in the biosynthesis of LPS structures which are not expressed by Nm strain MC58, which can express only the L3 or L8 immunotype.

Here we present a study of lgt loci in the Nm IT typing strains. We first isolated the lgt locus of Nm strain 126E, the L1 IT type strain (2), and determined the nucleotide sequence. This locus was similar to that described by Gotschlich in Ng (8), but with two significant differences: 1. In Nm 126E there had been a 1.5kb deletion between the *lgt*A and *lgt*B genes which has removed 81.5% of the *lgt*A coding region and 82.6% of the *lgt*B coding region. This deletion presumably renders these genes, required for the biosynthesis of LNT, non-functional. Colony immunoblots to detect the LNT structure has confirmed that it is not made by Nm 126E. 2. The Nm 126E *lgt*D gene contains a short homopolymeric tract of 8 guanosines (Ng has 11) which has put the gene out of reading frame. In Ng *lgt*D is proposed to be involved in the terminal modification of LNT with N-acetylgalactosamine (8). As Nm 126E cannot make LNT even a functional *lgt*D gene would presumably have no effect on LPS biosynthesis.

Using a set of hybridization probes, based on the *lgt*ABE genes from Nm MC58 and the *lgt*C and *lgt*D genes from Nm 126E, we had surveyed the IT strains for the presence of these genes. This survey revealed that the Nm IT strains were not like the Ng F62 example (8) in that none of them contain all 5 lgt genes - consistent with a more restricted repertoire of terminal structures which can be made by an individual Nm strain. For example, the *lgt*C gene is present only in the L1 and L8 IT strains, *lgt*D is only present in the L1 IT strain, and *lgt*A is present in all strains except L1 and L10. This survey has allowed the prediction of the phase variation repertoire of individual strains based on the lgt genes present. The predicted phase variation repertoire of a selection of these strains has been confirmed using colony immunoblot with IT specific monoclonal antibodies. Refinement of this system with new probes to other key LPS biosynthetic genes may lead to an improvement of the IT system such that individual strains may be classified by the potential repertoire of LPS structures that may be expressed.

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Detection of opsonophagocytosis of *Neisseria meningitidis* by chemiluminescence with demonstration of the effect of immunotypes L3,7,9 which can be sialylated and L1,8,10 which cannot be sialylated on the process

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It has long been understood that bactericidal antibodies provide immunity to meningococcal infection (1). Other aspects of host immunity are less well understood but it is thought that opsonophagocytosis is a critical defense mechanism in non-immune individuals (2). In one outbreak most case isolates were of immunotype L3,7,9 which can be sialylated whilst carrier isolates were immunotype L1,8,10 which cannot be sialylated (3). In gonococci, sialylation delays opsonophagocytosis (4). This study investigated whether possession of the L3,7,9 immunotype enabled *N. meningitidis* to evade phagocytosis with implications both for the pathogenicity of and immune response to meningococcal disease.

Sera and white cells from 5 healthy volunteers were reacted with 9 meningococcal strains of LOS immunotype L3,7,9; L1,8,10 or a combination of the two and representing case and carrier isolates. Chemiluminescence was used to investigate opsonophagocytosis by detection of oxygen radicals produced by activated neutrophils.

Phagocytosis of all three of the L3,7,9 strains irrespective of whether they were case or carrier isolates was significantly delayed in comparison with 3 strains with the combination immunotype. In the 3 groupable case strains, phagocytosis was also delayed in the L3,7,9 strain when compared with the L3,7,9 L1,8,10 strains. In nongroupable carrier strains possession of the L3,7,9 immunotype also delayed phagocytosis and, in comparison with the L1,8,10 strain, also diminished the peak chemiluminescence value. Possession of a capsule also affected the results with a groupable case strain of both immunotypes being more resistant to phagocytosis than non-capsulate, carrier strains.

This study demonstrated that possession of an immunotype which can be sialylated may reduce opsonophagocytosis of *N. meningitidis* thereby enhancing its surviving in the bloodstream following invasion.

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Molecular characterization of antibodies specific for meningococcal lipooligosaccharide

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The lipooligosaccharides (LOS) of *Neisseria meningitidis* are not only important major surface antigens but also play a critical role in the pathogenesis of meningococcal disease (1). Antibodies specific for the hydrophilic oligosaccharide component of LOS may offer protection by neutralizing the effects mediated by LOS (2) and sialylation of LOS has been shown to modulate the serum resistance of meningococci (3). The oligosaccharide portion is also structurally and antigenically variable providing the basis for the immunotyping of meningococcal isolates. For the purposes of characterizing meningococcal LOS, a number of murine hybridomas that produce monoclonal antibodies recognizing epitopes in the oligosaccharide have been isolated (4). Studies of antibody interactions with protein antigens has made rapid progress thanks largely to the availability of simple methods for epitope mapping, whereas studies of antibody interactions with carbohydrate antigens which are more complex has proceeded relatively slowly. The interaction between meningococcal LOS and LOS-specific antibodies provides a model system for studying carbohydrate protein interactions. The LOS immunotype L3,7,9 is associated with organisms causing invasive disease and may contribute to the resistance of meningococci to complement-mediated lysis (5). The present study examines the interaction of two monoclonal antibodies with LOS of the L3,7,9 immunotype, purified from a case isolate.

The recent development of biosensor technology permits studies of molecular recognition, affinity and kinetics in real-time, without the need for labelling (6,7). The benefit of this approach is that it focusses on the biological activity rather than biological structure and, unlike fixed-endpoint assays, provides a dynamic representation of binding interactions. Previous comparison of these antibodies in ELISAs has shown that they have quite different reactivities with purified LOS of the L3,7,9 immunotype. The kinetics of antibody binding to purified LOS has been analysed using a resonant mirror optical biosensor, with biotinylated LOS captured on an avidin-aminosilane surface. The kinetic data obtained were consistent with the different reactivities of these antibodies observed in ELISAs and demonstrated that biosensor technology provides an important tool for the immunologist to study antibody interactions with carbohydrate antigens at the molecular level.

The nucleotide sequence of cDNA encoding the variable domains of the antibody heavy and light chains from two of the monoclonal antibodies against the L3,7,9 epitopes has been determined. The framework region sequences show that the heavy chains of antibodies 4A8-B2-L379 (J. Poolman) and 9-2-L379 (W. Zollinger) both belong to the

V_H family J558, although their low homology (86%) to each other suggests that they are probably from different subfamilies. The heavy chains showed similar levels of homology to the germline genes VMU3.2 and 186-2, and use J_H2 and J_H3, respectively. The light chains are from different families: Vκ8 and Vκ-ARS, respectively. Comparison of the nucleotide sequences with those of other murine antibody genes revealed that the 4A8-B2-L379 κ chain is 82% homologous to the D23 germline gene and uses the Jκ1 segment, whereas the 9-2-L379 κ chain is 84% identical to the germline gene 28.4.10A(κ) and uses Jκ2.

The heavy chains of these MAbs have a higher overall level of identity than do the light chains, differing primarily in their CDR3 sequences where different D segments are utilised. Given the overall similarity of the V_H segments, it is likely that the differences seen in the antigen binding affinity of these antibodies is either due to L chain association or the different V_H domain CDR3 sequences: CDR3 of the V_H domain is known to play an important role in antigen binding specificity and antibodies can show similar specificities despite differences in the amino acid sequence of this region. In the present study, the CDR3 regions of both MAbs consist of the same number of residues, and it has been suggested that the length of CDR3 in the V_H domain may play a fundamental role in the structure of the binding site, with amino acid sequence affecting the fine specificity and kinetics of antibody-antigen interaction. Current experiments with recombinant Fab fragments will help to resolve these issues.

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Interaction of a gonococcal sialyltransferase-deficient mutant with human epithelial cells and neutrophils

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Strain F62 of *Neisseria gonorrhoeae* (GC) is sensitive to normal human serum (NHS) unless exogenous CMP-NANA is present. Sialic acid (NANA) is transferred primarily to a 4.5 kDa terminal galactose (gal) residue in the gal β-1,4 N-acetylglucosamine (gal-glcNac-R) lipooligosaccharide (LOS) structure by a GC sialyltransferase (Stase) (1,2). Sialylation results in an increase in LOS M_r to 4.9 kDa. Sialylated GC resist killing by normal human serum (NHS), show reduced invasion into epithelial cells, and have reduced adhesion to and stimulation of human neutrophils (1,2,3). We asked the following question: Is Stase activity required for interaction of GC with host cells in the absence of exogenous CMP-NANA?

To address the first question we created ethyl methanesulfonate (EMS) mutants of strain F62 that failed to express Stase activity. EMS-treated GC were grown on CMP-NANA plates and screened with monoclonal antibody (mab)1B2-1B7, which is specific for gal-glcNac and reacts only with asialylated GC. We isolated five mutants having no detectable Stase activity compared to wild type (WT) F62. These mutants may have a mutation(s) in a regulator of Stase expression or in the structural gene, such that a truncated and/or non-functional enzyme is made. The LOS phenotype in these Stase null mutants was identical to WT F62, yet the mutants could not sialylate their LOS when grown with CMP-NANA: there was no increase in M_r of the 4.5 kDa species to 4.9 kDa. As expected, Stase null mutants remained serum sensitive even when grown with CMP-NANA. When these mutants were transformed with WT F62 chromosomal DNA and Stase-plus transformants were serum selected after growth with CMP-NANA, protection from serum killing at levels comparable to WT F62 was obtained. The kinetics of rescue from Stase-minus to Stase-plus suggest a single mutation. One Stase null mutant, ST94A, adhered to and invaded the human cervical epithelial cell line ME-180 at levels indistinguishable from WT F62 in the absence of CMP-NANA (adherence per epithelial cell- F62: 17.6, n = 3, ST94A: 16.8, n = 4; invasion per epithelial cell-F62: 0.82, n = 4, ST94A: 0.70, n = 5). ST94A also stimulated the oxidative burst in and adhered to human neutrophils at levels similar to WT F62 (adherence per neutrophil- F62: 15.1, n = 3, ST94A: 7.6, n = 3). In the absence of serum, ST94A and WT F62 were also phagocytically killed by neutrophils at similar levels in an Opa-dependent manner. These results indicate that expression of Stase activity is not required for interaction of GC with human cells.

The mutants deficient in Stase activity described here have numerous properties in common with the previously described *Tn 1545Δ3* derived Stase-deficient mutant, JB1

(6): all of these mutants lack Stase activity, and in the presence of CMP-NANA, cannot sialylate their LOS, are serum sensitive, and react with anti-gal-glcNac mabs. The only notable difference is that JB1 expresses altered amounts of several LOS species, whereas our Stase null mutants express similar amounts of individual LOS species compared to WT F62. The specific defect(s) in these mutants is unknown.

Once inside human cells, GC appear to re-sialylate themselves using host-derived CMP-NANA, but the specific location inside the cell that this occurs is not known. It does appear that sialylation of GC inside human cells occurs before GC escape into the extracellular environment and this is a necessary requirement for protection of sensitive strains to NHS (3).

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Identification of an *htrB* analog of *Neisseria meningitidis* serogroup B.

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Integrity of the lipid A structure in gram negative bacteria has been linked to growth at elevated temperatures. While searching for new heat-shock genes of *E. coli*, Karow identified and studied a non-heat shock-induced gene, designated *htrB*. *E. coli htrB-* demonstrate a number of phenotypic changes including the inability to grow in rich media at temperatures above 32.5°C, viability at lower temperatures, resistance to elevated levels of deoxycholate, and the LPS stained rusty brown rather than black on silver-stained SDS-PAGE (1, 2, 3). The *htrB-* strains also showed abnormal cell morphology when grown at high temperatures(1).

Studies done by Lee et.al (4) demonstrated that *Haemophilus influenzae htrB* mutants exhibited a similar set of phenotypes. The mutants were unable to grow at temperatures above 33°C, but were able to grow at 30°C; however, mutants transformed with a plasmid containing the *htrB* gene grew as well as wild-type at 37°C (which indicated that the function of the *NTHi* 2019 *htrB* is analogous to that of *E.coli htrB*.). The wild-type *NTHi* strain showed resistance to high levels of deoxycholate at both 30° and 37°C ; whereas, the *htrB-* demonstrated sensitivity to high levels of deoxycholate at both 30°C and 37°C. On a silver-stained SDS-PAGE the *htrB* mutant appeared rusty brown while the wild type band was black in color. The SDS-PAGE also demonstrated that the LOS from the *NTHi* mutant migrated faster than LOS from the parent strain. In addition, these studies predicted that *htrB* may be an acyltransferase responsible for substitutions of myristic acid at the 3' position of hydroxy myristic acid of the lipid A. Subsequent studies by Raetz et al. confirmed the observation that HtrB is a KDO dependent acyltransferase (personal commun). Mass spectroscopic analysis revealed an *H.influenzae htrB*$^-$ lipid A to be predominately tetraacyl with a structure similar to lipid IVA. Macrophages exposed to *H. influenzae htrB-* LOS demonstrated a marked reduction in TNF release when compared to exposure to *H. influenzae htrB+* LOS. Because of the importance of *htrB* in the synthesis and the possible toxicity of lipid A, we investigated the possibility that an *htrB* analog exists in *N. meningitidis*.

An *N. meningitidis* genomic DNA library was introduced via electroporation into the *E. coli htrB* mutant strain MLK217. The cells were plated onto LB-tet-kan plates and were grown overnight at 37°C, Colonies that grew at 37°C were patched onto a new LB-tet-kan plate and grown at 37°C overnight. Colonies that grew at 37°C were potential *htrB+* complements, and were subjected to further studies.

The MLK217 strains containing plasmid expressing *N. meningitis htrB+* candidates were grown a second time at 37°C and were patched onto 5% and 10% deoxycholate plates.

After an overnight incubation at 37°C, all of the colonies were able to grow on the 5% deoxycholate plates. All of the colonies except one were able to grow on the 10% deoxycholate plates. The colony that did not grow on the 10% deoxycholate plate, but did grow on the 5% deoxycholate plate exhibited a phenotype similar to MLK2 (htrB+). The colony, A3, was tested again on 5% and 10% deoxycholate plates, and similar results were obtained.

A standard plasmid preparation was done on one of the colonies that demonstrated the *htrB* + phenotype. Restriction digest analysis was performed on the pNMBA3 plasmid and a 2Kb DNA insert was identified.

LPS was prepared from MLK217 bearing the pNMBA3 plasmid and analyzed on a silver- stained SDS-PAGE. The SDS-PAGE gel demonstrated that the LPS of MLK2 and the LPS of MLK217/pNMBA3 had similar band migration; whereas, the LPS of MLK217 migrated at a slower rate. The LPS of MLK2 stained black , the LPS of MLK217 strain stained a rusty brown color, and the LPS of MLK217/pNMBA3 stained black similar to the LPS of MLK2. These data suggest that the LPS of MLK2 and the LPS of MLK217/pNMBA3 may be similar, further suggesting that pNMBA3 contains an analog of the *htrB* gene.

These experiments demonstrate that the NMB 2Kb insert in pNMBA3 was capable of conferring the *htrB*+ phenotypes when transformed into an *htrB*$^-$ *E. coli* mutant . The *htrB* mutant strain, MLK217, containing pNMBA3 was able to grow above 32.5°C. MLK217/pNMBA3 cells were unable to grow at high levels of deoxycholate. The LOS from MLK217/pNMBA3 was similar to the MLK2 LOS. These results suggest that NMB may possess an analog of the *htrB* gene, and this gene is present on pNMBA3.

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Structure of the lipooligosaccharide (LOS) of pathogenic *Neisseria meningitidis* serogroup B

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The oligosaccharide and lipid A were obtained following mild acid hydrolysis of the lipooligosaccharide from a cerebrospinal fluid isolate of *Neisseria meningitidis* serogroup B(serotype 2b:P1.2,5). Their structures were determined using composition and glycosyl linkage analyses, NMR spectroscopy and mass spectrometry. The oligosaccharide has a structure similar to that previously reported for immunotype L2 (1) i.e., the lacto-*N*-neotetrose group which is attached to heptose I (Hep I), and the *N*-acetylglucosamine and glucose residues attached to Hep II in the inner core. Phosphoethanolamine (PEA) was also found to be attached to *O*-6 or *O*-7 of the Hep II residue. However unlike immunotype L2, this serogroup B oligosaccharide contains one *O*-acetyl substituent per oligosaccharide, and is partially substituted with sialic acid. In contrast to the previous description (2) of *N. meningitidis* lipid A, electrospray mass spectrometric analysis of the de-*O*-acylated LOS indicates that the 4'-phosphate is missing in a large proportion of the lipid A molecules of this isolate. These data emphasize the structural heterogeneity of both the oligosaccharide and lipid A of meningococcal LOS.

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Characterization of terminal NeuNAcα2-3Galß1-4GlcNAc sequence in lipooligosaccharides of *Neisseria meningitidis*

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Neisseria meningitidis is serologically divided into 12 immunotypes based on lipooligosaccharide (LOS) antigen. *N. meningitidis* LOS may be sialylated with N-acetylneuraminic acid (NeuNAc) or non-sialylated at the nonreducing end (1,2). Structural analyses revealed that NeuNAc is 2->3 linked to Gal in the L3 LOS from strain 6275 (3,4). Using lectins which bind different sialic acid-galactose sequences as probes, six of the 12 LOSs (L2, L3, L4, L5, L7, and L8) bound specifically to *Maackia amurensis* leukoagglutinin (MAL) which recognizes NeuNAcα2-3Galß1-4GlcNAc/Glc trisaccharide sequence, but not to *Sambucus nigra* agglutinin which binds NeuNAcα2-6Gal sequence. The LOS-lectin binding was abolished when the LOSs were pretreated with Newcastle disease virus neuraminidase, which cleaves specifically α2->3 linked sialic acid. Thus, NeuNAc is 2->3 linked to Gal in these MAL-binding LOSs including the L3 LOS mentioned above. Methylation analysis of a representative LOS (L2) confirmed that NeuNAc is 2->3 linked to Gal. When desialylated, the six MAL-binding LOSs have a common terminal lacto-N-neotetraose (LNnT, Galß1-4GlcNAcß1-3Galß1-4Glc) structure with Gal at the nonreducing end as revealed by previous structural analyses (3,5) and also by epitope analysis using LNnT-monoclonal antibodies (2,6). Therefore these LOSs possess a terminal NeuNAcα2-3Galß1-4GlcNAc trisaccharide sequence, if sialylated, which is responsible for the binding the LOSs to the MAL lectin. These LOSs structurally mimic glycolipids, paragloboside (LNnT-ceramide) and sialylparagloboside, in having LNnT structure with or without NeuNAc at the nonreducing end (7). They also mimic some glycoproteins which have N-acetyllactosamine sequence with or without an α2->3 linked NeuNAc. The molecular mimicry of the LOSs may contribute to the virulence of *N. meningitidis* by assisting the organism to evade immune defenses in man.

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Systemic survival of *Neisseria meningitis* serogroup B depends on sialic acid of both the capsule and the sialylated oligosaccharide

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The capability of *Neisseriae* to survive the host defense mechanisms has been shown *in vitro* to depend largely on the incorporation of the sialic acid (NeuNAc) into the capsule as well as its use as a terminal modification of the neisserial lipooligosaccharide (LOS) (1,2). In the present study we investigated the *in vivo* contribution of the polysialic-acid capsule and the terminal LOS-modification by sialic acid to the pathogenicity of *Neisseria meningitidis* serogroup B using a set of defined isogenic mutants of the wild type strain B1940. The mutants were deficient in either the capsule synthesis (B1940*sia*D$^-$) or LOS-sialylation (B1940*gal*E$^-$). A spontaneous capsule deficient variant (3) (B1940*sia*D$_{SSM}$) was also used which is capable of switching on the capsule synthesis *in vitro* in a frequency of $3x10^3$. Infection of infant rats with the wild type strain revealed a high potential of to cause bacteremia. An infective dose of 100 CFU applied intraperitoneally resulted in a bacteremia of $10^{3.5}$ CFU/ml after 9 h of infection. This potential was attenuated 100-1000 fold in the spontaneous capsule deficient variant (LOS sialylation$^+$) which was capable of switching on the capsule synthesis in a frequency of $3x10^{-3}$ *in vitro*. All reisolates of B1940*sia*D$_{SSM}$ from the blood or the peritoneal fluid were encapsulated. Using a mutant irreversibly deficient in capsule synthesis, but nevertheless sialylating its LOS, bacteremia could only be achieved using 10^6 times higher numbers of bacteria when compared to the wild type strain. All reisolates of this mutant from the blood and the peritoneal fluid were proven to be unencapsulated suggesting that defense mechanisms directed against unencapsulated meningococci were exhausted using very high doses. Interestingly, bacteremia could never be achieved when the encapsulated, LOS-sialylation deficient mutant B1940*gal*E$^-$ was inoculated into the newborn rats, probably because LOS sialic acid more potently than the polysialic-acid capsule protects against the action of the rat complement system which cannot be exhausted. Microscopic analysis of peritoneal cells revealed that the mutants B1940*gal*E$^-$ and B1940*sia*D$^-$ were phagocytosed in contrast to the wild type strain suggesting that both the capsule and the LOS sialic acid are prerequisites for the resistance of meningococci against phagocytosis by rat intra peritoneal phagocytes. In conclusion our study demonstrates that in the infant rat model of meningococcal infection both forms of sialic acid on the bacterial cell surface are indispensable for systemic survival.

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Complement factor C3b deposition via the classical pathway of complement activation on surfaces of isogenic sialic acid mutants of *Neisseria meningitidis*

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The incorporation of sialic acids into cell surfaces has been proven to interfere with the activation of the complement system (1). *Neisseria meningitidis* serogroup B expresses sialic acids as α-(2→8) linked homopolymers which form the capsule polysaccharide and as a terminal modification of the lipooligosaccharide (LOS). Although it is established that the defense mechanisms of meningococci against the action of the complement system largely depend on the surface exposure of sialic acids (2), it remains unclear which form of sialylation of the bacterial surface is directed against specific host defense mechanisms in the meningococcal infection, i.e. bacterial lysis by the classical or alternative pathway of complement activation and opsonophagocytosis. In the present study we used isogenic mutants of *Neisseria meningitidis* serogroup B (strain B1940) deficient either in capsule expression, LOS sialylation or both to analyze the classical pathway (CP) mediated deposition of the complement factor C3b on neisserial surfaces in correlation to their sialic acid expression. C3b deposition was analyzed by immunoblotting using the C3 specific monoclonal antibody 755 (3). Complement factor C8 deficient human serum was used as the complement source in order to allow prolonged incubation of the bacteria without induction of lysis. Furthermore, C8 deficient serum has been shown to inhibit the amplification loop of the alternative pathway of complement activation (4). Accordingly, the use of 10% C8 deficient serum proved to promote predominantly the activation of the complement cascade via the CP as could be shown by blocking the CP using EGTA and magnesium, since the deposition of C3b and its cleavage products was drastically reduced under these conditions. Interestingly, C3b deposition on the surfaces of meningococci from C8 deficient human serum via the CP occurred only in mutants defective in their capability to sialylate the LOS, irrespective of the capsule phenotype. The effect was detectable after three minutes of incubation and reached a maximum after 15 min. Using 10 % normal human serum survival of the meningococci in the bactericidal assay again depended on the expression of LOS sialic acid irrespective of the capsule phenotype. Bactericidal assays using 40 % serum, however, proved both the capsule and the LOS sialic acid to be indispensable for serum resistance. This finding suggests that either pathway of complement activation interacts differentially with meningococcal sialic acids.
We conclude that C3b deposition and bacterial lysis via the CP of complement activation is exclusively inhibited by the expression of LOS sialic acid irrespective of the capsular phenotype.

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Lipooligosaccharides, Poster 35

Cloning of the Lipooligosaccharide α-2,3-sialyltransferase from the bacterial pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae.*

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The genes encoding the α-2,3-sialyltransferases involved in LOS biosynthesis from *Neisseria meningitidis* and *N. gonorrhoeae* have been cloned and expressed in *Escherichia coli* . A high sensitivity enzyme assay using a synthetic fluorescent glycosyltransferase acceptor and capillary electrophoresis was used to screen a genomic library of *N. meningitidis* MC58 L3 in a "divide and conquer" strategy. The gene, denoted *lst*, was found on a 2.1 kb fragment of DNA , and its sequence was determined and then used to design probes to amplify and subsequently clone the corresponding *lst* genes from *N. meningitidis* 406Y L3, *N. meningitidis* M982B L7 and *N. gonorrhoeae* F62. Functional sialyltransferase was produced from the genes derived from both L3 *N. meningitidis* strains, and the *N. gonorrhoeae* F62. However the *N. meningitidis* M982B L7 gene contained a frameshift mutation which renders it inactive. The expression of the *lst* gene was easily detected using the enzyme assay, and the protein expression could be detected when an immunodetection tag was added to the C-terminal end of the protein. Using the synthetic acceptor N-acetyllactosamine-aminophenyl-(6-(5-(fluorescein-carboxamido)-hexanoic acid amide), the α-2,3 specificity of the enzyme was confirmed by NMR examination of the reaction product. The enzyme could also use synthetic acceptors with lactose or galactose as the saccharide portion. This study is the first example of the cloning, expression and examination of α-2,3-sialyltransferase activity from a bacterial source

Neisseria gonorrhoeae must express the paraglobosyl LOS in order to invade human genitourinary epithelial cells

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Introduction. Adherence to and invasion into host genitourinary epithelial cells are the first steps in infection by the *Neisseria gonorrhoeae. N. gonorrhoeae* make LOS that have glycose moieties that are the same as those of human lactosyl [Lac-R or Gal(ß1-4)Glc-R], paraglobo- [LacNAcß1-3Lac or Gal(ß1-4)GlcNAc(ß1-3)Gal(ß1-4)Glc-R] and ganglio-series (GalNAcß1-3LacNAc-R) glycosphingolipids. Intraurethral challenge of male volunteers with LOS variants showed that only those gonococci that make paraglobo- and ganglioside-like LOS can cause urethral leukorrhoea. In the present study, we investigated the role of these two gonococcal (LOS) in this process of parasite-directed engulfment of the bacteria by human genitourinary epithelial cells.

Materials and Methods. We used MS11mkA, a gonococcal variant that makes only lactosyl LOS, MS11mkC, a variant that makes paraglobosyl and gangliosyl-like LOS, F62 and FA1090. The latter two strains makes paraglobosyl and gangliosyl-like LOS.

We subcultured organisms onto GC agar base supplemented with IsoVitaleX®(BBL, Cockeysville, MD) (GC agar) and used standard visual criteria to select piliated and opaque.

Purified MS11 A LOS and C were prepared by our standard methods. *N*-Acetyllactosamine, α-Lactose, ß-Lactose, or Lactose, *N*-Acetyl-D-Glucosamine, Glucose, D-Mannose, Lacto-*N*-tetraose, *N*-Acetylneuraminyl-Lacto-*N*-neotetraose, Lacto-*N*-neotetraose, GM1, GM2, GT1b and GMmix were used as inhibitors.

MAb 1B2 and 2D4 were obtained from the ATCC Hybridoma Bank. MAb 1B2 is specific for the terminal galactose of paraglobosyl LOS. MAb 2D4 binds gangliosyl LOS made by some, but not all, gonococcal strains. MAb 6B7 binds a basal epitope of lactosyl LOS.

All the adherence and invasion experiments were performed in 24 well cell culture plates, $2x10^5$ epithelial cells were seeded into each well and incubated for 16 hours before the addition of $2x10^7$ bacteria to each well. The inoculated cells were incubated for 2-6 hours, and then rinsed with PBS 3 times. For assays of invasion, gentamicin (50μG/mL) was added to each cell and the plates incubated for two hours in order to kill extra cellular bacteria. The wells were again rinsed with PBS 3 times. Cells then were dislodged from the wells with trypsin and lysed with 1% saponin in PBS. Lysates

diluted 1:10 and 100μL were inoculated onto GC agar. Bacterial colonies were counted after overnight growth.

Results and discussion. We found that MS11mkC, which has both paraglobosyl and gangliosyl LOS, invaded HEC-1-B cells, whereas MS11mkA which has only lactosyl LOS did not. There was no difference in the ability of these two variants to adhere to the cells.

LOS purified from the C variant, but not that purified from the A variant, inhibited invasion.

MAb 1B2 which binds paraglobosyl LOS reduced invasion into HEC-1-1B and PC3 by strains MS11mkC and FA1090 by 40-60%. MAb 6B7, which binds a LOS basal epitope, did not. MAb 2D4, which binds strain F62 gangliosyl LOS, did not prevent this strain from invasion.

None of the monosaccharide components of lacto-*N*-neotetraose, nor its LacNAc and Lac disaccharide subunits, inhibited the adherence or invasion. Lacto-*N*-neotetraose, but not sialylated Lacto-*N*-neotetraose, nor Lacto-*N*-tetraose, completely inhibited invasion of, but not adherence of HEC-1-B cells by MS11mkC. The GM2 ganglioside, which binds MAb 2D4, had not inhibitory effect.

Conclusion. These data show that it is the paraglobosyl LOS structure, not the gangliosyl LOS structure that is necessary for gonococcal invasion of human genitourinary epithelial cells.

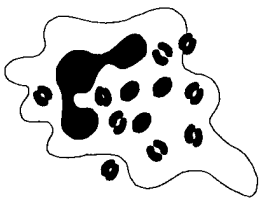
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Sialylation of LOS inhibits gonococcal killing primarily through an effect on classical pathway activation.

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Sialylation of LOS has been shown to inhibit the *in vitro* complement-dependent killing of gonococci (Gc) isolated from patients with symptomatic mucosal infection. Our laboratory has been interested in the mechanism by which sialylation exerts its inhibitory effect on the susceptibility of Gc to IgM mediated, complement-dependent killing. In order to assess the impact of sialylation on complement activation we examined the relationship between the extent of LOS sialylation and Gc susceptibility to killing by different concentrations of intact serum as well as serum in which only the classical or alternative pathways were intact. LOS sialylation increased in a dose dependent manner between 0 and 20 μg/ml CMP-NANA in the growth media and was accompanied by a dose-dependent inhibition of killing (2.53 to 0.21 logs) in 10% pooled human serum (PHS). A similar decrease in killing occurred when sialylated organisms were incubated in 25% PHS, but in contrast to the situation in 10% PHS, significant killing (1.2 logs) remained under conditions of maximal LOS sialylation. Killing of unsialylated Gc in serum depleted of C1q, factor D and properdin (qDP-) and thus lacking both a functional classical or alternative pathway was minimal (-0.33 logs) but was restored to normal by the addition of C1q alone but not factor D plus properdin. CMP-NANA inhibited C1q dependent killing in a dose dependent manner (4.92 to 1.77 logs). In order to determine which component of the classical pathway was affected by LOS sialylation specific studies of C4b BP, C1q, C4, C2 and C3 binding or consumption were performed. Sialylation had no effect on C4b BP binding but displayed progressively greater effects on C1q and C4 binding, and C2 consumption which culminated in a major difference in C3 binding and the rapid inactivation of bound C3. These findings suggest that the effect of sialylation is to inhibit the progressive enzymatic amplification of classical pathway activity beginning with C1q binding. Bactericidal IgM binding was unaffected by Gc sialylation and Western blots indicated that the LOS species to which IgM bound was different than that which was sialylated. However, the quantity of bound IgM necessary to achieve 50% killing increased with CMP-NANA input and extent of LOS sialylation. Together these data indicate that Gc killing in PHS normally proceeds via IgM activation of the classical pathway and that though sialylation does not affect the quantity of IgM binding, it does exert a qualitative effect on the function of bound IgM, perhaps by interfering with the ability of this pentameric molecule to engage sufficient epitopes to undergo the putative change in shape required for effective complement activation. As a consequence, complement activation on sialylated Gc, demonstrates increasingly apparent differences at each step in the classical pathway as the enzymatic amplification that occurs with the activation of each component becomes progressively impaired.



Noncapsular Vaccines

Clinical trials with outer membrane protein vaccines and PorA recombinant vaccines

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General childhood immunization against systemic infectious diseases such as meningitis caused by encapsulated bacteria has come within reach. The general introduction of a capsular polysaccharide protein conjugate vaccine has eliminated infectious diseases caused by Hib (Haemophilus influenzae type b).A major effort is now needed to ensure a maximum global health benefit through the Expanded Program on Immunization (EPI). The development of conjugate vaccines against infectious diseases caused by pneumococci and group A and C meningococci is in an advanced stage of development, including the performance of efficacy studies. An efficacious group B meningococcal vaccine is still lacking, because the group B capsular polysaccharide is poorly immunogenic. During the last decade a number of efficacy trials were undertaken with meningococcal outer membrane protein vaccines in Cuba, Brazil, Chile and Norway. The outcome of these studies indicated efficacies in the range of 50-80%, revealing no protection in the very young, showing a rapid decline in the antibody response over time. The results are summarized in Table 1.

The studies reveal a remarkable similarity in outcome. The differences in efficacies in the placebo controlled, double-blinded studies appear to be related to the study period, indicating waning immunity.
Two questions are important:

i) The specificity of protective antibodies
ii) The lack of protective immunity in the very young with the Finlay and WRAIR vaccines

In relation to question i) it will be extremely hard to correlate volunteers immune responses with protection while working with a multicomponent vaccine. The best we can achieve relates to the analysis of the specificity of bactericidal antibodies by *in vitro* methods.

The age-related differences with respect to protection as found in the Chilean and Brazilian studies appeared to be reflected by the bactericidal titers as measured in the laboratory (4,5). These results show that the Finlay and WRAIR vaccines do not induce bactericidal antibodies in the very young.

With respect to the specificity of the vaccine induced bactericidal antibodies the following has been found/published so far: the Chilean and Brazilian studies revealed evidence for the importance of antibodies against PorA, the class 1 OMP (5,6). The

Norwegian study demonstrated the importance of Opc and PorA with respect to the induction of bactericidal antibodies (7). This study also demonstrated the beneficial effect of a third immunization, 4-5 yrs after the initial series of two. In addition to Opc and PorA, other antigens were found to induce bactericidal antibodies after the third immunization, albeit in a minority of the vaccinees, but with a tendency of cross-reactivity towards strains with varying serosubtype composition (7).

Table 1. Efficacy trials with meningococcal OMP vaccines

Vaccine	Study	Efficacy
Purified total OMP in proteoliposome with added high mol. wt OMP (Finlay) 50 μg (B:4:P1.19,15)	Cuba, teenagers, randomized at school level, double-blinded placebo control, two immunizations, 16 mo study period	83% (Ref. 1)
Same vaccine	Brazil, case-control, two immunizations, 12 mo follow-up	-37% (3-23 mo) 47% (24-47 mo) 74% (48-83 mo) (Ref. 2)
Total OMP in vesicle (OMV) formulation (SIFF) 25 μg (B:15:P1.7,16)	Norway, teenager, randomized at school level, double-blinded, placebo control, two immunizations, near to three year study period	57.4% (Ref. 3)
Purified class 1, 3, 4 containing OMP (WRAIR) 100 μg (B:15:P1.3)	Chile, 1-21 yrs, randomized, double- blinded, placebo control, two immunizations, 20 mo follow-up	-39% (1-4 yrs) 70% (5-21 yrs) (Ref. 4)

Quo vadis? The results obtained with the first generation OMP vaccines are very promising. Improvement of these vaccines can be achieved in two ways:

I) to focus on the antigens with demonstrated ability to induce bactericidal antibodies

ii) to further investigate other vaccine candidates with special emphasis onto cross-reactive immunogens.

With respect to antigens with demonstrated ability to induce bactericidal antibodies, the class 1 OMP deserves further attention. The Opc protein can be considered as well, however, most case isolates do not seem to express appreciable amounts of this protein. Vaccines preferably will have to induce a consistent immune response in the vast majority of vaccinees. In order to achieve this, vaccines were constructed to contain

PorA for the greater part (8). Since the serosubtype-specificity is a critical factor, a hexavalent PorA vaccine in vesicle formulation was prepared and immunization studies in adults, infant monkeys and infants were carried out.

The induced bactericidal antibodies were dependent upon PorA as proven by the use of various target strains having defined deletions/mutations within porA (9).
During the conference, I will also give data about infant and infant monkey studies with the multivalent PorA vaccine as well as the Norwegian OMP vaccine. Results obtained with the Norwegian OMV vaccine after immunization of Icelandic teenage volunteers will be discussed. A set of strains with defined deletions/mutations in Opc/PorA/PorB were used to characterize the bactericidal antibodies. One important outcome of these studies is the observation that the OMV vaccine formulation allows for the induction of bactericidal antibodies in the very young. The bactericidal antibodies are totally dependent upon PorA in case of the Dutch multivalent PorA vesicle vaccine. Results obtained with sera from vaccinees having received the Norwegian vaccine, indicate the critical importance of PorA although other antigens play a role.

The patterns of bactericidal antibody activity found with the multivalent PorA vaccine suggest some antigenic competition amongst PorA's within a vesicle (three PorA's are expressed simultaneously into one vesicle). The bactericidal antibodies were further analyzed by using target strains with deletions and point-mutations within the relevant PorA epitopes.

Other antigens/protection assays. Research is ongoing towards other possible vaccine antigens. In addition to the in vitro bactericidal assay, other protection assays can be of help in the finding of potential protective antigens. Such assays relate to the mechanism of phagocytosis or the combination of phagocytosis and direct bactericidal mechanism. In addition to the established value of the in vitro bactericidal assay, the following methods deserve further development:

i) an in vitro opsonophagocytosis assay
ii) an in vitro whole-blood assay, combining the bactericidal and opsonophagocytosis mechanisms
iii) passive immunization with vaccinees antibodies and challenge in an appropriate animal model.

A number of antigens in addition to PorA were identified to be able to induce bactericidal antibodies:

LPS.

Conflicting results have been obtained with respect to bactericidal activity of human antibodies against meningococcal LPS. Because of the abundance of expression and the inherent stability of saccharide epitopes, LPS still deserves further attention. The availability of well-defined mutants (10) allows a detailed analysis of human antibodies.

A critical factor will be the potential cross-reactivity of LPS-specific antibodies with human tissue because of the structural similarities.
Studies with a collection of monoclonal antibodies in relation to bactericidal activity, tissue-crossreactivity and specificity as defined with mutant strains will be described in my presentation.

The definition of the LPS composition and selection of appropriate variants by way of these well-defined monoclonal antibodies, appears to be critically important in relation to the bactericidal assay.

Opa and Opc.

As mentioned, the Opc protein is associated with the induction of bactericidal antibodies. Since Opc is expressed by many carrier isolates, the question comes to mind if the induction of bactericidal antibodies against Opc will be able to kill the bacterium before Opc-non expressing bacteria enter the bloodstream.

In this context, the issue of parenteral versus mucosal immunization also appears relevant. As far as we understand the epidemiology and pathogenesis of meningococcal disease, carriage seems to be a self-limiting event by way of inducing bactericidal antibodies. The description of a highly conserved 22 kDa OMP that induces cross-reactive bactericidal antibodies (11) may relate to Opc. Until now, the feeling is predominant that the variability of Opa will be too high to be of vaccine relevance. However, the extent of this variability has not been delineated.

PorB.

PorB is the major OMP of the meningococcus and because of that a likely vaccine candidate. However, PorB appears not to be able to induce a consistent, relevant bactericidal antibody response in volunteers being immunized with vaccines containing appreciable amounts of this protein. The growth of meningococci under glucose limitation with an effect on LPS composition (sialylation most likely) completely eliminated the bactericidal effect of PorB-specific monoclonal antibodies (12). Monoclonal antibodies against PorB given passively to infant rats before challenge, were poorly protective (13).

Fe-limitation inducible OMPs/exotoxins.

Particularly Tbp2 and FrpB are interesting vaccine candidates (14,15). However, Tbp2 and FrpB are highly variable and it needs to be investigated how many types will be needed to ensure broadly reacting bactericidal antibody activities (16,17). Interestingly, meningococci appear to be able to express a LbpB with homology to Tbp2, indicating that the binding of lactoferrin resembles the binding of transferrin, involving two lactoferrin-binding proteins (18). Further studies are needed to investigate the vaccine potential of LbpB. Iron-limitation also appears to induce the expression of two proteins, FrpA and FrpC, which reveal homology to the RTX family of bacterial exotoxins (19).

The role in pathogenesis as well as the potential vaccine implications still have to be established.

Still other antigens.

An overview is given in reference 20. Cross-reactive antigens such as Rmp, H8 have revealed disappointing results as vaccine candidates; Pili were found to be extremely variable. In addition to the porin functions (PorA, PorB); adhesion/invasion mechanisms (pili; Opa; Opc); Fe-uptake mechanisms (LbpA,B; Tbp1,2) there is some lack in our knowledge with respect to the OMPs involved in protein secretion. On the basis of homology to enterobacterial proteins, the Omc protein seems important in this respect (21).

In conclusion. We have reached a critical stage in menB vaccine development. Outer membrane proteins do induce protective immunity as found with the first generation OMP vaccines. These vaccines however contain many nonprotective components (i.e. not inducing bactericidal antibodies) and vaccinees respond in a scattered manner because of the complexity of the vaccine. PorA was found to be the most relevant antigen within first generation OMP vaccines. Developments with respect to homogeneous PorA containing vesicle vaccines are promising since a PorA dependent bactericidal antibody response can be induced, even in infants. Further studies, including aiming at efficacy, are needed with such vaccines. Laboratory research indicates that further human immunization studies are indicated with a few other vaccine candidates such as Tbp2, FrpB, LbpB, Opc. Because of the inherent stability of saccharide structures, research towards the B polysaccharide as well as LPS needs to continue.

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Recombinant and synthetic antigens from meningococcal class 1 protein

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Meningococcal class 1 outer membrane is an important candidate antigen for incorporation into vaccines designed to prevent infection by serogroup B meningococci. Monoclonal antibodies directed against the class 1 protein are highly bactericidal for meningococci and also passively protect infant rats in vivo against meningococcal infection (1). Most importantly, recent data from the Norwegian trial has shown that the presence of bactericidal activity in vaccinees' sera correlates with the presence of antibodies directed against the class 1 protein (2).

The cloning and sequencing of the *porA* genes which encode the class 1 protein (3) have permitted structural and antigenic studies which have led to a model of the organization of the protein within the OM (4). This predicts a structure composed of 16 amphipathic β -strands which traverse the outer membrane and generate eight surface exposed hydrophilic loops. Sequence variation is largely confined to two discrete variable regions designated VR1 and VR2, which are located in the longest surface-exposed loops 1 and 4 respectively. This restricted antigenic diversity is the basis for the sero-subtyping classification of meningococci. In addition, epitope mapping with synthetic peptides has localized the epitopes recognized by the bactericidal and protective subtype-specific mAbs to the apices of these loops (5).

Further studies on the protective effect of class 1 protein are hampered by the presence of additional components in the vesicle preparations so that the immune response to the different components varies between individuals. As an alternative, in this study a series of recombinant and synthetic antigens have been used to investigate optimal presentation of class 1 protein antigens for immunization strategies designed to induce biologically functional antibodies.

The *porA* gene encoding class 1 protein has been cloned in *E. coli* into the high level expression vector p-GEMEX^{TM}-1 in which the class 1 protein was expressed as a fusion with the bacteriophage T7 gene 10 capsid protein. The class 1 protein has also been expressed without the gene 10 leader peptide and with a poly (His) tag to facilitate purification by metal ion affinity chromatography. The recombinant proteins have been used in experiments designed to investigate refolding of the protein for immunization, including the use of liposomes incorporating the additional adjuvants monophosphoryl lipid A and muramyl dipeptide.

As an alternative strategy synthetic peptides have been used to focus the immune response to the protective epitopes. Previous studies utilized a multiple antigen peptide

(MAP) containing a protective class 1 protein B-cell epitope together and promiscuous Th-cell epitope (6). Immunization using Freund's adjuvant produced a bactericidal immune response. In the current study this MAP has been used to investigate the use of adjuvants both acceptable for human immunization and with ability to induce relevant bactericidal IgG subclasses

With each of the antigens a good immune response to the immunizing agent was obtained but the antibodies produced differed markedly in antigenic specificity and their ability to activate complement mediated killing of the meningococci. The magnitude of the bactericidal effect could be correlated with production of a sero-subtype specific immune response, demonstrating that the presentation of the antigen in a conformation resembling the native protein produced the most functionally effective immune response. These data emphasize the importance of designing vaccination strategies to produce the most biologically effective immune response by optimizing epitope specificity, antibody subclass and avidity.

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Infant rat meningitis passive protection assay and protection evoked by human group B OMV vaccine induced antibodies.

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Infant rat meningitis model has previously been found useful for studying the passive protection afforded by mouse antibodies to meningococcal surface antigens (1). It has been used for comparison of the protective activity of monoclonal antibodies directed to capsular polysaccharide, lipopolysaccharide, PorB and PorA (2). Furthermore, the infant rat passive protection model has been used as a guide in our developmental work of PorA-based recombinant vaccine (3).

In this model 4 to 6 day-old pups are injected intraperitoneally with bacteria two hours after the passive administration of antibodies. The development of bacteremia and meningitis are followed by culturing blood and CSF sample 6 hours later.

We have now optimized this model for use with human sera. In these studies the i.p. challenge strain was rat passaged B:15:P1.7,16 (IH5341) (3). Doses of 10^5 and 10^6 cfu/pup proved most useful. The larger dose produced bacteremia and meningitis in all pups while the lower dose induced bacteremia in all animals and meningitis in a majority of them. Four human sera (bactericidal titers, SBA, <1:2, 1:4, 1:32 and 1:64, respectively) obtained after immunization with a group B meningococcal outer membrane vesicle vaccine (4) (OMV) were used for protection experiments. The sera were inactivated and diluted 1/10, 1/30 and 1/100; 100 µl were injected i.p. Saline was used as a negative and monoclonal antibody to group B capsular polysaccharide (2 µg/pup) as a positive control. There were 6 pups/group and to test the reproducibility of the assay, most of the experiments were repeated once or twice.

The reproducibility of the assay was good and results of the two to three repeat experiments were combined. The non-bactericidal serum (SBA <1:2) did not protect for either bacteremia or meningitis. Serum with weak SBA (1:4) showed protection against the lower dose, but not against the higher dose. Serum with the SBA titer 1:32 protected at the 1/10 dilution against both doses. The 1/30 serum dilution did not protect against higher challenge dose. However, with the lower dose of bacteria the majority of the animals were bacteremic but with low numbers of cfu (4 per cent of the negative control) recovered from the blood; correspondingly only 18 % had bacteria in the CSF. Serum with the highest SBA (1:64) protected at the 1/10 and 1/30 dilutions against both doses. No sera gave protection at the dilution 1/100. The development of meningitis clearly correlated with the number of bacteria in the blood; when the cfu in the blood exceeded 10^5/ml, the majority of the pups had bacteria also in CSF, whereas no bacteria were found in the CSF of animals with low grade or no bacteremia.

We conclude that the assay is entirely feasible with human sera, and its reproducibility is good. Two studies using this model with human sera are in progress: an evaluation of the model as a surrogate of protection in humans by using the sera from the "Icelandic study" (5), and a study of the ability of human antibodies evoked by group B outer membrane vesicle vaccines (4,6) to protect against B:15:P1.7,16 variants with point mutations in the loop 4 of PorA (6).

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Immunization with a low molecular weight meningococcal outer membrane protein protects against lethal experimental infection

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Even though meningitis caused by *Neisseria meningitidis* is currently an important problem in both developed and developing countries, there is presently no effective vaccine available that can stimulate a long-lasting protective immunity in young children (1). Efforts are being made to improve the current polysaccharide vaccines or to find alternative meningococcal surface antigens that could serve as effective vaccinogens. In this respect, the great interstrain antigenic variability of the major meningococcal outer membrane proteins (OMP) could be restricting their protective efficiency to a limited number of antigenically related strains. Here, we report that the immunization of mice with a newly identified highly conserved OMP with an apparent molecular mass of 22 kDa called NspA, protects against lethal experimental infection.

Monoclonal antibodies (MAbs) specific for the NspA protein were generated (2). These MAbs were used to demonstrate that the NspA protein is exposed at the surface of all the meningococcal strains tested. These strains represented all major serological groups. In the presence of complement, two of the MAbs, Me-1 and Me-7, exhibited *in vitro* bactericidal activity against the four meningococcal strains tested: two strains of serogroup B and one strain from each serogroup A and C. These two bactericidal MAbs also protected mice against experimental *N. meningitidis* infection (3). In fact, the injection of ascitic fluid containing the NspA-specific MAbs 18 hours before the bacterial challenge increased the rate of survival of Balb/c mice from 8% observed in the control groups to 70%. This data clearly indicates the protective potential of antibodies directed against the meningococcal NspA protein.

The gene coding for the meningococcal NspA protein was identified and cloned into the expression plasmid vector pWKS30 (4). Similarity searches using the nucleotide and the deduced amino acid sequences of established databases confirmed that this protein has never been described previously. The affinity-purified recombinant NspA protein was then used to immunize Balb/c mice in order to evaluate its ability to confer protection against a bacterial challenge with a lethal dose of *N. meningitidis* strain of serogroup B. The mice were injected subcutaneously three times at three weeks intervals with 10 or 20 µg of affinity-purified recombinant NspA protein or control antigen preparations. Serum samples were obtained after each injection and the titers of these sera were determined by ELISA using meningococcal outer membrane preparations as the coating antigen. The results clearly showed that the purified protein is immunogenic when administered with an adjuvant such as QuilA. The serum titers varied from 1/2,000 to 1/51,000. The serum titers obtained in the control groups were below 1/200. Western immunoblotting

experiments showed that the antibodies present in the sera obtained from the immunized mice recognized the recombinant NspA protein, but more importantly reacted strongly on the nitrocellulose membrane with the native meningococcal NspA protein. In both groups of mice injected with the purified recombinant NspA protein, 80% of the mice survived the bacterial challenge compared to 0 to 40% in the control groups. Analysis of the sera indicated that the NspA immunized mice who died following the bacterial challenge had the lowest serum titers suggesting that there is a correlation between the specific antibodies titers and the observed protection.

In conclusion, the newly identified meningococcal NspA protein can induce an immune response that can protect mice against a lethal challenge. This protein is antigenically highly conserved among meningococcal isolates and is exposed at the surface of intact meningococcal cells, where it is accessible to the antibodies. For all these reasons we believe that this protein possess all the important characteristics to be considered a potential candidate for the development of a new broad-range vaccine against meningococcal disease.

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Anti-endotoxin activity of monoclonal antibodies against meningococcal LOS and the memory response to LOS incorporated into liposomes

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In our previous study we showed that incorporation of the native lipopolysaccharide (LOS) from *Neisseria meningitidis* followed with decrease of toxicity and increase of immunogenicity (1). The bactericidal and endotoxin neutralizing activity of antibodies to meningococcal LOS is still subject of discussions. A potential disadvantage of using complete meningococcal LOS is the presence of structures which are also found in the human host (2). Moreover, LOS as a thymus-independent antigen is generally thought to be poor at inducing secondary immune response.

Murine monoclonal antibodies (MAbs) against meningococcal L3,7,9 *Neisseria meningitidis* LOS were tested for their endotoxin neutralization properties. The MAbs were characterized by using a set of LOSs from meningococcal strain H44/76 having defined stepwise truncations - mutants *lgtB, galE* and PB4 (3,4). Four groups of epitopes on the oligosaccharide part of LOS could be identified (terminal, inner and two middle). MAbs specific to terminal lacto-*N*-tetraose unit and deep structures of LOS were broadly cross-reactive with human tissues and mast cells respectively. Activity of outer membrane blebs and chemically extracted LOS was amplified with plasma to stimulate IL-6 production in human blood cells. Neutralization of purified LOS and blebs was demonstrated with MAbs specific to middle part of oligosaccharide and in both cases inhibition of IL-6 stimulation appeared to be up to 500 times less active in the presence of plasma derived factors. Besides endotoxin neutralization, one of these MAbs demonstrated bactericidal activity. Avidity of anti-LOS MAbs seems not to be related to neutralizing activity and interaction of plasma factors with both LOS and blebs in most cases improved the interaction with MAbs.

LOS preparations isolated from meningococcal wild strain H44/76 (L3,7,9) and mutant strains *lgtB* and *galE* were incorporated into liposomes and used for immunization. Primary immune response was similar in all tested strains of mice (BALB/c, C57BL/6, AKR and nude) and followed with high elevation of IgM antibodies. Compared to the primary response, the secondary anti-LOS response is characterized by the appearance of IgG antibodies (IgG3>IgG1>IgG2b>IgG2a). Booster effect was lower in AKR mice and significantly higher in C57BL/6. The study of liposomes prepared with different ratio of LOS/phospholipids showed that the secondary immune response corresponded with

epitope density and did not require B-cell mitogenicity or cytokine stimulation (TNF-α, IL-6, IFN-γ). The priming effect of *lgtB* LOS was cross-reactive with wild H44/76 and *galE* LOSs and persisted for at least 8 weeks.

Thus antibodies to truncated L3,7,9 LOS can be expected to be more beneficial in induction of bactericidal and endotoxin neutralizing response against meningococcal infection. Our observations strongly support the hypothesis that memory response can also be induced with thymus-independent antigen.

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Design and production of meningococcal vaccine based on transferrin binding proteins

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Transferrin-binding protein 2 (Tbp2) from *Neisseria meningitidis* is involved in iron capture in the infected host and elicits bactericidal antibodies in animals (1); this is similar to what has been reported for recombinant TbpB from *Actinobacillus pleuropneumoniae* which was shown to induce protective immunity in pigs (2). When Tbp2 is purified to homogeneity from *Neisseria meningitidis* strain B16B6 (B:2a, P1.2) grown in 30l fermentors in an iron poor media (Mueller Hinton broth containing 30 µM EDDA) yields are low not exceeding 0.5mg/ml under our experimental conditions. The gene encoding Tbp2 has been cloned and sequenced and recombinant lipidated Tbp2 (67kDa) was produced in *E. coli* using the arabinose-inducible expression vector (3). A purification scheme was designed which involved the preparation of outer membranes, two ion exchange chromatographies and a polishing gel filtration step. The product obtained retained its ability to bind human transferrin (hTf) and was devoid of contaminants. Mice were immunized with different amounts of rTbp2 on days 0, 21 and 35 and bled on days 21, 35 and 42. The animals developed high IgG titers and bactericidal antibodies. The antisera were bactericidal versus strains expressing a 67kDa Tbp2 but did not induce the lysis of strains expressing a 85kDa Tbp2, these results were quite comparable to those reported earlier with Tbp1-Tbp2 purified from meningococcal cells (4) while others have described a broader cross reactivity (5). Because high molecular weight Tbp2s (>80kDa) are more divergent than low molecular weight (67kDa) Tbp2, we analyzed different truncated forms of Tbp2 before designing the vaccine antigen. Full length Tbp2 (85kDa) from strain M982 (B:9,P1.9) was produced in *E. coli* using the pMAL-c2 expression system. The fusion protein retained its ability to bind hTf and the system allowed to produce a series of truncated proteins including N-terminal domain (2-351) and C-terminal domain (352-691). The fusion proteins were purified by chromatography on amylose; the different Tbp2 molecules were analyzed for their ability to bind hTf and were inoculated to rabbits to produce antisera. We confirmed with this expression system that the N-terminal domain bound hTf as described earlier (6) but moreover identified a hTf binding site in the C-terminal domain of the protein. The analysis of the different antisera showed that the C-terminal domain induced more cross reactive antibodies than the N-terminal domain. These results allowed to design a rTbp2 from strain M982 which induced bactericidal antibodies broadly cross reactive against many strains of meningococci. Clinical grade Tbp2 will be tested in humans in the near future.

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Immunogenicity and safety of intranasal vaccination with meningococcal native outer membrane vesicles in mice and rabbits.

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Efficacy trials with meningococcal group B outer membrane protein (OMP) vaccines have demonstrated the potential of the OMPs to induce protective immunity (1,2,3,4). The OMP vaccines evaluated in efficacy trials, however, have been poorly protective in children under the age of 4 years (3,5). In these children, serum antibody responses as measured by ELISA were high, but bactericidal antibody titers were low. We have approached this problem by attempting to present the OMPs and the LOS in a lipid or native outer membrane environment. One of our strategies is to use native outer membrane vesicles (NOMV), never exposed to detergents, as an intranasal vaccine. The NOMV is an excellent antigen but has been considered too toxic to use as a parenteral vaccine in humans. We believe, however, that NOMV prepared from a genetically optimized vaccine strain can be a safe and effective vaccine if given intranasally (i.n.). This approach mimics the process of natural immunization and has the potential to induce a local antibody response against the OMPs that act as adhesins and invasins (6) as well as a serum bactericidal antibody response. NOMV were prepared under GMP from a mutant group B strain, 9162(-:15:Pl.3:P5.10,?:L3,7,9), deficient in sialic acid synthesis and therefore lacking capsule and sialylated LOS. This mutant was prepared using published sequence information (7) by deletion of a portion of the SynX gene and insertion of a Kanamycin resistance gene. For vaccine production the cells were grown on iron-deficient medium which resulted in the expression the iron regulated proteins. Two Opa proteins were expressed, but Opc was not expressed at significant levels. A vaccine strain expressing the L3,7,9 immunotype was chosen in spite of the presence of the lacto N-neotetraose group because of evidence we have obtained that bactericidal antibodies to this structure are often present in human sera following natural infections.

The NOMV vaccine was found to be non-pyrogenic in the rabbit when given intranasally (i.n.) at a dose of 400 μg protein (88 μg LOS). When given intravenously (i.v.) the maximum non-pyrogenic dose was about 0.05 μg/rabbit.

The mucosal and systemic immunogenicity of NOMV was determined in mice and rabbits. Mice were immunized intraperitoneally (i.p.) or i.n. with NOMV at day 0, and boosted i.n. at day 28. Mice developed serum bactericidal antibodies as well as high levels of specific serum IgA (4 μg/ml) and IgG (1-3 mg/ml) by day 42 as determined by ELISA. Mice immunized i.n. also responded with high levels of IgG and IgA antibody-secreting cells in the lungs as determined by ELISPOT. Rabbits immunized intranasally with three 100 μg doses of NOMV at days 0, 28, and 56 developed high serum bactericidal antibody titers (>512), and mean increases in serum IgG and IgA levels in excess of 450 and 370 μg/ml, respectively, at day 70. Western blot analysis of the rabbit

sera showed IgG antibody responses to the Opa proteins, Tbp2, LOS, class1,3, and 4 OMPs, and several proteins in the 45-60 kDal range. ELISA vs purified LOS showed geometric mean levels of antibodies against L3,7,9 and L8 LOS increased from < 0.05 μg/ml to > 10 μg/ml. The results of these animal experiments demonstrate that NOMV given intranasally in animals is safe and induces both serum bactericidal antibodies and a mucosal immune response which may help prevent meningococcal infections by interfering with adhesion and/or invasion. We conclude that these results warrant further intranasal studies with NOMV in human volunteers.

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Opsonic activity induced by a monoclonal antibody against the Lip (H.8) antigen.

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Introduction: The Lip antigen (formerly called H.8) is an outer membrane protein expressed on all pathogenic *Neisseria* species. The gene has been cloned and sequenced and reveals an unusual protein built up of several pentapeptide repeats (1). However, the functional role of the Lip protein is unknown. Unlike many other outer membrane proteins on *Neisseria*, the Lip protein display no variability among strains and might thus be an interesting vaccine antigen. Also, patients with meningococcal or gonococcal infections readily make antibodies against the Lip antigen.

Several mAbs against Lip have been made, but none have revealed any bactericidal activity (2). Affinity-purified human polyclonal antibodies against Lip are also negative in bactericidal activity (3). After these observations were published, much of the interest concerning Lip protein as a vaccine antigen has declined. Phagocytosis is an other effector function that may confer protection against meningococcal disease. The aim of this study was to characterize the opsonophagocytic activity of a mAb specific for the Lip antigen.

Experiments: We have recently made a mouse mAb (denoted 215,C-1, of IgG2a isotype) against Lip, which reacted identically with mAb 2-1-CA2 (W. Zollinger) on Western blot. The 215,C-1 mAb did not induce any bactericidal activity, which is in accordance with the other reported anti-Lip antibodies. However, when tested against ethanol killed *N. meningitidis* strain 44/76 (B15: P1.7,16) for opsonophagocytic activity, 215,C-1 revealed a very high activity. The mAb was also opsonic against other meningococcal strains: G1963, 8069, B385, all previously alcohol killed and stored at -20 °C. However, when tested against live 44/76 or B385 the activity was almost negative and phagocytosis was induced in only about 20% of the neutrophils and few bacteria were ingested per cell. This low/negative opsonic activity remained also after ethanol fixation of the same inoculi.

The expression of the Lip antigen on the opsonic+ and opsonic– ethanol killed strains was further tested in ELISA, immuno electron microscopy (IEM) and by Western blot (WB).

In whole cell ELISA, using the 215,C-1 mAb, a much stronger binding against the opsonic+ preparation than against the opsonic– preparation was observed.

IEM of whole cells was carried out using an on-grid immunogold-labeling technique incubating with 215,C-1 as primary antibody and goat anti-mouse conjugated to 10 nm colloidal gold particles as secondary antibody. The opsonic+ preparation of strain 44/76

showed an even, dense labeling of all bacteria, as opposed to the opsonic– preparation of the same strain, which showed a strong variation in labeling density from moderately labeled cells to unlabeled cells. The results from ELISA and IEM might indicate that the Lip antigen is hidden within the membrane structure and scarcely exposed at the surface on live and recently ethanol killed bacteria, and that prolonged storage might expose the 215,C-1 epitope of the Lip protein. However, this is unlikely since in WB the bacterial preparations that induced high opsonic activity showed strong staining of the Lip band, whereas the preparations that were negative in opsonic activity showed much fainter staining. This suggests that there is a difference in the total amount of the Lip protein between the opsonic+ and the opsonic– preparations, and that this difference is metabolically regulated, and not a result of live vs killed or stored bacteria. How the Lip antigen is expressed on the surface of in vivo growing meningococci remains to be elucidated.

	Phagocytosis		Bactericidal		ELISA		IEM		WB	
					Mab against					
Batch	Lip	P1.16	Lip	P1.16	Lip	P1.16	Lip	P1.16	Lip	P1.16
44/76-94	+++	+++	n.t.	+++	+++	+++	+++	+++	+++	+++
44/76-95	+	+++	–	+++	+	+++	–/++	+++	±	+++

Conclusion: These studies show that the Lip antigen is strongly opsonic *per se*, but that it may show a restricted and variable expression at least after in vitro cultivation.

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Expression and purification of meningococcal class 1 porin from *E. coli*: influence of adjuvants on specificity of the immune response to native protein.

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Currently licensed meningococcal vaccines based on group specific capsular polysaccharides offer no protection against serogroup B strains which currently cause the majority of European outbreaks, since the group B polysaccharide is non-immunogenic in humans, probably due to the structural similarity with human glycoproteins (1). Alternative sub-capsular antigens are being investigated as potential vaccine candidates. The class 1 porin protein is one of the two most abundant proteins in the outer membrane and the gene which encodes it, the *porA* gene has been cloned and sequenced (2).The class 1 protein is immunogenic (3) and antibodies raised against it are bactericidal to meningococci *in vitro* (4) and protect against infection *in vivo* (5).

The gene for the class 1 protein was isolated from a P1.16 strain (MC50) and ligated, in frame, into an appropriate 'XPRESS PRSET' vector (Invitrogen) overnight at 14°C. The Xpress system is designed for high level production and purification of recombinant proteins which are fused to a short leader peptide containing a polyhistidine sequence which has a high affinity for divalent cations enabling the protein to be purified by affinity chromatography on Ni^{+2} agarose. The plasmid gives high level expression of recombinant protein from a T7 promoter. T7 RNA polymerase can be introduced to the system via a M13 phage carrying the T7 polymerase gene, or by using *E. coli* JM109(DE3) which has its own T7 RNA polymerase. Thus, the ligated plasmid containing the *porA* gene was transformed into JM109 and expression was induced by the addition of IPTG. Crude cell lysates were analysed by SDS PAGE gel electrophoresis for the presence of class 1 protein. Large amounts of class 1 protein were present in cell lysate pellets, which was solubilized and purified by affinity chromatography on a Ni^{+2} agarose column. The effects of the protein on the immune response were studied using various adjuvants and liposomes.

Liposomes composed of L-α-phosphatidylcholine and cholesterol were prepared by a dialysis-sonication method (6). Recombinant class 1 protein was solubilized in 0.2% SDS and incorporated into liposomes both on its own and with the additional adjuvants muramyl tripeptide-phosphatidylethanolamine (MTP-PE) or monophosphoryl lipid A (MPLA) both at 1mg/ml concentrations. In each case immunogold electron microscopy with a P1.16 specific monoclonal antibody showed that the protective epitope was located on the surface of the liposomes. In addition solubilized class 1 protein was also adsorbed to alum, incorporated into an emulsions with (a) Ribi adjuvant containing MPLA, cell wall cytoskeleton and trehalose dimycolate, and (b) squalane and pluronic-block copolymer L121.

Groups of Balb-C mice and New Zealand half-lop eared rabbits were immunised at 0, 14, 28 and 42 days with 20 μg recombinant class 1 protein liposomes in a volume of 100μl (in PBS pH 7.2). In addition, purified class 1 protein in alum, Ribi and pluronic emulsions were used in simultaneous immunisations. Animals were bled pre-immunisation, at regular intervals throughout the immunisation schedule and for a further three months after the last injection.

Sera from the animals given the emulsions and alum gave the greatest response in ELISA against native protein and homologous outer membrane preparations with pluronic giving the best titres. The addition of adjuvants to the liposomes produced significant increases in response to the purified class 1 preparation and against homologous outer membrane preparations. In Western blots against homologous outer membranes, all the sera reacted specifically with the class 1 protein.

The sera were analysed for their IgG subclass specificities. Sera from animals immunised with the Ribi and pluronic emulsions and liposome preparations containing MPLA gave antibodies of IgG2 subclass associated with bactericidal activity. Sera produced from all the other preparations only gave IgG1 subclass antibodies.

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Expression of the outer membrane protein complex of *Neisseria meningitidis* group B in different culture conditions.

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Neisseria meningitidis express outer membrane proteins complex (OMPsC) which are surface antigens of vaccine interest (1). The majority proteins can be present in numbers from 3 to 5 and with molecular weights between 25 and 46 KDa (2). Other outer membrane proteins of larger size molecules between 70 and 110 KDa were expressed when cultivated with iron limitation (3). The expression of OMPC is variable depending on the genetic features of the strain, medium and culture conditions.

The objective of this paper is to assess the potentiality of the expression of Neisseria meningitidis group B in fermentation with different culture conditions and concentrations of Fe^{+3} in the medium.

Cultures of strain B:4:P1.15 were carried out with successive passes in fermentors of up to 35L, in supplemented Frantz medium with dialyzed yeast extract (FM) under oxygen and pH controlled or uncontrolled conditions. The kinetics of expression of high molecular weight proteins was determined in FM medium and the character of the iron regulated were assessed by adding or depleting Fe^{+3} in the FM medium. Harvest took place at the end of the logarithmic growth phase. The OMPC was extracted with 0.2 M Lithium Chloride or 0.5 % Sodium Deoxycolate and was separated by ultra-centrifugation at 100,000 g. The proteins were analyzed through SDS-PAGE and quantified by laser densitometry.

The growth kinetics in the cultures conducted in FM medium with controlled pH showed larger growth speed. Harvest was accomplished in the maximum of feasible microorganisms. The yield of biomass and the composition of OMPsC were similar for each studied condition. The proteins present in the complex, class 1, 3, 4, 5, 70 kDa and 80 kDa, were expressed with a uniform composition. The kinetics of expression of the 70 kDa and 80 kDa proteins were related to the concentrations of Fe^{+3} present in the culture. In the iron depleted FM medium these proteins were expressed since the very first hours of culture, whilst the medium with enough Fe^{+3} were not expressed which shows their iron regulation.

The assessed culture conditions did not influence the OMPsC expression. Results were that high molecular weight proteins are iron regulated, therefore controlling this, OMPsC of well defined and uniform composition of these proteins can be obtained. This fact is of major importance for vaccines based on OMPsC (4).

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Consistency in the large scale production of the outer membrane protein complex of group B *Neisseria meningitidis.*

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GMP's establish controls for each and every stage of the vaccine production processes to assure quality, safety and efficacy of the final product and production consistency. Among all, the culture production processes is the main stage in the expression of target antigens.

In antimeningococcal vaccines that are based on outer membrane protein complex (OMPsC), it is very important that the compound contain proteins that will induce broad spectrum antibodies, because the antigenic diversity of the meningococcus, makes it dificult to know which are directly responsible for the control of the disease. The defined composition of the complex and their presence in outer membrane vesicle (OMVs) are crucial for them to confer immunity (1). Using detergents, OMVs should be extracted from the biomass obtained in rational harvest time and well defined fermentation processes. (2-3).

The objective of this paper is to show large scale consistency in the expression of OMPsC of group B Neisseria meningitidis and characterize the purified antigens regarding their composition, structure and contaminant levels.

Thirty fermentation processes of strain B:4:P1.15 were studied in Frantz medium supplemented with yeast extracts dialyzed at a scale of 300 litters. The extraction of OMVs was carried out with 0.5% sodium deoxycolate and was separated by ultra-centrifugation. After purification, the composition and structure of OMPsC were studied. Protein analyses were conducted using Lowry method, SDS-PAGE and laser densitometry. Electronic microscopy was used to determine vesicle composition. Lipopolysaccharides (LPS) were quantified by KDO method. Polysaccharide and nucleic acids contaminants were assessed by determining the sialic acid and 260 nm spectroscopy, respectively.

Uniform and vesicle structured proteins class 1, 3, 4, 5, 70 kDa, and 80 kDa forming part of the complex were shown during fermentation. The purification of the OMVs ensured stable concentrations of LPS. The level of contaminant polysaccharides and nucleic acids were kept below the acceptance limits.

The proteins present in the studied OMVs concurred with those reported in the batch of VA-MENGOC-BC studied in Iceland (4). Contrary to other papers (5 - 6) iron regulated proteins 70 kDa and 80 kDa were consistently produced and under control.

The large scale production of OMPsC met the established requirements for their use as vaccine antigens of VA-MENGOC-BC and had a well defined composition in the form of vesicles and having low contaminant levels.

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Recombinant Opc reconstituted into liposomes elicits opsonic antibodies.

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Opc is an antigenically conserved outer membrane protein of *Neisseria meningitidis* which is widely distributed among different serogroups. This protein is highly immunogenic in humans and induces bactericidal antibodies (1). The expression of Opc is regulated at the transcriptional level (2), with the amount of expressed protein ranging from very high in bacteria isolated from the nasopharynx to low in bacteria isolated from blood or cerebrospinal fluid. Opc seems to function as a bacterial adhesin, mediating some step of the meningococcal interaction with host epithelial cells (3).

The *opc* gene from the *N. meningitidis* strain B385 (B:4P1:15) has been cloned in *E. coli* fused to the first 44 amino acids of the P64k protein . The resultant 32 kDa protein was expressed as inclusion bodies, and after a washed pellet procedure it was obtained in soluble form with more than 80% of purity (4). In order to renature the recombinant polypeptide into a conformation resembling its native state, we have included it into synthetic liposomes by freeze-drying and analyzed the immunogenicity of the resulting preparation. Briefly, a mixture of empty vesicles and Opc was frozen and lyophilized three times, obtaining 30% incorporation of the protein into dried-rehydrated vesicles (DRV). Opc was inserted in the lipid bilayer rather than enclosed in the vesicles; and surface exposed as demonstrated by protease treatment of the liposome suspension. Furthermore, the intact vesicles bound the human monoclonal antibody LuNmO3, which recognizes a conformational epitope on natural 5C (5).

To further investigate the immunogenicity of this preparation, DRVs containing Opc mixed with the beta subunit of cholera toxin (CTB) and $Al(OH)_3$ as adjuvants were used to immunize Balb/c mice. The resulting sera showed high titers against pure recombinant 5C, as well as against OMPs of meningococci belonging to different serotypes. Moreover, it recognized $5C^{++}$ meningococci in colony blots, demonstrating that at least a portion of 5C in the immunizing liposomal preparation was in a native-like conformation. The antibodies, however, were not bactericidal, although they did show opsonic activity on a Fluorescence-Activated Cell Sorter (FACS) based assay. The reasons for their lack of bactericidal activity are currently under investigation.

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Influence of adjuvants on the humoral immune response towards a synthetic peptide containing a B-cell epitope from meningococcal class 1 protein.

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A major problem encountered in developing modern vaccines using chemical, recombinant or other technologies, is their weak immunogenicity. This has traditionally been overcome by the use of adjuvants, which are defined as compounds which can increase the humoral and/or cell-mediated immune responses to an antigen (1). A large number of structurally unrelated compounds, which can act via different pathways, are known to augment immune responses to weak antigens. Adjuvants commonly used or considered for human vaccines include mineral compounds, oil emulsions, natural structures and synthetic analogues derived from bacteria, saponins (of vegetal origin), surfactants, cytokines, and delivery vehicles such as liposomes, iscoms and microspheres.

Synthetic peptides as vaccines present a major problem because they are generally haptenic in nature and often require covalent linkage to carrier proteins to become effective immunogens. In addition, such highly purified peptide vaccines require very strong adjuvants to achieve early, high and long-lasting immune responses. In this study, a chimaeric synthetic peptide containing a meningococcal B-cell epitope in tandem with a defined tetanus toxin T-cell epitope, and assembled on a polylysine multiple antigen peptide core (BT-MAP) (2), was used as a model immunogen to assess the influence of various adjuvants on the humoral immune response. The adjuvants compared are either licensed preparations or have been used in human clinical trials.

Groups of mice were immunized with peptide and the following adjuvants:
i) adsorbed to aluminum hydroxide (Alhydrogel) and calcium phosphate gels, the only adjuvants licensed for clinical use in humans, and which have the longest history of proven use, ii) the saponin Quil A, iii) Ribi adjuvant (RAS) emulsion, containing monophosphoryl lipid A (MPLA), cell wall cytoskeleton and trehalose dimycolate, iv) Syntex adjuvant formulations (SAF). Syntex adjuvant contains muramyl dipeptide, the smallest unit of the mycobacterial cell wall which retains adjuvant activity, and a non-ionic block copolymer which is a simple linear polymer of hydrophobic polyoxypropylene (POP) and hydrophilic polyoxyethylene (POE), in a squalane-in-water emulsion (3). In addition, water-in-oil and oil-in-water Syntex emulsions were prepared with the addition of MPLA. For control immunizations, peptide was emulsified with Freund's complete and incomplete adjuvants, and with TitreMax, a squalane-in-oil emulsion containing a block copolymer which offers comparable adjuvanticity to Freund's emulsions but with greatly reduced toxicity. Also, peptide in saline alone and in a squalane-in-oil emulsion was used for immunization.

When administered in saline alone, the peptide induced a weak but significant humoral response against the B-cell epitope. This adjuvant effect is probably two-fold: a combination of increased epitope density on the peptide core with stimulation of Th-cells. In addition, immunization in a squalane-in-water emulsion alone also induced a similar response. However, when administered with adjuvants, the immune response to the peptide was markedly increased. The most adjuvant-active formulations were the oil-in-water Syntex emulsion containing MPLA and the water-in-oil Syntex emulsions and RAS. The mean ELISA titres with these adjuvants were significantly greater than those observed with aluminum hydroxide and Freund's adjuvant. The least adjuvant-active formulations were the TitreMax emulsion, Quil A and calcium phosphate. In addition, the anti-peptide IgG titers elicited by peptide with adjuvant(s) declined only slowly with time, thus fulfilling the criterion of inducing a long-lived response. The RAS and water-in-oil SAF-MPLA emulsions were also able to elicit early IgG antibody titres after 1-2 doses only. No differences were observed in the epitope specificity of antisera, as determined by reactivity with overlapping peptides synthesized on pins. In addition, all antisera also reacted with homologous outer membranes.

The antibody isotype profile was markedly influenced by the adjuvant used. When peptide was administered in saline, with squalane, the saponin Quil A, adsorbed to $Al(OH)_3$ or $CaHPO_4$, or emulsified with TitreMax, only antibodies of the IgG1 isotype were elicited. Freund's adjuvant, RAS and the Syntex formulations, however, were able to induce antibodies of the IgG2a and IgG2b subclasses, which may be important for protection (4). Indeed, significant differences were seen in the bactericidal activities of antisera, suggesting an important role for adjuvant in modulating the immune response towards production of functional antibodies.

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IgG antibodies specific to the linear B-cell epitope on the class 3 outer membrane protein can promote opsonophagocytic killing of *Neisseria meningitidis*

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The class 3 outer membrane protein (OMP) of *Neisseria meningitidis* has been reported to be one of the potential targets for bactericidal and opsonic antibodies in humans, which recognize mostly surface-exposed epitopes [1,2]. The serotype 15 class 3 OMP constitutes one of the components of Norwegian group B meningococcal outer membrane vesicle (OMV) vaccine, which was used recently to immunize about 90 000 adolescents in Norway [3,4]. The aim of this study was to identify linear epitopes occurring on the serotype 15 class 3 protein and to study the antigen-binding and effector properties of the specific antibodies.

Synthetic peptides spanning the entire sequence of the class 3 OMP from the vaccine strain 44/76 (B:15:P1.7,16) were synthesized on pins and screened with paired sera from vaccinees from the Norwegian vaccination trial and from patients with systemic meningococcal disease (SMD). Basing on the differences in reactivity patterns in pre- versus post-vaccination sera, an immunodominant epitope consisting of 14 residues (17-30) was identified on the putative loop 1 (VR1) region close to the N terminus of the molecule. To study specific immune responses in more detail, a soluble 23mer peptide D63b2 covering the VR1 region was synthesised. Then quantitative peptide D63b2-specific IgG responses were measured in sera from 27 volunteers immunized with the Norwegian group B OMV vaccine, and from 132 SMD patients. The 17-30 epitope was found to be efficiently recognized by post-vaccination sera taken after three doses of the Norwegian group B OMV vaccine in 74% vaccinees, while no clear linear epitopes were recognized by four different murine monoclonal antibodies [5]. In contrast, both the class 3 OMP and the 17-30 epitope were low immunogenic in the course of SMD, as judged from both immunoblotting studies (responded 24/132; 18.2%) and reactivity with peptide D63b2 (responded 18/132; 13.6%), respectively. Peptide D63b2 significantly inhibited IgG binding to the denatured PorB protein on immunoblots, suggesting that this B-cell epitope was one of the main linear epitopes on the PorB protein recognised by sera from vaccinees and some SMD patients.

To study antigen-binding and effector properties of the specific antibodies, we purified the class 3 OMP from the PorA-deficient isogenic mutant M14 [6]. The purified class 3 OMP and peptide D63b2 were immobilised on the highly cross-linked (6%) spherical agarose beads with N-hydroxysuccinimide (NHS)-activated 6 atoms-long spacer arms. Peptide- and protein-specific IgG preparations were affinity purified from a post-vaccination serum with high levels of peptide-specific IgG antibodies. To study antigen-antibody interaction in more detail, we measured the affinity constants characterizing binding of the specific IgG preparation to bacteria, to the OMV from strain 44/76, or to

peptide D63b2. Whatever the antigen studied, the peptide D63b2-specific antibodies possessed higher affinity compared to the class 3 OMP-specific antibodies, which supports the interpretation that the superior accessibility of the target epitope on peptide D63b2 selected for high-affinity antibodies during purification. The affinity constants characterizing IgG binding to bacteria were significantly higher than that for OMV complex or peptide D63b2, which may suggest higher target epitope density on the bacterial cell surface. Both the class 3 OMP- and peptide D63b2-specific IgG preparations were found not bactericidal, but enabled complement-dependent opsonophagocytosis of *Neisseria meningitidis*, measured as respiratory burst of human neutrophils and/or internalization of opsonized meningococci by PMNL. Blocking experiments clearly suggested involvement of both FcγRII (CD32) and FcγRIII (CD16) into the observed antibody-dependent receptor-mediated phagocytosis. Taken together, the data presented here indicates that the PorB-specific antibodies may contribute to vaccine-induced protection against systemic meningococcal disease via the opsonophagocytic route of pathogen clearance.

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Immune response of mice to *Neisseria meningitidis* serogroup B protein antigens

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Neisseria meningitidis is one of the most common meningeal pathogens which causes systemic disease in humans. Depending on the clinical manifestations, the disease shows mortality rates of 10 to 70% (1). The identification of meningococcal antigens which induce functional protective antibodies constitutes an important approach to vaccine development (2). The polysaccharide antigens in general show age dependent protection, an immune response of short duration and no induction of immunological memory. In addition, the polysaccharide of the B serogroup shows poor immunogenicity relative to the other serogroups. This situation has stimulated interest in protein antigens and conjugated vaccines (3). In our laboratory, the immune response of Swiss mice to 0.1, 1.0 and 2.5 μg doses of outer membrane vesicles (OMVs) obtained by Sodium desoxycholate (DOC) treatment of the cells of *N. meningitidis* serogroup B was studied (4). The response was evaluated in terms of total antibodies and their bactericidal activity against prevalent Brazilian strains. The two higher doses induced high titers of total and functional antibodies with no statistical difference between them. These antibody levels were sustained during a four month period. The animals receiving the lowest dose received a booster dose after one month and showed an immune response equivalent to the high dose animals. These results suggest that OMVs protein antigens represent potentially protective components of a vaccine against *N. meningitidis* serogroup B.

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Vaccine potential of meningococcal transferrin binding proteins: mouse protection studies.

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There is much interest in the vaccine potential of *Neisseria meningitidis* transferrin binding proteins (Tbp1 and Tbp2) which are possessed by all meningococcal strains and are involved in the uptake of iron from transferrin (1). They have been shown to elicit protective and bactericidal antibodies against the homologous *N. meningitidis* strain in laboratory animals (2,3). There is, however, heterogeneity of molecular size and lack of a cross-reactive immune response in laboratory animals, particularly for Tbp2, which would appear to limit the usefulness of Tbps as vaccine antigens. In contrast, human antibodies to meningococcal Tbps have been shown to be highly cross-reactive (4), strengthening the case for their inclusion in a vaccine against meningococcal disease. We have used a mouse IP model of meningococcal infection to determine the protective potential of transferrin binding proteins (Tbp1 and Tbp2) against challenge by homologous and heterologous strains.

Tbp1+2 were purified from detergent extracts of iron-limited bacteria using transferrin-Sepharose affinity chromatography (5) and separate Tbp1 and Tbp2 were obtained by ion exchange chromatography on MonoS (Pharmacia). Both of the isolated proteins retained the ability to bind transferrin.

Mice (CAMR-NIH) were immunized with Tbps on days 1, 21 and 28 and challenged on day 35. Mice received 10mg human transferrin IP with the challenge and a further 10mg at 24h. Bacteria for the challenge were grown under iron-limited growth conditions. Animals immunized with Tbp1+2 isolated from serogroup B meningococci were protected against challenge with other serogroup B organisms, with greater protection seen with the homologous strain and strains expressing a Tbp2 with a similar molecular weight. Little or no protection was observed against challenge with meningococci possessing Tbp2 with a very different molecular weight. Mice immunized with Tbp1+2 from a serogroup B strain were also protected against infection with a serogroup C but not a serogroup A strain.

IgG1 was the predominant immunoglobulin type induced in response to Tbp1+2 immunization and antibodies cross-reacted with purified Tbps with a similar Tbp2 molecular weight. Mouse serum also strongly inhibited transferrin binding to homologous Tbps, determined by ELISA.

The protective potential of separate, functionally active, Tbp1 and Tbp2 was also determined. Previously, protection with Tbp1 was not investigated as it did not elicit bactericidal antibodies (3) but this may be because the purification methods used

produced Tbp1 that no longer bound transferrin. It was found that Tbp2 provided protection against the homologous strain which was equivalent to that provided by Tbp1+2 or recombined Tbp1+Tbp2. Tbp1 provided a much reduced degree of protection compared with Tbp2. The cross-reactivity of sera raised against separate Tbp1 and Tbp2 (of both high and low molecular weight) will be presented. Preliminary results indicate that Tbp1 in the separate form is less immunogenic compared with Tbp1 in the Tbp1+2 complex. This may account for the apparent lack of protective potency of Tbp1 alone. These results strengthen the case for inclusion of Tbps in a meningococcal vaccine but confirm that, in mice, Tbp2 is the predominant protective antigen.

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Prospects for a nasal vaccine against group B meningococcal disease

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Outer membrane vesicles (OMV) from *N. meningitidis*, the main constituent of the Norwegian vaccine against group B meningococcal disease (1), have proved to be strongly immunogenic when administered intranasally in mice (2). Based on these findings, similar studies were initiated in humans. We have searched for evidence that non-proliferating mucosal vaccines might be effective without the use of toxins as so-called mucosal adjuvants.

Twelve volunteers were immunized with OMV in the form of nose drops or nasal spray four times at weekly intervals, followed by a fifth dose six months later. Each dose consisted of 250 μg protein (i.e. 10x the intramuscular dose) in 0.5 ml PBS, with half the amount given on either side. Blood for serum and peripheral lymphocytes, nasal fluid and whole saliva were collected for up to seven months.

Five of the vaccinees responded to the first series of four immunizations with at least a two-fold rise in serum IgG antibodies to OMV as measured by ELISA. Five individuals also developed more than four-fold increases in serum bactericidal titers to the vaccine strain, but these results were concordant with the IgG ELISA results only for two of them. On the other hand, eight of the vaccinees showed increases in serum bactericidal activity against a variant of the vaccine strain expressing the Opc protein, which correlated well with the increases in serum IgG antibodies. The Opc protein may thus be important for induction of immunity via the mucous membranes.

Following the first four immunizations, all 12 vaccinees responded with at least two-fold increases in IgA antibodies specific to OMV in nasal fluid, and eight of them also developed such antibodies in saliva. Thereafter the antibody levels in the secretions decreased gradually although significantly increased levels persisted for five months, until the fifth dose were given. No significant IgA response in secretions was detected in a control group of 11 vaccinees given the OMV vaccine, with aluminum hydroxide, twice intramuscularly. Thus, the nasal vaccine induced mucosal antibodies most consistently at the site of stimulation.

On immunoblots, serum antibody responses to the nasal vaccine were mainly directed against the class 1 and 5 meningococcal protein and LPS, which are also strong immunogens by intramuscular vaccinations (3). The IgA antibodies in nasal fluid and saliva from some vaccinees were mainly directed against the class 1 protein.

After the fifth intranasal immunization dose it appeared to be signs of a booster effect on the antibody levels in serum, as well as in secretions. However, the changes in antibodies following this last dose were not statistically significant

A transient proliferative response of peripheral lymphocytes from 10 of the 12 vaccinees could be detected against the class 1 protein two-to-four weeks after start of the nasal vaccinations. This indicates that an antigen specific T-lymphocyte response was also elicited.

Our results indicate that the administration to human volunteers of a meningococcal OMV vaccine directly onto the mucous surfaces, either as nose drops or nasal spray, can stimulate local mucosal as well as systemic immune responses. These effects which were not dependent on the addition of any other adjuvant, might eventually be shown to convey protection against invasive meningococcal disease.

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Analysis of the human Ig isotype response to individual Tbp1 and Tbp2 from *Neisseria meningitidis*

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There is as yet no vaccine available for prevention of serogroup B meningococcal disease. The poor immunogenicity and low affinity response to the group B capsule (1) has focused attention on meningococcal subcapsular antigens as vaccine candidates for group B disease (2). Meningococcal transferrin-binding proteins are currently being investigated for their vaccine potential (3). This study examined the human isotype antibody response to purified meningococcal Tbp1 and Tbp2 from two strains (SD and B16B6) expressing both high and low molecular weight Tbp2s (4).

The IgM, IgG and IgA responses to meningococcal Tbps in seventy well-defined sera from proven cases of meningococcal disease were examined by ELISA.

Tbp1 isolated from both strains was recognized more frequently and produced higher ELISA absorbance values than Tbp2s from either strain. This antibody response was independent of the serogroup or serotype of the infecting meningococcal strain. Tbp1 from strain B16B6 produced the most pronounced reactivity and was the most frequently recognized antigen. The reactivity of all four proteins was highly variable between individuals and differed significantly between all four antigens.

The reactivity of the majority of sera examined and the durability of the anti-Tbp1 response may indicate a role for these antigens in a vaccine. However, the variability of immune responses to each class of Tbp from the two strains suggested that a successful vaccine would need to include a combination of Tbps of varied specificities.

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Inducement and duration of cellular response to VA-MENGOC-BC® in babies and children.

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VA-MENGOC-BC® is the commercial name of a Cuban vaccine against *Neisseria meningitidis* of serogroups B and C. The vaccine consists of purified proteins from the outer membrane of group B meningococci enriched with proteins of "High Molecular Weight", which form a proteoliposome with controlled amount of phospholipid and lipopolysaccharide. The whole complex is mixed with C polysaccharide and then adsorbed to aluminum hydroxide gel (1,2).

The preclinical and clinical (phase I and II) studies showed the vaccine to be innocuous, safe and induce antibodies against different pathogenic serotypes (3). A double blind placebo-vaccine trial had been conducted in secondary students (11-16 years), with an efficacy of over 80 % (4). A second field trial with children from 5 months to 24 years of age had an efficacy of 83-90 %. This vaccine was included in the Cuban National Immunization Scheme at the age of 3.5 months and a mass vaccination campaign was organized in 1989-90 in babies under 6 months, with an efficacy of 92.5 % (2). This vaccine induces long-lasting specific IgG and bactericidal antibodies against some of the most frequent serotype B pathogens. After a 3rd dose these antibodies increased various times, showing an excellent anamnestic specific response, which allows a high efficacy (2).

VA-MENGOC-BC® has been applied to over 40 millions of persons in Cuba and other countries (Brazil, Colombia, Argentina, etc.). The Cuban strategy made it possible for the morbidity and mortality rate caused by *Neisseria meningitidis* B to drop since 1988, due to the vaccine application in provinces (5). Nevertheless, the cellular evaluation induced by VA-MENGOC-BC® has been less studied, so our group working in this field has shown the existence of Lymphoproliferative response *in vitro* in vaccinated mice and in humans (6). Also, and even more important, is the presence of delayed-type hypersensitivity (DTH) response that was determined in vaccinated humans (7) and this response was transferred by cells in mice (unpublished results).

Methods. Immunization. A population of nursing Infants (3.5 months old), children (from 2 to 6 years old) and teenagers (from 11 to 13 years old) were included to carry out the present work. The ages were selected regarding the time of vaccination (from 0 to 7 years). All were immunized or had been vaccinated with a two dose scheme of VA-MENGOC-BC®, spaced from 6 to 8 weeks apart, accordingly to producer indications. The teenagers were challenged with a third dose.

Prospective evaluation. To evaluate the induction of a cellular response, the Nursing Babies were studied in terms of the dermal test of DTH, before the vaccination program started, after the first dose (6 months of age) and 28 days after second dose.

Retrospective evaluation. To determine the duration of induced cellular response, children and teenager previously vaccinated between 2 and 7 years ago were evaluated. A dermal test was carried out in all, and in addition, in teenagers two blood extractions were carried out for the evaluation of Lymphoproliferative (LP) response and antibody forming cells (AFC), using ELISPOT techniques.

Dermal test of delayed-type hypersenitivity. This test was carried out by multi-puncture, using 14 μg of proteoliposome (protein component of VA-MENGOC-BC®) diluted in glycerol-PBS-phenol, taking previous aseptic and antiseptic measures. Determinations were carried out at 4, 12, 24 and 48 hours, with delimitation of hardness with a pen, transferring it to paper and measuring its diameters (mm).

ELISPOT The peripheral mononuclear cells (MNC) were obtained from the extraction of 10 ml of blood, before (t = 0) and after a third dose (t = 7, 14 and 21 days), by a Ficoll-Hypaque cushion. MNC were employed in the trial. Antigen (proteoliposome) was fixed at 10 μg/ml on a 96 nitrocellulose bottomed-well plate, incubated at 4 °C over-night and then cells were added at variable concentrations. Incubation took place during 4 hours at 37 °C in a 5 % CO_2 atmosphere. Finally, horseradish peroxidase labeled conjugated was added, being revealed with Diaminobenzidine. The reading was conducted in a stereo-microscopy and the results were expressed as AFC per million of MNC. The mean and standard deviations were calculated and the T test was applied to compare the results between experiments, considering that $p < 0.05$ indicates a significant difference.

Lymphoproliferation (LP). The MNC were cultured in complete medium RPMI-10 % FCS and were faced *in vitro* with proteoliposome at 2, 5 and 10 μg/ml for 5 days. The culture were pulsed with [^{3}H] Thymidine during the last 18 hours, harvested and counted their incorporation in a ß liquid scintillation counter. The mean count per minutes (CPM) of triplicates and Stimulation Index (SI) were determined. The SI > 2 were considered positive.

Ethical features. Because of the inclusion of children in this work, the authorization by National Group of Pediatrics and Health Municipality was necessary, once the characteristics of innocuity were known. Furthermore, the agreement by writing of each father or guardian was included.

Results. The cellular immune response is very important to be evaluated, since T cells are the principal orchestrator of any kind of immune response, intervening in and directing the specificity (by determination of which antigen and epitope will be in fact recognized); the effector mechanism selection; helping in the proliferation of effector cells selected; increasing phagocytic functions and activating other effector cells;

allowing Ig isotope switching; participating in the long-lasting memory inducement and intervening in suppression. T Cell Response, according to the cytokines profile produced, can be split into T_H1 (IL-2, IFNγ) and T_H2 (IL-4, IL-5 and IL-10) subsets.

DTH is an *in vivo* test that translates an induced T_H1 cellular immune response. In previous projects the presence of DTH in Balb/c mice was demonstrated and proteoliposome concentrations were standardized for their use in human adults. Because of this, the evaluation in children was indispensable, even more when in Cuba, this vaccine is included in the National Program of Immunization. The first dose beginning in nursing babies 3.5 months after birth, with a coverage of over 98 %.

The absence of DTH in nursing babies before vaccination, being positive after the first dose and its subsequent increase after this, emphasizes that VA-MENGOC-BC® induces a strong cellular response, not only in adult, but also in nursing infants. Finally, no response was observed 4 and 12 hours after DTH test application, and the maximum hardness was after 48 hours. These results explain the good and previously observed antibody response in these ages and that the dermal response is mainly DTH.

The T cell response duration, evaluated by DTH, was maintained for 2 to 7 years, without meaningful differences ($p < 0.5$) between them. These results evidence a long-lasting memory induced by the vaccine or their natural booster or by the cross-reactive stimulation of other microorganisms.

T Cell response was also evaluated *in vitro* for Lymphoproliferation, and the B cell response by AFC with ELISPOT. For teenager vaccinated 5 years ago, both responses were determined in kinetic. Twenty six percent were LP positive ($SI > 2$) before the third dose and then was a decreased in the subsequent determinations. This decrease could be associated with the recruitment of specific T lymphocytes toward the site of immunization. The specific AFC were absent, and there was a positive conversion in 78% ($p > 0.05$) of those evaluated at 7 days. The rest of the determinations were negative. These activated cells, after proliferation and differentiation may be directed to the effector sites of the immune response.

Taking into account the results in the teenagers vaccinated 5 years ago, the *in vitro* immune response in the seven year group was only determined before and 7 days after challenge. The LP was positive before the challenge in a 34 percent, and B cell response was positive in a 12 percent before and 98 percent 7 days after the challenge. It should be emphasized that the number of AFC in the positive population was very low at the beginning (0.73×10^6 MNC); but showed a greater increases after challenge (67.68 vs 32.24×10^6 MNC). Furthermore, the number of AFC in teenagers vaccinated 7 years ago (73.9×10^6 cells) was significantly higher ($p<0.001$) compared to those vaccinated 5 years ago (3.5×10^6 cells).

The greater LP and AFC response of children after 7 years (Holguín), than those after 5 years (Havana City), could be due to a different traffic of *N. meningitidis* or other cross-reactive microorganisms circulating in both groups (provinces). This result was

analogous to the epidemiological and microbiological studies developed previously to vaccination. We are currently working on the monitoring of this hypothesis.

The presence of a meaningful percentage of teenagers with positive Lymphoproliferation, all with positive dermal tests, and the absence or low percentage of responders at B cell level in those vaccinated 5 and 7 years ago, allow us to suggest that the long-lasting response (memory) induced by VA-MENGOC-BC® is mainly T cell-mediated.

Conclusions.

1. VA-MENGOC-BC® induces a T-cell immune response, not only in adults, but also in children and nursing babies.
2. The response induced by VA-MENGOC-BC® is long-lasting, at least 7 years, and is fundamentally T cell-mediated.
3. The ELISPOT was shown to be a useful technique for humoral response evaluation at the cellular level.

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Anamnestic B cell response by ELISPOT after a challenge in humans vaccinated with VA-MENGOC-BC® 5 years ago.

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Introduction. Meningococcal diseases is a health problem in many South American countries, not only because of its substantial incidence, but also because of its high lethality in children (1). Until recently, no vaccine had been available for the prevention of infection with *Neisseria meningitidis* B (2). The Finlay Institute developed and carefully tested a vaccine against *N. meningitidis* B and C serogroups, that mainly consists of outer membrane proteins from group B meningococci enriched with protein from the High Molecular Weight Complex, integrating a well defined and stable proteoliposome with a controlled amount of LPS (3). In 1989 and 1990, children under the age of 6 years were vaccinated in a national campaign, with a great decrease in the incidence of the disease.

Limited studies about duration of the immune response after vaccination have been carried out. The present study was conducted considering the existence of immunological memory and cellular migration that allow antigen recognition and rapid proliferation in the inflammatory sites due to the expression on the cell surface of accessories and adhesion molecules. All this was examined in order to assess the effects of the vaccination in the B cell compartment of the population immunized several years before, and the kinetics of occurrence of these cells in peripheral blood, using mononuclear cells (PBMC).

The objectives are to evaluate B cell immune response, at cellular level, in teenager immunized with VA-MENGOC-BC® 5 years before and after a third dose, and to determine the kinetics of occurrence of B cells recirculating in peripheral blood, after a third dose of VA-MENGOC-BC®.

Methods. Immunziation. A population of 80 teenagers, age ranging from 11 to 12 years, were included in this study. All were immunized with two doses of VA-MENGOC-BC® 5 years before, following manufacturers' instructions, and challenged with a third dose.

Elispot. Extraction of 10 ml of peripheral blood were carried out, before and after the third dose of VA-MENGOC-BC®. The kinetics for the study of the occurrence response of peripheral Antibody Forming Cells (AFC) were made with the evaluation of the response at 0, 7, 14, 21 and 28 days after challenge. The universe was separated in four groups (I-IV) of 20 individuals each, measuring all responses at time (t)=0 days and at t = 7 (Group I); t = 14 (Group II); at t = 21 (Group III) and t = 28 (Group IV). The PBMC

were purified by a Ficoll-Hypaque gradient and were used at 5 x 10^5 per well, in Nitrocellulose 96 bottomed-wells plates. Antigen (proteoliposome 10 µg/ml) was fixed, incubated at 4 °C over-night and the cells were added and incubated during 4 hours at 37°C, in 5% CO_2 atmosphere. The peroxidase labeled conjugate was added and exposed to diaminobenzidine. The reading of the wells was made in a stereo-microscope and the results were expressed as AFC per million of PBMC. The mean and standard deviations were calculated, and the T test was applied to compare the results between experiments, considering that $p < 0.05$ indicates a significant difference.

Results. The ELISPOT is a new method that uses the properties of B lymphocytes, carrying a surface immunoglobulin as antigen receptor for its recognition and specifically proliferates after stimulation, with the production of antibodies molecules that are measured by the formation of spots. In this study, we report the results after the evaluation of AFC response in teenagers' PBMC challenged with a third dose of VA-MENGOC-BC®.

The evaluation showed the absence of specific circulating B lymphocytes response in the whole group that was vaccinated 5 years ago (t = 0) and their conversion in 78 % of cases 7 days after the challenge in group I. This was the only group with a positive response after vaccination; group I (t = 14), group II (t = 21) and group III (t = 28) was negative when AFC in PBMC was measured. This lymphocytes behavior suggests that B cell memory is non-long lasting, at least 5 years after of primary vaccination (two doses of VA-MENGOC-BC®) Due to their complexity, proteoliposome induce the T cell compartment (4, 5) with a T cell-mediated long-lasting memory. Significant lymphoproliferation was observed 5 years after vaccination (6) that could explain the B cell response conversion after a third dose. T cells cooperate with B cells, inducing their activation and proliferation with a high antibody response, not only in animal but also in humans. The presence of some antigen-specific memory B cells in germinal center in lymph nodes (LN) is also possible, and may explain IgG-producer B cell as early as a week in peripheral blood. The cells proliferated specifically against the antigen taken and carried by APC toward the LN, with their differentiation in AFC after undergoing affinity maturation (7).

The expression of adhesion molecules or homing receptors on cell surface, allow cellular migration from LN to the inflammatory sites (8, 9). This cellular migration can be determined as optimal around 7 days after vaccine booster. After that, the determination of B cell in blood is almost impossible, maybe because cells leave the vascular stream toward the inflammatory sites.

Conclusions.

1. For the first time anti-proteoliposome AFCs of humans vaccinated with VA-MENGOC-BC® were evaluated.
2. The occurrence of recirculation of B cell response after a third dose of VA-MENGOC-BC®in teenager was determined, with an absence of circulating B lymphocytes before vaccination and with a great increase after vaccine booster.
3. ELISPOT was shown to be a useful method to measure B cell response in humans.

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IgG subclass response after systemic immunization with the Norwegian outer-membrane vesicle vaccine against group B meningococci.

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IgG is the main antibody class in serum and represents the secondary immune response with raise in antibody affinity and the generation of immunological memory. There is both overlapping and distinct protective effector functions attributed to each of the four human IgG subclasses (1). Thus, analysis of the IgG subclass response after vaccination will give valuable information concerning anticipated protective effect of the vaccine.

Two groups of ten volunteers were analyzed for IgG subclass response against OMV after vaccination. The first group was given a third vaccine dose after having received two doses 4-5 years before. The second group consisted of previously unimmunized volunteers and selected for low antibody level against group B meningococci. This group was given three doses of the vaccine by a schedule of 1 month between the first and the second dose and 9 months between the second and the third dose. We measured the antibody response in gravimetric units based on monoclonal chimeric, mouse V and human C, hapten antibodies of all four human IgG subclasses as calibrators (2).

There was a substantial individual variation among the vaccinees. The overall dominating subclass was IgG1.

Nine of ten in group one had measurable IgG1 level (range 1-14 mg/ml), while undetectable IgG3, 4-5 years after the second vaccine dose. All vaccinees in the first group showed at least two fold increase in the maximum IgG1 level achieved four weeks after the third dose (range 5-41 and median 15.5 mg/ml). Eight of ten in this group showed a measurable IgG3 response with maximum at two weeks after the third dose (range 2-25 and median 6.5 mg/ml). The IgG1 level prevailed for at least 14 weeks, while the IgG3 level quickly vanished and was almost not detectable at 14 weeks. Only two of ten in this group showed an IgG2 response (range 2-5-mg/ml), while none showed any IgG4 response.

For the second group of previously unvaccinated individuals, only two of them had detectable level of IgG1 (1 mg/ml). In this group, all responded at least two fold in IgG1 level after the first vaccine dose with a maximum after two weeks (range 1-24 and median 8.5 mg/ml). There was also a good booster effect on the IgG1 level of the second dose with maximum response after two weeks (range 4-50 and median 11.5 mg/ml). The highest median IgG1 level after the third dose for this group was fifteen weeks after the immunization (range 7-43 and median 14.5 mg/ml). In this group only three of ten gave an IgG3 response after the first dose, while eight of ten gave an IgG3 response after the second dose (median 3 mg/ml two weeks after the third dose), which was undetectable

39 weeks after the second dose. The maximum IgG3 response in this group was two weeks after the third dose (range 0-50 and median 7 mg/ml) with only one of ten as non responder. Only one vaccinee in group two gave a strong IgG2 response and one vaccinee gave a measurable IgG4 response.

There was similarities but also differences in subclass response after the third dose for both groups, indicating that the immunological memory with regard to IgG subclass response lasted for at least 5 years. The IgG1 response seemed to last longer for group two than for group one indicating an other memory response in this group.

The IgG response after the first dose in group two could represent the beginning of a secondary respond as a result of prior exposure or as a result of prolonged immunological stimulation due to the adjuvant.

The kinetics of the antibody response was quite different among the subclasses. The IgG3 responded fastest but also gave the most rapid fall, while IgG1 responded slightly slower, but lasted longer. IgG2 had a very slow respond, but remained for a long time. IgG4 was only formed for a few vaccinees.

We also measured by a capture ELISA technique the total level of the IgG subclasses showing that the total IgG subclass levels did not change during the test period. Thus, the vaccination did not lead to a detectable polyclonal B-cell activation. There was neither any striking correlation between the total IgG subclass level and the corresponding OMV-vaccine response within each separate subclass.

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Epitope specificity and functional activities of human and murine antibodies against class 4 outer membrane protein from *Neisseria meningitidis*.

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The class 4 protein of *Neisseria meningitidis* is a highly conserved outer membrane protein (OMP), closely related to protein III (PIII) of *Neisseria gonorrhoeae*. Human IgG antibodies against PIII and some murine monoclonal antibodies (mAbs) against PIII and class 4 OMP have been reported to block bactericidal activity (SBA) of other antibodies (1, 2). Furthermore, in volunteers with previous gonococcal infection, who were immunized with gonococcal protein I vaccine, containing < 10% PIII, had a fall in bactericidal activity after vaccination. This fall in bactericidal activity was associated with the development of anti-PIII antibodies.

In order to investigate if antibodies against class 4 OMP elicited after vaccination with the Norwegian group B outer membrane meningococcal vaccine (3) block the bactericidal activity of other antibodies, sera from 8 vaccinees were selected. Six had antibodies, on immunoblots, against class 4 OMP before and after vaccination and 2 showed an increase in such antibodies after vaccination. In our experiments, we also used three new mAbs against class 4 OMP generated at National Institute of Public Health (185H-8, 155B-4, 173G-1), one mAb generated at Fundação Oswaldo Cruz, Rio de Janeiro, Brazil (AE3) and six mAbs against PIII, generated by J. Heckels *et al.* (SM50-SM55) (4).

Specific antibodies to class 4 OMP were purified by absorption with OMP from a strain lacking class 4 OMP (44/76 rmp-), after isolation the immunoglobulin (Ig) fraction from serum using protein G-Sepharose chromatography. After ultracentrifugation, the supernatant was precipitated with 50 % ammonium sulfate and the pellet resuspended and dialyzed against PBS.

Purified Igs reacted well in immunoblots against the OMP, recognizing only the class 4 protein, while no band was observed when OMP from 44/76 rmp- strain was used. However, when ELISA experiments were performed with OMP and whole cells from 44/76 strain, only weak reactions were observed, indicating that purified antibodies reacted with the parts of the molecule that were not exposed.

In order to detect linear epitopes and identify specificity of the mAbs, peptides of 14 residues were synthesized on polyethylene pins, using a commercially available kit (Cambridge Research Biochemicals). According to the predicted amino acid sequence of class 4 OMP, peptides spanning the entire molecule were synthesized with adjacent

peptides overlapping by 7 residues. We found that the epitopes recognized by the mAbs raised against class 4 OMP from meningococci were different from those recognized by mAbs against PIII developed by Heckels *et al.* The amino acid sequences of peptides that these antibodies recognized were: SM50 and SM51: **^{43}NYGECWKNAYFDKA^{56}**, AE3 **^{92}DETISLSAKTLFGF^{105}**, 185H-8 and 155B-4: **^{197}GAKVSKAKKREALI^{210}** and for 173G-1: **^{190}EAEVAKLGAKVSKA^{203}**. MAbs SM52-SM55 and purified human anti-class 4 OMP Igs were also tested and showed binding to multiple peptides, indicating that these antibodies were reacting mainly with conformational epitopes.

Functional activities of the class 4 OMP specific antibodies, were assayed in bactericidal and opsonic tests. Neither of the human Igs were bactericidal or opsonic against strain 44/76. Only mAbs 155B-4 and 185H-8 were weakly opsonic, while no SBA were observed for any mAbs.

To investigate the blocking activity of purified Igs, SBA were performed in two ways. In the first one, the inoculum was incubated with dilutions of the purified anti-class 4 OMP Igs, after 15 min, the appropiate dilution of the 151F-9 P1.16 mAb (as a bactericidal antibody) and the human complement were added, followed by incubation for 30 min. In the second procedure, dilutions of the purified Igs were mixed with the P1.16 mAb before the inoculum and complement were added, followed by 30 min incubation at 37°C. After this time for both assays, agar was added and the plates were incubated overnight at 37°C, in 5% CO_2, and the colonies counted. In this experiment, one of eight Igs revealed a blocking effect in the highest concentration (1/5 dilution, 200 μg/ml total Ig concentration), while the other Igs did not block the bactericidal effect of P1.16 mAb. In the concentration used, neither of the mAbs inhibited the bactericidal activity of the effector P1.16 mAb.

The Norwegian group B vaccine contains about 10% class 4 OMP. Only a few vaccinees responded against the class 4 OMP after vaccination with this vaccine. In those who responded, no decrease in SBA titers were observed after vaccination and there was no negative correlation between anti-class 4 OMP IgG in immunoblots and bactericidal SBA titers (5). These studies give no evidence for induction of blocking antibodies against class 4 OMP after vaccination with the Norwegian group B outer membrane meningococcal vaccine.

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Recombinant Opc meningococcal protein, folded *in vitro*, elicits bactericidal antibodies after immunization.

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The meningococcal Opc protein, whose expression is restricted to a large subset of *N. meningitidis* strains(1), may mediate attachment to both endothelial and epithelial cells(2). Meningococci which lack capsular polysaccharide not only adhere to, but can also invade human cells when they express large amounts of Opc protein. Moreover, bacteria expressing large amounts of Opc protein are more commonly isolated from the nasopharynx than from the cerebrospinal fluid or bloodstream of patients.

The Opc protein is highly immunogenic in humans and stimulated bactericidal antibodies after vaccination with the Norwegian meningococcal group B outer membrane vesicle vaccine(3). Taking this fact into account, we wanted to evaluate this antigen as a vaccine candidate. For this purpose, the *opc* gene was cloned and expressed in *E. coli*. Then, the Opc protein was produced as a fusion protein with the N-terminal part of the high molecular weight meningococcal protein P64k as a stabilizer (4, 5).

The protein was found as inclusion bodies and after cell disruption and successive washing of the insoluble fraction of the cells, the proteins were solubilized in presence of the chaotropic agent Guanidium hydrochloride. The extract was applied on a Reverse Phase High Performance Liquid Chromatography (RP-HPLC) C4 column, for a further step of purification. The so obtained recombinant Opc protein was refolded *in vitro*, by adding several compounds to the solution where it was suspended. After several periods of time, the progress of the folding was tested by immunoblot with the human monoclonal antibody LuNm03 against the meningococcal Opc protein. LuNm03 recognizes a conformational epitope. The two unique conditions that favoured the folding were: (I) 1 M Urea, 400 µg/mL of PEG and 0.2 M L-Arginine and (II) 0.5 M GuHCl, 400 µg/ml of PEG and 0.4 M L-Arginine.

To evaluate the immunogenicity of the recombinant polypeptide, Balb/c mice were immunized subcutaneously with three doses of 0.5 mL injections, containing 20 µg of the refolded recombinant protein in Freund's adjuvant, at two weeks intervals. The immune serum was collected two weeks after the last injection. In this case other variants were used as controls: (III) Opc protein in 1 M Urea, (IV) Opc protein in 0.5 M GuHCl and (V) recombinant Opc protein after 4°C storag, in phosphate balanced salt solution (PBS), for 2 months at 100 µg/mL.

Sera collected during the immunization schedule were studied by western blotting, colony blotting and ELISA experiments. In colony blots, the natural protein was

recognized only by the antibodies elicited against the two refolded variants, and the protein stored in PBS. In contrast, antibodies elicited against all variants recognized the Opc protein from strain H44/76 in western blotting. Likewise, the recombinant protein was recognized in western blotting by all collected sera.

Antisera elicited against all variants recognized the recombinant Opc protein in ELISA, prior to folding. Antisera against the refolded proteins recognized the Opc protein in the OMP preparation, while preimmune sera and antisera elicited against conditions III and IV did not show any binding at all. A correlation between ELISA and colony blot experiments was observed. The natural protein was recognized by the sera of the variants where the Opc protein was refolded.

To study the functional activity of the antibodies elicited against the hybrid recombinant protein, sera were tested in a bactericidal assay, against strains having high, low and no level of expression of the Opc protein. Complement mediated bacterial killing was observed only when sera against refolded proteins were assayed. Strains with a higher amount of Opc protein were more complement-killed by the antibodies elicited, than strains having lower amounts of this antigen. A correlation was seen between the bactericidal titer of the antisera and the level of expression of this antigen on the surface of meningococci. This is in agreement with the results published by others (3), where bactericidal antibodies can only kill Opc^{++} bacteria and not Opc^{+} or Opc^{-} variants. Variable expression of Opc protein is due to phase variation at the transcriptional level (6).

These experiments described here, indicate that is feasible to fold properly the recombinant Opc protein produced in the *E. coli* system. It could be confirmed by the bactericidal activity of the murine antibodies elicited against it. Taking together these results with those reported by Rosenqvist *et al.*, attention should be paid to this meningococcal antigen, as a vaccine candidate.

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Induction of antigen specific human T cell responses after nasal immunization with the Norwegian group B meningococcal outer membrane vesicle vaccine.

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Systemic (intramuscular) vaccination with the Norwegian group B meningococcal outer membrane vesicle (OMV) vaccine has earlier been shown to induce protection against disease (1). We have studied the ability of this OMV vaccine to induce systemic human T cell responses when administered without adjuvant on the mucosal surface of the nose. A group of 12 vaccinees were given doses of 250 ug OMV once a week for 4 weeks (primary immunizations) and one single dose (250 μg) 6 months later. Peripheral blood mononuclear cells were assayed at several intervals for proliferative response (thymidine incorporation) against OMV, purified class 1 protein, class 3 protein as well as control antigens. In the majority of the vaccinees mucosal immunizations with OMV induced a significant increase in the specific T cell response to the meningococcal class 1 protein antigen, wheras only two vaccinees responded to whole OMV as antigen. Only one vaccinee showed a weak increase in the antigen specific T cell response to the class 3 protein antigen. The last dose given 6 months after the primary immunizations did not result in a proliferative response level higher than obtained within the first immunizations as far as all three antigens tested are concerned. In conclusion, we have shown that it is possible to induce vaccine antigen specific T cell responses after nasal administration of OMV in humans, but the condition required to obtain immunological memory remains to be eluciated.

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Cellular immune response after immunization with the Norwegian group B *Neisseria meningitidis*outer membrane vesicle vaccine.

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Vaccination with protein antigens will usually result in a both a cellular (T cell) and humoral (B cell) immune response. B-cells are dependent on help from T cells in order to produce antibodies efficiently (1). T cells are also necessary for the generation of immunological memory. For protection against extracellular bacterial infections like *Neisseria*, antibodies are of crucial importance while T cells will have a more indirect influence by regulating the antibody response. T cells can also indirectly induce killing of bacteria by activating phagocytes. Detailed mapping of the cellular immune response after immunization with bacterial vaccines is largely lacking, but such studies can lead to valuable information. We have therefore analyzed the cellular immune responses after vaccination with the Norwegian outer membrane vesicle (OMV) vaccine made from B:15:P1.7,16 meningococcal epidemic strain (2). The vaccine has previously been shown to induce protection against disease, after two doses given intramuscularly (3). In this study 10 volunteers, selected for low IgG antibody levels against group B meningococci, were given two doses of vaccine (25 μg outer membrane proteins) 6 weeks apart and a third dose after 9 months. Peripheral blood mononuclear cells (PBMC) were isolated before vaccination, 2 and 6 weeks after the 1. and 2. dose, and 1, 2 and 6 weeks after the 3. dose. The cellular immune response was analyzed by measuring T cell proliferation (thymidine incorporation) of freshly isolated PBMC from the vaccinees against OMV, purified class 1 and class 3 outer membrane proteins as well as the vaccine antigen BCG as control. Although individual variations occurred, we observed a significant primary and secondary T cell response. The response was usually highest against purified class 1 protein with stimulation indexes (SI) between 40 and 500 after the 1. dose (median SI = 155). The response against OMV gave SI values between 30 and 1100 after the 1.dose (median=80) and a further increase after the 2. dose with SI values up to 1300 (median=100). The response against the class 3 protein was highest after the 2. dose and varied from SI = 6 up to above 200 (median=38). The effect of the 3. dose is not yet evaluated. Furthermore, ELISPOT was used in parallel experiments to detect PBMC producing OMV-antibodies of IgM, IgA and IgG isotypes and IgG subclasses. This response was highest one week after vaccination. Analysis to correlate the corresponding serum antibody responses and cellular responses will be presented.

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Analysis of the continuous epitopes recognized by antibodies against a recombinant meningococcal high molecular weight antigen.

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Neisseria meningitidis is a pathogen responsible for a serious invasive disease throughout the world (1). The lack of an effective vaccine against all serogroups constitutes a problem in the control of meningococcal disease (2). Several outer membrane proteins (OMPs) have been examined as vaccine candidates (3). Besides the major OMP, other surface proteins are under investigation in several laboratories, in special highly conserved OMPs that could potentially confer protection against meningococcal disease in humans (4).

Our group has previously isolated , cloned and expressed in *E. coli* the gene coding for a high molecular weight protein (P64k, LpdA) which is common to many meningococcal isolates (5). To further characterize this meningococcal antigen at the molecular level, we have evaluated its immunogenicity in mice, rabbits and monkeys. The antigen was highly immunogenic in all three animal species (6), inducing antibodies that recognized the natural antigen in the membrane of intact meningococci. Besides, we have generated a group of monoclonal antibodies (Mabs) against the recombinant protein which recognize 4 non-overlapping epitopes , as shown using competition assays with biotinylated Mabs (7).

To characterize the epitopes on P64k recognized by these Mabs and by antisera obtained in the three species, multiple overlapping peptides were synthesized on pins and screened for binding by the antibodies (8). The complete P64k sequence was synthesized as 20 amino acid peptides overlapped by 10 a.a. stretches.

Also, the sequences involved in antibody binding were located in the previously determined 3-D structure of the protein (9). All 8 Mabs reacted with some of the overlapping peptides, indicating that they recognized linear epitopes.

A number of continuous epitopes were detected in mouse, rabbit and monkey sera, when immune and preimmune bleeds were compared. For mouse and monkey sera there appear to be four or five major antigenic regions, the response of the rabbit antisera being much more heterogeneous. In rabbits the epitopes seem to be less precisely defined. Despite variation in the exact location of continuous epitopes defined by different anti-P64k sera , we found an immunogenic core region within the molecule. Its sequence is AETGR. Consistently, these five residues are located in a β hairpin loop which is exposed to the solvent. This region is protruding, accessible to an sphere of 9 Å radius.

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Evaluation of the antigenic and molecular conservation of a new neisserial low molecular weight outer membrane protein

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An antigenically conserved protein that would confer protection against all *Neisseria meningitidis* isolates would be the antigen of choice for the development of a more efficient meningococcal vaccine. However, the conserved proteins already described, such as the class 4 or the Lip proteins do not elicit bactericidal or protective antibodies (1). Here, we present findings clearly indicating that a newly identified meningococcal protein, called NspA, is not only antigenically highly conserved among all *Neisseria meningitidis* strains, but is also present at the surface of certain *N. gonorrhoeae* and *N. lactamica* isolates. We also present results showing that the gene coding for that protein can be found among all *N. meningitidis, N. gonorrhoeae* and *N. lactamica* strains.

The antigenic conservation of the NspA protein was clearly established with monoclonal antibodies (MAbs). In a dot immunoassay done on intact bacterial cells, a series of six MAbs specific for this meningococcal protein recognized more than 94%, or even all, in the case of Me-7, of the 71 meningococcal strains chosen to represent the major serological groups (2). Five of these MAbs also reacted with 2 out of the 16 *N. gonorrhoeae* tested. The five MAbs reacted also with at least one out of the 5 *N. lactamica* strains. These results indicate that the protein is also present on closely related neisserial species. Further testing using Western immunoblotting experiments indicated that the protein is present on *N. gonorrhoeae* strains that were not reactive in the dot immunoassay. A radioimmunobinding assay which is used to evaluate the surface exposure of antigens confirmed that the protein is only accessible to the MAbs at the surface of intact bacterial cells on a limited number of *N. gonorrhoeae* strains. This protein was not detected by the MAbs on any other non pathogenic *Neisseria* species.

To corroborate these results, a DNA dot blot hybridization assay was used to evaluate the molecular conservation of the gene coding for the NspA protein among *Neisseria* isolates. First, the *nspA* gene was cloned from a meningococcal DNA library derived from strain 608B (B:2a:P1.2) and sequenced. The nucleotide sequence revealed an open reading frame of 525 nucleotides coding for a polypeptide of 174 amino acid residues, with a predicted molecular weight of 18,000 and a pI of 9.93. A comparison between the predicted amino acid sequence and the one obtained by N-terminal sequencing of the native meningococcal outer membrane protein showed the presence of an 19 amino acid residues leader peptide which is typical of outer membrane proteins. Similarity searches using the nucleotide and the deduced amino acid sequences of established databases confirmed that this protein has never been described previously.

The *nspA* gene was amplified by the polymerase chain reaction. After DNA dot blot hybridization using this probe, all the 71 *Neisseria meningitidis* strains tested were shown to have the *nspA* gene in their genome. This latter result is in perfect agreement with the observed reactivity of MAb Me-7 which indicated that the NspA protein is produced by all the meningococcal strains and is accessible at the surface of intact cells. The DNA probe also hybridized with the chromosomal DNA of each of the 16 *N. gonorrhoeae* and 5 *N. lactamica* strains tested, but did not hybridize to the chromosomal DNA of any other neisserial species. Further studies are presently under way to determine whether the lack of reactivity of the NspA-specific MAbs with the gonococcal strains is due to the lack of expression of the *nspA* gene in most gonococcal strains, or to the absence of epitopes recognized by the NspA-specific MAbs on most gonococcal NspA proteins.

The *nspA* gene was cloned from two other meningococcal strains, one strain from each of serogroup B and A and from one *N. gonorrhoeae* isolate in order to further evaluate the level of molecular conservation. Alignment of the four *nspA* genes and the predicted amino acid residues sequences revealed very high degrees of identity of 90% and 95% respectively. Moreover, the observed variations were not clustered in a particular region of the sequences. These results clearly indicate that the *nspA* gene is highly conserved not only among *N. meningitidis* isolates, but also among *N. gonorrhoeae* strains.

The facts that the NspA protein is highly conserved, expressed at the surface of intact bacterial cells and that it can elicit the production of bactericidal and protective antibodies (2) greatly emphasize its potential use in a broad-range vaccine.

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Bactericidal effect of human neutrophils on meningococci incubated in pre- and post-vaccination serum of complement deficient patients.

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Of 45 Russian patients with late complement component deficiency (LCCD) who experienced one-to-five meningococcal infections, thirty-three were immunized with meningococcal polysaccharide vaccine (A+C+W135+Y) and followed for one to five years. The partial protective efficacy of vaccination was demonstrated (1). As far as bacteriolytic activity should be absent in LCCD plasma and serum, the potential bactericidal effect of human neutrophils on meningococci of groups A, W135 and B was studied in LCCD serum samples collected from vaccinees.

When meningococci were incubated at 37°C in 50% LCCD serum alone, exponential growth of meningococci occurred despite the presence of meningococcal antibodies. In LCCD serum the concentration of meningococci increased 1.5-3 times per hour, while meningococci were rapidly killed in all 20 control samples of 50% serum with normal complement activity. After the addition of human neutrophils ($5x10^6$ cells/ml) to meningococci ($5x10^3$ cells/ml) in LCCD serum, the growth was partly inhibited or even reversed to bacterial elimination. These experimental conditions were selected specially to simulate the conditions of initial bacteremia in meningococcal disease, to exclude some methodical errors, and to discriminate better the situations of high and low bactericidal effects. In all experiments there were used neutrophils of the same healthy donor, having heterozygous combination of Fcgammma-RIIa-receptors (Fc-RIIa-R131/Fc-RIIa-H131).

The rate of bactericidal effect of neutrophils depended on type of serum and group of meningococci. Group A, W135 and B meningococci were killed efficiently by neutrophils in 37% prevaccination LCCD serum samples. 84% of serum samples, collected one month to one year after vaccination, promoted the bactericidal effect of neutrophils against group A and W135 meningococci ($p < 0.05$). Three years after vaccination only 58% of LCCD serum samples were "bactericidal" in these conditions. 91% of serum samples, collected during one year after revaccination, promoted the bactericidal effect of neutrophils against group A and W135 meningococci. In contrast, no increase of bactericidal effect against group B meningococci was found in post-vaccination serum samples. Interestingly, bactericidal effect against both groups A, W135, and B was observed in 68% of serum samples collected from LCCD patients 3 months after systemic meningococcal disease. The latter effect was not polysaccharide-specific and possibly was caused by cross-reacting antibodies. These samples were excluded from the analysis below.

The rate of bactericidal effect of neutrophils correlated with the concentration of serogroup-specific IgM and, to some extent, IgG. For example, 88% of 54 LCCD serum samples, having more than 1.5 mg/l anti-group A IgM and/or more than 5 mg/l anti-group A total Ig ("high" levels), supported the killing of group A meningococci. In contrast, only 31% of 35 samples, having specific antibodies under these limits ("low" levels), were "bactericidal" ($p < 0.05$). The same effect was observed also in experiments with group W135 meningococci. The cross-reacting antibody might function in synergy with polysaccharide-specific antibody. We measured the level of antibodies to inner core of lipopolysaccharide of gram-negative bacteria in the same LCCD serum samples. 92% of LCCD serum samples, having both high level of anti-polysaccharide antibody and high level of anti-core antibodies supported the killing of meningococci. Only 25% of samples, having low levels of both antibodies, were bactericidal. The samples, where one antibody was in high concentration and another in low one, exhibited the intermediate bactericidal effect. No significant bactericidal effect was demonstrated by neutrophils in heat-inactivated serum samples in spite of the concentration of specific antibodies, suggesting that the action of antibodies was complement-mediated. The data of Schlesinger et al. (2), who studied three C7-deficient vaccinees, agreed in general with our observations.

In conclusion, neutrophils could kill meningococci upon incubation in LCCD serum; this effect increased after vaccination and depended on specific antibody and complement content. Protection obtained from vaccination might be caused by an increased killing capacity of neutrophils.

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Neisseria meningitidis LOS micelle-based vaccine

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Endotoxin (LPS/LOS) is a glycolipid considered as one of the most significant antigens expressed by Gram negative bacteria and responsible for the toxic effects leading from endotoxicosis to septic shock. Toxicity is associated with the induction of endogenous cytokines like tumor necrosis factor (TNF) through the interaction of the conserved lipid A moiety of LPS with cell receptor proteins like CD14. We have recently elucidated the features needed by peptide structures to competitively bind and detoxify the conserved lipid A structure (1). These studies, using synthetic anti-endotoxin peptides (SAEP) to inhibit the toxicity of LPS systemically and in organ tissues, have revealed that a fundamental factor related to the full expression of the biological toxicity of LPS resides in its micellar architecture (2). Studies investigating the molecular architecture of LPS by Nuclear Magnetic Resonance (NMR) have come to similar conclusions (3). Lipid A is responsible for the micellar configuration of LPS and any approach which tends to chemically modify or eliminate the lipid A structure leads to the lack of the supramolecular architecture of LPS with the consequent reduction or elimination of toxicity. There is very limited but significant information about the importance that such supramolecular architecture may also have for optimal expression of antigenic and immunogenic activity (4,5). Since meningococcal group B LOS can be considered an important vaccine candidate, we have studied a vaccine formulation which is based on the use of purified LOS micelles detoxified by complex formation (1) with an appropriate amount of SAEP in order to reach the necessary level of safety, for investigation of the role of anti-LOS antibodies in conferring protection against bacteremia and endotoxemia. LOS purified from N. meningitidis A1, which is cross-reactive with Group B LOS but that does not contain the lacto-N-neotetraose structure similar to human glycolipids (5), has been therefore reacted with a synthetic cyclic peptide (SAEP-2). In selected experiments, after complex formation, the bound peptide has been "locked" into the lipid A binding site by covalent cross-linking with tailored bifunctional spacers in order to achieve its irreversible binding to the lipid A moiety. For either model of vaccine, the non-covalent or covalent complex, detoxification was ascertained in a variety of assays which included inhibition of LPS-induced LAL clotting, systemic and local TNF release by LPS challenge in mice, inhibition of LPS-induced local hemorragic dermonecrosis in rabbits. The vaccines and a control of purified LOS were then injected subcutaneously in SW mice at the dose of 5 ug/mouse in various schedules of treatment in order to follow the kinetic of the induced antibodies.

Sera were obtained from the animals on weekly basis and the anti-LOS antibodies induced were quantitated by ELISA and characterized for isotype (IgG and IgM) and sub-isotype (IgG1, IgG2a, IgG2b, IgG3). The antibody response induced in all groups of animals contained essentially IgG antibodies which peaked after three injections with end-point titers in the range of dilution 10^{-4}-10^{-5}. The sub-isotype present within the IgG population of the animal groups were mainly IgG2 (44 %, equally distributed between 2a and 2b) followed by IgG1 (36 %) and IgG3 (20 %). Interestingly, the sub-isotype IgG distribution induced by either the vaccines or native LOS in mice was similar to that reported in febrile patients affected by typhoid to LPS of Salmonella typhi (6). The murine anti-LOS antibodies induced were biologically functional in fixing and activating guinea pig complement thus resulting in the lytic activity on N. meningitidis A1 LPS-coated sheep erythrocytes at serum dilution of 1:200. Analysis of immunochemical specificity of the induced antibodies for different antigenic regions of A1 LOS, performed by inhibition ELISA, revealed that all antibodies were directed against the carbohydrate region and none of them recognized the lipid A moiety. Accordingly, no cross-reactivity of the anti-A1 LOS antibodies was detected against heterologous LPS purified from P. aeruginosa, S. typhosa, S. enteritidis, S. flexneri, H. influenzae and B. pertussis. In contrast, the induced antibodies were cross-reactive with purified N. meningitidis group A and B LOS as well as with three bacterial strains (Group A, strain A1; Group B, strain BB431; Group B, strain 44/76) sharing the immunotype L8 determinant (5).

These results show that peptide-detoxified LOS represents a novel new method for safely administering LOS/LPS in micellar configuration which induces an immunogenic response in mice comparable to that qualitatively and quantitatively induced by native (toxic) LOS, in contrast to lipid A-deprived LOS conjugated to carrier proteins that have shown a lower level of immunogenicity when compared to native LPS (5). Studies are in progress for investigating in appropriate animal models the efficacy of the anti-LOS antibodies for prevention of meningococcal bacteremia and endotoxemia.

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Heterogeneity of *tbp2* genes among *Neisseria meningitidis* B strains belonging to the ET-5 complex

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Since 1974, serogroup B meningococci of the ET-5 complex have caused epidemics in Europe, Cuba and South America; these epidemics elevated disease rates for many years (1,2) and led to sustained efforts for vaccine development. The aim of this study was to assess the level of variability of *tbp2* genes encoding Transferrin binding protein 2 (Tbp2) among 23 strains of the ET-5 complex representing different serotypes and subtypes and nine different geographic origins. The size of *tbp2* genes of these strains determined after PCR amplification was 2.1 kb, indicating that they all belonged to the M982 family according to the classification previously described (3). We cloned and sequenced the *tbp2* gene of a strain isolated in Chile in 1987 (strain 8680) and the homology was only 65.5% with the nucleotide sequence of the reference strain M982. The multialignment of nucleotide sequences of five M982-like strains (4, 5), this ET-5 strain (8680) and another strain of the ET-5 complex, BZ83, previously described (5) allowed us to design two oligonucleotides in the N-terminal part of the gene giving a *tbp2*, the length of which was characteristic for three different types (type M982, type BZ83, type 8680). These primers were used to type the *tbp2* gene of each strain and this classification was confirmed with an analysis of the fragment length restriction pattern using specific enzymes. We found that ET-5 complex strains are heterogeneous for the *tbp2* gene as four different types were found. While types M982 and BZ83 were the most represented (10 and 9 strains out of the 21 studied respectively), we only found one other 8680-like strain. Among 7 Chilean strains isolated from 1986 and 1987, the four different types were represented. Overall, these data indicate that the *tbp2* gene is variable within the ET-5 complex.

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Noncapsular Vaccines, Poster 61

Human B- and T-cell responses after three doses of a hexavalent PorA meningococcal outer membrane vesicle vaccine

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Adult volunteers were immunized three times with 100 μg of a hexavalent PorA outer membrane vesicle (OMV) vaccine. This vaccine consists of OMV preparations derived from two trivalent meningococcal vaccine strains, each one of them expressing three different PorAs [1-2]. Previously, a phase I safety study was conducted in adult volunteers, who received one immunization of 50 or 100 μg of the vaccine [3]. This study investigates human B- and T-cell responses as a consequence of three consecutive immunizations. The vaccine was given with intervals of two and six months. Blood samples were taken weekly to study B- and T-cell responses. Antibody responses against the six PorAs as present in the vaccine were analyzed by ELISA using the trivalent vaccine OMVs as antigen. Six isogenic PorA target strains, each carrying a different PorA in an identical meningococcal background, were constructed to investigate the bactericidal immune response against individual PorAs [3]. Subsequently, PorA loop-deletion and point-mutation variant strains carrying PorA subtypes P1.7,16 or P1.5c,10 were constructed to investigate the epitope specificity and cross-reactivity of the induced antibodies by using such strains as targets in the bactericidal assay [4 and this study]. Lymphocyte proliferation assays were carried out to study the T-cell responses in both vaccinated and non-vaccinated donors. Monovalent OMV preparations of six wild type meningococcal strains, each one of them carrying one of the PorAs as present in the vaccine, and purified PorA P1.7,16 were used to study the antigen-specific stimulation of human peripheral blood cells. The first immunization induced a strong B-cell response resulting in high IgG levels in OMV ELISA and at least a four-fold increase in bactericidal activity as compared to pre-vaccination titers. Clonal specificity was observed in the bactericidal assay for one of the three PorAs as expressed by the two trivalent vaccine strains. The second and third immunizations did not induce booster responses. This was confirmed by the T-cell proliferation responses. A decline over time with respect to PorA specific antibodies was observed after each immunization. This was also reflected in the T-cell responses.

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Kinetics of bactericidal antibodies and specific IgG and IgA antibodies elicited by a meningococcal B:4:P1.15 outer membrane protein vaccine.

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Meningococcal B:4:P1.15 outer membrane proteins (OMP) vaccine produced in Cuba (VA-MENGOC-BC) was administered in two doses to 220 male recruits at an interval of 8 weeks. Thirteen serum samples were obtained in different times along one year. Specific IgG and IgA antibodies and serum bactericidal activity (SBA) elicited by the vaccine were measured by enzyme-linked immunosorbent assay (ELISA) and serum bactericidal assay, respectively. Arithmetic means and SD were calculated for all variables. Also a multivariate profile analysis was done. Nasopharyngeal carriage of meningococci was tested by a t-test for independent groups to compare meningococcal carriers with non-carriers with respect to specific IgA and IgG antibodies. After the second dose the higher increments in SBA and specific IgG were obtained. A significant decrease in these variables was observed from week 12 to week 52, although the negative slope showed by them after the second dose was smaller than that observed after the first one. In contrast to the kinetics showed by IgG, the increase of IgA was greater after the first dose. The kinetics of IgA and IgG stimulated by the vaccine reflect the presence and persistence of immunological memory at B cell level which could be protective since a similar behavior of SBA and specific IgG was observed. No significant effect of the carrier state on IgA and IgG was found.

Cloning and expression of human immunoglobulin gene encoding anti-meningococcal P1.7 epitope antibody SS269

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Introduction. Human monoclonal antibody SS269 (γ3,λ) reacts with linear peptides containing NGGAS which is located in VR1 region, corresponding to serosubtype P1.7, of meningococcal PorA protein (1). This antibody has been shown to react with P1.7,13a and P1.7,16 PorA proteins but not with P1.7,10 protein, suggesting that SS269 recognizes a conformational epitope which is affected by the sequence of the VR2 region of PorA protein (1). As to function, SS269 stimulates inefficient bactericidal killing possibly due to its low avidity but mediates efficient opsonophagocytosis (1). In contrast, a murine monoclonal antibody targeting meningococcal P1.7 epitope reacts with all P1.7 epitope-containing PorA proteins, shows high avidity and mediates efficient bactericidal killing (1). It will be important to determine the structural basis for such differences and to determine whether these differences would have any implications in the design of meningococcal vaccine, because the PorA protein has been recommended as one of the components of a new meningococcal vaccine based on experiments with murine mAbs (2). Phage-display of immunoglobulin genes is a powerful technique which was originally designed for performing quick screening of phage-displayed antibody libraries (3). We now report on the use of Phage-display technology to clone and express human immunoglobulin genes of hybridoma SS269.

Materials and Methods. Total RNA was prepared from 10^6 hybridoma cells. 5 μg of total RNA was used for a first strand cDNA synthesis reaction. Each Hot-Start PCR was performed in a 50 μl volume containing 2.5 units of Extend™ Long Template DNA polymerase. PCR products were obtained by using primers gaggtgcagctcgaggagtctggg and tgtgtcactagttggggttttgagctc for heavy chain and acaggbtcybkskccgagctcrwrbtgacdca and gcattctagactattatgaacattctgtaggggc for light chain after denaturing at 94°C for 3 min., 40 cycles of 92°C for 40 sec., 55°C for 40 sec. and 72°C for 1.5 min., and a long extension time of 5 min.

The heavy chain and light chain PCR products were sequentially ligated into a phagemid vector pComb3 after double digestions by *Xho* I and *Spe* I and *Xba* I and *Sac* I, respectively. Both cloned inserts were then sequenced. Soluble Fab was prepared according to a standard protocol (4) and tested in ELISA as described before (5).

Results and discussion. DNA sequence analysis indicated that heavy and light chain variable regions of SS269 belong to immunoglobulin gene family VH3a and family Vλ

1-b, respectively. Amino acid sequences of Fd and lambda chains of SS269 are shown below .

Fd fragment (1-98: VH, 99-116: J and 117-221: CH1):
VKLLEESGGGVVQPGRSLTLSCAASGFTFSSYGMYWVRQAPGKGLEWVAVIWY
DGTKKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVRDQYYGSG
WGQGTLVTVSSASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRV
ELKTPTS

Lambda chain (1-98: Vλ, 99-111: J and 112-216: Cλ):
ELVVTQPPSASGTPGQRVTISCSGSISNIGINSVYWYQQLPGTAPKLLFYRNNQRP
SGVPDRFSVSKSGTSASLAISGLRSEDEADYYCAGWDDSLSGWVFGGGTKLTVL
GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVET
TTPSKQSNNKYAASSYLSLTPEQWKSHKSYSCQVTHEGSTVEKTVAPTECS

Whole cell ELISA showed that both the soluble Fab_{SS269} and its parental mAb SS269 reacted with P1.7,16 meningococcal strain 44/76, but not with P1.7,10 strain 29019 and P1.2 strain 7379. This result indicates that Fab_{SS269} has the same binding specificity as its parental antibody.

The same procedure is being undertaken for three murine mAbs which are all directed against the P1.7 epitope. A comparison of DNA sequences and of variable region 3-D structures between the human and murine Fabs will help us in understanding how the differences in DNA sequences result in differences in their binding specificity and function.

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IgG antibody activity against meningococcal class 1 and 3 outer membrane proteins in patient sera: Comparison between immunoblot and ELISA analyses

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Previously, quantitative IgG antibody activities against purified class 1 and 3 outer membrane proteins from strain 44/76 (B:15:P1.7,16) in sera from patients, falling ill with meningococcal disease, have been determined in ELISA (1, 2). We wanted to compare these results with those obtained after immunoblotting of the same sera and density scanning of the blots. Outer membrane vesicles from 44/76 served as antigens, and all sera were incubated with and without Empigen BB to improve renaturation of boiled antigens (3). The sera were routinely used at 1:200 dilution, but those giving strong immunoreactive class 1 (P1.7,16) or class 3 (serotype 15) protein bands at this dilution, were diluted up to 1: 40 000. To control for variations between the blotting experiments, four strips with a reference serum were included on each blot. IgG binding was determined with peroxidase-conjugated rabbit anti-human IgG antibodies. Densities of immuno-reactive class 1 and 3 protein bands were measured with a video camera and a software program from Kem-En-Tec A/S, Denmark. In all, 56 acute and convalescent sera from 25 patients were studied; seven of the patients had been vaccinated with the Norwegian group B meningococcal OMV vaccine (4).

When 1:200 dilutions of the sera were used for immunoblotting, the Spearman rank correlation coefficient between IgG binding in ELISA (μg/ml IgG) (1, 2) and densities of the scanned class 1 protein band on blots was 0.38 ($p < 0.004$). The correlation coefficients for the vaccinees and non-vaccinees were 0.10 ($p = 0.72$) and 0.53 ($p < 0.001$), respectively. The low correlation coefficient for sera from vaccinees could possibly be related to a prozone effect , as the antibody binding was weaker at a 1:200 dilution compared to higher dilutions. Six patients had class 1 protein bands with scan values > 1 000, corresponding to medium or strongly stained bands. They were all infected with strains expressing subtype proteins different from that of the vaccine strain. Three were vaccinees, and their convalescent sera bound to protein P1.7,16 in the absence of Empigen BB, whereas the non-vaccinees' sera showed a strictly detergent-dependent binding. These results indicate that antibodies against more linear epitopes were preferentially raised after disease of the vaccinees.

When distinct class 3 protein bands were obtained on blots, they were generally much stronger than the corresponding class 1 protein bands. Eight patients had scanned class 3 protein bands > 1 000; seven of these were infected with serotype 15 strains and six were vaccinees. Anti-class 3 porin antibodies in the convalescent sera from these patient either

showed a detergent-independent binding or a partial detergent-dependent binding, indicating that the antibodies were mainly directed against linear epitopes (5) or against conformational ones renatured during the incubation steps with a smaller contribution of antibodies against epitopes refolded by the detergent. The correlation coefficient for IgG binding with all sera to class 3 porin with the two methods was 0.49 ($p < 0.001$). For vaccinees and non-vaccinees, the coefficients were 0.63 ($p = 0.007$) and 0.16 ($p = 0.32$), respectively. Distinct prozones were observed for sera with high anti-class 3 protein levels. When such sera were tested in 1:2 000 dilutions, the correlation coefficient was 0.89 ($p = 0.002$).

In conclusion, immunoblot analyses of IgG binding to the class 1 and 3 proteins of strain 44/76 with 1:200 dilutions of patient sera corresponded weakly to IgG levels measured by ELISA using purified class 1 and 3 proteins. The low correlation coefficients may be due to prozone effects observed at this serum dilution, as a high correlation was obtained at 1:2 000 dilution. Even though the use of Empigen BB allowed a differentiation of antibody activity directed mainly against conformational or linear epitopes, a possible loss of IgG antibodies against conformational epitopes using the blotting technique has to be taken into account. From a practical point of view, more work is involved in making serum dilution curves with the blotting method as compared to ELISA. These factors limits the use of scanned immunblots as a method to determine accurate antibody levels against class 1 and 3 proteins.

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Immunoblot analyses of antibody responses in sera, saliva and nasal fluids from volunteers immunized nasally with the Norwegian group B meningococcal vaccine.

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The immune responses in sera, saliva and nasal fluids from 12 volunteers, who received the Norwegian group B outer membrane vesicle (OMV) intranasally, were studied by the immunoblotting method. The immunizations were given as nose drops or nasal spray four times at weekly intervals followed by a fifth dose six months later. Samples for blotting analyses were taken before vaccination, two weeks after the fourth dose and two weeks after the fifth dose. OMV from the vaccine strain 44/76 served as antigen, and all samples were incubated with and without Empigen BB to renature boiled antigens (1). In sera, the IgG response was analyzed, whereas in saliva and nasal fluids the IgA responses were determined. All sera were also studied for crossreactive IgG antibodies against OMV from the Cuban vaccine strain 385/83.

Prevaccination sera from four volunteers showed distinct antibody activity against high molecular weight proteins (HMWP), class 1 porin and/or LPS of 44/76 OMV. In contrast, nine volunteers had similar bands against 385/83 OMV, in addition to class 5 proteins and lower molecular weight protein bands. Thus, preexisting antibodies crossreacting with antigens in the Cuban vaccine strain were more prevalent than those against the Norwegian vaccine strain.

Following nasal immunization, seven volunteers showed a distinct increase in antibody binding to one or more OMV antigens. Three vaccinees responded to class 1 porin, four to class 5 proteins (including Opc) and four to LPS of 44/76. Only one vaccinee had a weak response against the class 3 protein. None showed increases in IgG binding to HMWP as usually observed after intramuscular vaccination (2). The fifth immunization dose gave no distinct changes in antibody patterns. The vaccine-induced class 1 protein antibodies showed negligible crossreaction with 385/83, indicating a subtype-specific antibody response.

Serum bactericidal assays (SBA) were performed with strains 44/76-SL and 44/76-1, expressing low and high levels, respectively, of the Opc protein. Among five vaccinees, who developed more than 4-fold increases in serum bactericidal titers against 44/76-SL after immunization, three showed distinct class 1 porin bands, one a distinct LPS band, and the remaining a weak class 5 protein band. Postvaccination sera demonstrating more than 8-fold increases in bactericidal titers when SBA was performed with 44/76-1 compared to 44/76-SL, gave distinct bands in the class 5 protein region. Thus, for most post-vaccination sera there was a correlation between distinct class 1 or class 5 protein bands (including Opc) and titers in SBA.

After vaccination, a rise in IgA binding to class 1 and 5 proteins with nasal fluids from six volunteers was found. Compared to IgG binding patterns with sera, nasal fluids showed no distinct IgA antibodies against HMWP or LPS, and only three vaccinees demonstrated reactions matching those seen with serum IgG antibodies. IgA binding to class 4 protein was observed in two volunteers both before and after vaccination; corresponding IgG activity was not found in their sera. In saliva, only three volunteers demonstrated induction of IgA antibodies against the class 1 protein, and similarly to nasal fluids, no HMWP or LPS responses were detected.

Generally, sera giving distinct immunoreactive bands on blots had high IgG levels. A similar correspondence was also observed IgA in nasal fluids, but not for saliva. A correlation between distinct LPS bands on blots and high levels as measured in ELISA was also noted.

In conclusion, nasal immunization induced serum IgG antibodies against class 1 and 5 proteins and LPS in 58% of the vaccinees. Compared to intramuscular vaccinations, little or no increase in antibodies against class 3 porin and HMWP were found. Fifty percent of the vaccinees showed a vaccine-induced IgA response against the class 1 and 5 proteins in nasal fluids, while 25% demonstrated class 1 protein IgA antibodies in saliva. The IgA binding patterns in secretion generally did not reflect those seen with serum IgG antibodies.

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Liposomes as a vaccine delivery system for neisserial transferrin binding proteins

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There is currently much interest in the use of transferrin binding proteins (Tbps) as antigens for incorporation into a meningococcal vaccine. It has previously been shown that antibodies elicited to Tbps are bactericidal (1) and anti-Tbp antibodies that prevent binding of transferrin (TF) to Tbp can inhibit growth of *N. meningitidis* when TF is the sole source of iron (2). Two proteins have been identified that form the TF binding complex; Tbp1 (M.W. 95 kDa) and Tbp2 (M.W. 68-85 kDa). They are associated with the outer membrane, and Tbp2 has been found to be a lipoprotein (3).

The use of liposomes as a delivery system for Tbps desirable because a) Tbp will be presented to the immune system in a potentially native state mimicking *in vivo* orientation and b) liposomes can better target protein antigens to antigen presenting cells (APC). This was shown by Gregoriadis (4) who demonstrated that liposomes injected subcutaneously are retained at the site of injection and are taken up by APCs.

In this study, liposomes consisting of synthetic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in a 4:1 ratio were used. Lipids were rotary evaporated, shell dried and reconstituted in Tbp solubilized in 10% octylglucoside. Tbp was purified from detergent extract of *N. meningitidis* by affinity chromatography on TF-Sepharose 4B (2). Liposomes were formed by removing the detergent by dialysis against PBS. A homogeneous population of small unilamellar vesicles was obtained by extruding the Tbp/liposomes through 0.2 μm filter (5).

We found that intact Tbp/liposomes bound TF in a liquid-phase, detergent free binding assay, indicating that the Tbp complex was in a transmembrane location and in a functional conformation. The immunogenicity of the Tbp/liposomes was compared to Tbp alone, Tbp/Freunds and Tbp/alum in mice (n = 5). All preparations, containing 50 μg Tbp, were administered subcutaneously three times at two week intervals. Using purified Tbp as the coating antigen in an ELISA assay, we found that Tbp alone elicited equivalent amounts of IgG antibody to Tbp/Freunds and elicited more IgG antibody than Tbp/alum and Tbp/liposomes preparations. However, the Tbp/liposome antisera contained a higher percentage of whole cell-specific Tbp antibodies (16.4%) as compared to Tbp alone (5.2%), Tbp/Freunds (8.0%) or Tbp/alum (10.1%) antisera determined by whole cell ELISA. The only vaccine that elicited cross-reacting antibodies to *N. meningitidis* of different serosubtype was the Tbp/alum preparation.

In summary, liposomes proved to be an effective way to present TBP in a more

native state since intact TBP liposomes bound TF and compared to the other TBP vaccine preparations produced the highest percentage of antibody to native TBP on whole cells. In addition, TBP alone was immunogenic without additional adjuvant. Further studies regarding functional activity of antibodies elicited from the various TBP preparations are required.

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Antibody response of rabbits to intranasally administered meningococcal native outer membrane vesicles

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Delivery of vaccines via the intranasal route has gained much interest in recent years as a way to induce a mucosal antibody (IgA) response that may play a role in inhibiting the attachment or invasion of pathogenic microorganisms to mucosal surfaces. Many investigators have used the mouse as a model to examine effectiveness of intranasally delivered vaccines (1, 2). Investigators typically administer 20-50 µl of vaccine intranasally in mice. Much of the vaccine reaches the large mucosal surface area of the lungs and typically induces a good mucosal response. In humans, however, it may not be desirable to have lung involvement, especially with vaccines containing native endotoxin. We have used a larger animal model, the rabbit, as being more representative of intranasal immunization in humans with regards to vaccine disposition. We have found using dye experiments that intranasal delivery of 0.5 ml of vaccine in the rabbit provides good coverage of the nasopharyngeal region of the rabbit without getting to the lungs.

Immunizing with meningococcal native outer membrane vesicles (NOMV) would be an excellent way to present meningococcal antigens in their native conformation; however, NOMV does contain native endotoxin that would cause unwanted local and systemic reactions if given intramuscularly (i.m.) in humans. Endotoxin is highly pyrogenic, and we have found that in rabbits as little as 0.1 µg of NOMV caused a pyrogenic response when delivered intravenously. When administered intranasally, however, 400 µg of NOMV in rabbits caused no pyrogenic or other untoward responses.

In this study we investigated the immunogenicity of NOMV administered intranasally in rabbits. Rabbits were immunized 3 times at 28-day intervals. Vaccines that were used included: 1) NOMV from the parent strain 9162 (B:15:P1.3:P5.10,?:L3,7,9); 2) a capsule-deficient mutant of the parent 9162 strain; or 3) a vaccine containing detoxified meningococcal L12 LOS complexed with meningococcal (9162) outer-membrane protein (OMP-dLOS). We used two immunization schemes: 1) i.n. three times, or 2) i.m. once with OMP-dLOS followed by two i.n. boosters of parent or capsule-deficient NOMV. Vaccines were administered to unanesthetized New Zealand White rabbits i.m. at 25 µg protein or i.n. at 100 µg protein in a 0.5 ml volume.

Rabbits immunized i.n. three times with capsule-deficient NOMV had the highest serum bactericidal titers (mean $\log_2$ reciprocal endpoint titer having > 50% kill was > 9), highest serum IgG levels (500 µg/ml), and highest serum IgA levels (> 350 µg/ml). The NOMV from the encapsulated strain induced lower serum bactericidal activity (mean $\log_2$ reciprocal endpoint titer = 7.0) and lower serum IgG (400 µg/ml) and serum IgA

(190 μg/ml) levels. Rabbits immunized i.n. three times with OMP-dLOS or i.m. once with OMP-dLOS and twice with two i.n. NOMV boosters had similar lower levels of serum bactericidal activity (mean $\log_2$ reciprocal endpoint titers of 5.7-6.2), and lesser amounts of serum IgG (110-230 μg/ml) and IgA (30-220 μg/ml). Immunoblots showed that day 70 sera reacted with a wide variety of immunoreactive bands, including class 3 and 4 proteins, both class 5 proteins, as well as L3,7 LOS.

We have shown that intranasally administered NOMV are safe and induce good antibody responses against a variety of immunoreactive proteins in rabbits. Three i.n. doses of NOMV from a capsule-deficient mutant induced the best bactericidal and overall antibody response. It is not presently known if intranasal immunization in humans will induce high serum bactericidal activity as we observed in rabbits. We believe that the most effective strategy to immunize humans against group B meningococcal disease may involve a two-pronged approach using an intranasal vaccine to induce a local mucosal response and a parenteral vaccine to induce high titers of serum bactericidal antibodies.

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Immune response of mice to intranasally administered meningococcal native outer membrane vesicles

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The human nasopharyngeal region provides the sole natural habitat for *Neisseria meningitidis*. We believe that stimulating production of a local mucosal (IgA) antibody response may aid in preventing systemic meningococcal disease by interfering with meningococcal adhesion to and invasion of the mucosal surface. Secretory IgA has been shown to inhibit adherence of *Streptococcus* strains to human epithelial cells (1). Other investigators (2, 3) immunized humans intranasally with a purified M protein vaccine against group A *Streptococcus* and found reduced colonization and clinical illness after challenge with homologous streptococci. Furthermore, fewer vaccine side effects were observed in intranasally immunized subjects compared to parenterally immunized subjects (3). An added advantage of mucosal immunization is that vaccines containing lipopolysaccharide may be able to be delivered safely via the mucosal route without the adverse side effects seen with parenteral delivery (4).

We have conducted studies using an intranasal mouse model to examine the immunogenicity of meningococcal antigens delivered via the intranasal route. Meningococcal native outer-membrane vesicles (NOMV) were prepared from a mutant group B strain 9162 (--:15:P1.3:P5.10,?:L3,7,9) deficient in capsule and sialylated LOS. The NOMV were delivered intranasally (i.n.) to mice at a 20 μg dose of protein in a 25 μl volume using a micropipettor. For comparison, some groups of mice received one or both doses of NOMV intraperitoneally (i.p.) at a dose of 1 mg protein. Mice were immunized at days 0 and 28. Numbers of anti-meningococcal antibody-secreting cells (ASCs) were measured in lungs and spleens at day 33 using an ELISPOT assay. Antigens delivered intranasally in mice do reach the lungs; we therefore examined the histology of formalin-fixed H&E-stained mouse lungs collected at days 1, 2, 4, and 7 post i.n.-immunization.

Mice immunized and boosted i.p./i.n. or i.n./i.n. had 90 and 525 IgA ASCs per 10^6 lung lymphocytes, respectively, in lung tissue at day 33, whereas mice immunized i.p./i.p. had no detectable IgA ASCs in lung tissue. Mice immunized i.n./i.n. had 190 IgA ASCs per million spleen lymphocytes, compared to 30 and 43 IgA ASCs per 10^6 spleen lymphocytes, respectively, for mice immunized i.p./i.p. or i.p./i.n. Mice immunized i.p./i.p. or i.n./i.n. had similar high numbers of IgG ASCs in the spleen (105 and 91 per 10^6 spleen lymphocytes, respectively). In the lung, however, only 70 IgG ASCs per 10^6 lung lymphocytes was observed in the i.p./i.p group, compared to over 300 IgG ASCs per 10^6 lung lymphocytes in the i.n./i.n. group. Few IgM ASCs were observed in spleen and lung lymphocytes of any group. Serum bactericidal activity was highest in mice immunized i.p./i.n. (mean $\log_2$ reciprocal endpoint titer having > 50% kill = 8.8),

followed by the i.p./i.p. group (log_2 mean = 7.8) and the i.n./i.n. group (log_2 mean = 7.0). Mice immunized intranasally had serum IgG antibodies directed against several immunoreactive proteins including class 1 and class 5 proteins as observed on immunoblotting. Serum IgA antibodies which bound to class 5 proteins were also evident.

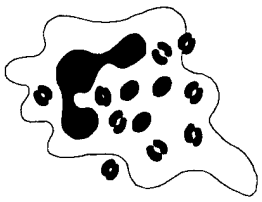
Histological evaluation of mouse lungs on days 1 and 2 post-i.n. immunization revealed a predominantly granulocytic inflammatory response, which by day 7 had significantly diminished in severity and in general consisted of small aggregates of perivascular and peribronchiolar mononuclear cells. The inflammatory reaction we observed in the lungs was not unexpected since NOMV contains native endotoxin. Mice that received an intranasal dose of 20 μg of NOMV had an average weight loss of 1.8 g by day 3, probably due in part to the poor condition of the lungs; mice recovered to pre-immunization weights by day 7.

Our results show that intranasal administration of meningococcal NOMV can induce a mucosal IgA response in mice. Importantly, intranasal immunization also induced production of serum bactericidal antibodies in mice. However, NOMV does cause unwanted inflammatory responses in the lungs when administered intranasally to mice. We have therefore initiated further intranasal experiments in a larger animal model, the rabbit, in which intranasally administered antigens do not reach the lungs but do provide good coverage of the nasopharyngeal region. We believe that the rabbit model will provide a better physical model than the mouse with respect to disposition of intranasally delivered vaccines.

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(Two additional abstracts by AK Lehmann, et al., and J Tappero, et al. on the topic of *Noncapsular Vaccines* are found on pages 596 and 598)



Conjugate and Polysaccharide Vaccines

Conjugate and Polysaccharide Vaccines, Plenary Review

Meningococcal polysaccharide-protein conjugate vaccines

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Meningococcal polysaccharide vaccines are available for prevention of invasive diseases caused by *Neisseria meningitidis*, serogroups A, C, Y and W135. Protection correlates with the ability of vaccination to induce serum complement-mediated bactericidal antibody(1) In general, the antibody responses to these unconjugated polysaccharides are age-dependent: these vaccines are highly effective in adults (1), but they elicit negligible or incomplete and short-lived protection in infants and preschool children (1,2). Polysaccharide vaccines for prevention of disease caused by serogroup B meningococcal strains are not available. The group B polysaccharide is a poor immunogen at all ages, possibly because of immunologic tolerance induced in the host by the presence of cross-reactive polysialyated glycoproteins in fetal and adult tissues (4).

Most polysaccharides appear to elicit antibody responses largely without the need for T-cell help (i.e., they are thymic-independent, or TI antigens). Conjugation of a polysaccharide to a protein carrier profoundly alters the immunologic properties of the polysaccharide, converting it from a TI to a thymic-dependent (TD) antigen. In the resulting TD conjugate, the immunogenicity of the polysaccharide is greatly enhanced, giving rise to IgG anticapsular antibodies and memory B cells.

Experience with *Haemophilus* type b conjugate vaccines. Much has been learned about human immunity to polysaccharide-protein conjugate vaccines from studies of *Haemophilus influenzae* type b (Hib) vaccines. Compared to unconjugated Hib polysaccharide, the immunogenicity of the conjugated Hib polysaccharide in infants is greatly enhanced, and repeated injections elicit IgG booster responses, whereas such responses are not observed after repeated doses of unconjugated Hib polysaccharide (1). Serum antibody to the type b capsule confers protection against invasive Hib disease. In addition, Hib conjugate vaccination primes for long-term immunologic memory to the Hib polysaccharide, a property not elicited by vaccination with unconjugated Hib polysaccharide or even after recovery from Hib disease (1). The ability to develop memory B cells, which leads to rapid IgG anticapsular antibody responses upon encountering Hib organisms, may be an important alternative mechanism of protection against developing disease in vaccinated individuals who either have shown subprotective antibody responses to Hib conjugate vaccination (1), or whose serum antibody concentrations have declined to below the protective level (1).

Hib conjugate vaccines are effective in two other important ways: first, the anticapsular antibody elicited by repeated injections of Hib conjugate vaccines undergoes affinity

maturation (author's unpublished data). The resulting higher avidity antibodies are more efficient at activating complement-mediated bacteriolysis or opsonization of Hib than lower avidity antibodies (1,2). Second, immunization with Hib conjugate vaccines not only protects the individual from developing invasive Hib disease by inducing protective immunity, but also lowers the rate of nasopharyngeal colonization and transmission of Hib in the population (1,2). By this mechanism, Hib conjugate vaccination can have a better effect on decreasing the incidence of Hib disease in the population than would be predicted based on vaccine coverage (1).

Experience with meningococcal polysaccharide-protein conjugate vaccines. Meningococcal oligosaccharide- and polysaccharide-protein conjugate vaccines have been prepared for prevention of diseases caused by serogroups A, B, and C organisms (1,2,3,4,5). To date, data from humans are limited to serogroup A and C meningococcal oligosaccharide-protein conjugate vaccines. A first-generation prototype vaccine was prepared at Chiron Biocine using meningococcal A and C oligosaccharides that are independently coupled to CRM_{197} carrier protein (a cross-reactive nontoxic mutant diphtheria toxin) (17). The conjugation method is based on selective end-reducing group activation of oligosaccharides and subsequent coupling to the protein through a six-carbon "spacer" molecule, adipic acid. In adults, the immunogenicity of the first-generation meningococcal A and C conjugate vaccine appeared to be similar to that of a control unconjugated meningococcal polysaccharide vaccine (1). A "second-generation" meningococcal C vaccine was prepared using similar chemistry except that very small oligomers (degree of polymerization [Dp] less than six monomers in length) were removed prior to conjugation (1). In phase I and II clinical trials in humans, both vaccines have been shown to be very safe in infants, toddlers and adults (1,2,3,4,17,18,19). Expanded clinical trials with the second-generation meningococcal C conjugate vaccine are currently in progress.

Immunogenicity. Toddlers. In a study of US toddlers 18 to 23 months of age, conducted at the University of California, Los Angeles (UCLA), two doses of the Chiron Biocine meningococcal A and C conjugate vaccine given two months apart elicited 50- to >100-fold higher bactericidal antibody responses to both group A and group C strains, compared to those observed in control toddlers vaccinated with two doses of unconjugated meningococcal polysaccharide vaccine (Table 1) (21). Interestingly, the relative differences in immunogenicity between the conjugate and unconjugated vaccines in this study were much less striking when the antibody responses of the toddlers were measured by an ELISA, performed using a consensus protocol developed at the Centers For Disease Control (i.e., relative differences in antibody responses of 2-fold, instead of 50- to >100-fold, as determined by the bactericidal assay). These results suggest that the anticapsular antibody elicited by this conjugated meningococcal oligosaccharide vaccine is qualitatively different from that elicited by unconjugated meningococcal polysaccharide vaccine and, on a μg/ml basis, the conjugate-induced antibodies have a higher specific functional activity. Further, the ELISA is insensitive to these qualitative antibody differences, which may be important in protection against developing meningococcal disease.

Table 1. Serum bactericidal antibody responses of US toddlers vaccinated with a meningococcal A and C oligosaccharide-CRM conjugate vaccine*

Meningococcal Bactericidal Antibody	Geometric Mean Bactericidal Titer (Reciprocal)	
	Conjugate Vaccine (N = 42)	Polysaccharide Vaccine (N = 41)
Anti-A		
Pre-	8	7
Post-2	756	38
Anti-C		
Pre-	5	4
Post-2	3198	11

*Toddlers were given two doses of either meningococcal A and C oligosaccharide-CRM_{197} conjugate vaccine, prepared at Chiron Biocine, or meningococcal polysaccharide vaccine (Menomune, Connaught Laboratories, Inc.). The injections were separated by two months. Complement-mediated bactericidal activity was measured in serum obtained immediately before dose one (Pre-) and one month after dose two (Post-2). Adapted from Lieberman J, et al. JAMA 1996;275:1499–1503. (21)

The "second-generation" Chiron Biocine meningococcal C polysaccharide-protein conjugate vaccine, in which small saccharide oligomers are removed prior to conjugation, was recently evaluated in toddlers 12 to 23 months of age in a multicenter Canadian study (19). This vaccine also elicited much higher serum bactericidal antibody responses after one or two injections than those observed in sera of control toddlers vaccinated with unconjugated polysaccharide vaccine.

Infants. The safety and immunogenicity of Chiron Biocine meningococcal conjugate vaccines also have been investigated in infants less than six months of age.
The first study was performed with the combined A and C conjugate vaccine in Gambian infants immunized at 2, 3 and 4 months of age, or 2 and 6 months, or 6 months of age (20). The anticapsular antibody responses of the infants to the A component of the conjugate vaccine were of similar magnitude to those of a control group receiving an unconjugated meningococcal A and C polysaccharide vaccine. In contrast, the conjugate vaccine elicited two- to four-fold higher anticapsular antibody responses to meningococcal C polysaccharide than those observed in infants given the unconjugated polysaccharide vaccine. However, the elevated anti-C antibody concentrations in the conjugate group began to decline within three months after vaccination.

In the Gambian study, only ELISA antibody responses were reported. Based on the experience in the UCLA toddler study described above (21), the ELISA results may have underestimated the relative effectiveness of the conjugate vaccine as compared to unconjugated polysaccharide, had the responses been assessed by serum bactericidal antibody. In a study in the UK, 58 infants were vaccinated with the combined Chiron Biocine meningococcal A and C conjugate vaccine at 2, 3 and 4 months of age (23). To date, antibody responses to the group A vaccine have not been evaluated. However, the infants showed excellent anti-meningococcal C bactericidal responses after the first and second injections (geometric mean bactericidal titer of <1:10 prior to vaccination increasing to >1:100 after one injection, and >1:1000 after two injections). There was no further increase in titer after the third injection.

There are two other reports of the use of meningococcal C conjugate vaccines in infants. In one study, the "second-generation" Chiron Biocine meningococcal C polysaccharide-protein conjugate vaccine was given to UK infants at 2, 3 and 4 months of age (22). Preliminary results were limited to assays of sera obtained prior to vaccination and one month after the third injection. The infants showed 25-fold increases in anticapsular antibody concentrations, as measured by ELISA, and >50-fold increases in bactericidal titers. In the other study, a meningococcal serogroup C oligosaccharide-CRM_{197} conjugate vaccine was prepared by Lederle Praxis Biologics and administered to US infants at 2, 4 and 6 months of age (16). This conjugate vaccine appeared to be well tolerated and elicited significant increases in serum anticapsular antibody concentration after two or three injections, as assessed by ELISA.

Induction of immunologic memory. In the study of Gambian infants given the Chiron Biocine first-generation combined meningococcal A and C vaccine, serum antibody concentrations to both polysaccharides had begun to decline within three months after conjugate vaccination (20). An important question, therefore, is whether or not the conjugate vaccination induced memory B cells that might allow the infants to respond rapidly with an increase in serum anticapsular antibody concentration upon encountering group A or C meningococci. To examine this question, participants in this study were re-vaccinated at 18 to 24 months of age with an unconjugated meningococcal polysaccharide vaccine (1). Prior to the booster injection, the serum antibody concentrations to the A or C polysaccharides in the groups previously given the conjugate vaccine were not significantly different from those of toddlers of similar age who had not been previously vaccinated. However, as shown in Table 2, the toddlers primed with meningococcal conjugate vaccine at 2 and 6 months of age developed much higher anti-meningococcal C bactericidal titers after the polysaccharide booster vaccination than did toddlers of similar age immunized for the first time with unconjugated meningococcal polysaccharide vaccine. In contrast, the Gambian toddlers who had been primed with meningococcal unconjugated polysaccharide vaccine at 3 and 5 months of age showed significantly lower anti-C bactericidal responses to the booster injection than the control toddlers vaccinated for the first time (Table 2). This result confirms previous data suggesting that immunization at an early age with unconjugated meningococcal group C polysaccharide vaccine induces immunologic tolerance and impairs the ability to respond to a subsequent immunization with unconjugated

meningococcal polysaccharide vaccine (1). The clinical importance of this finding is unknown. However, immunologic tolerance to meningococcal C polysaccharide could enhance susceptibility to developing invasive meningococcal disease by impairing the ability of the child to develop a protective serum anticapsular antibody response upon encountering the organism.

Although the Gambian toddlers who had been given conjugate vaccine as infants were primed for memory antibody responses to meningococcal C polysaccharide, similar priming was not observed to the group A polysaccharide: that is, the magnitude of the anti-A antibody response to the booster injection of unconjugated polysaccharide vaccine in the conjugate-primed group was not significantly higher than that observed in Gambian toddlers vaccinated with unconjugated meningococcal polysaccharide for the first time (Table 2). Further, in contrast to meningococcal C, evidence of immunologic tolerance to meningococcal A polysaccharide was not observed in the toddlers previously given the unconjugated polysaccharide vaccine at 3 and 5 months of age (Table 2). Indeed, the toddlers previously vaccinated with unconjugated polysaccharide vaccine appear to have shown secondary antibody responses to the serogroup A polysaccharide. These data confirm the results of many previous studies indicating that group A and group C meningococcal unconjugated polysaccharide vaccines have very different immunologic properties (summarized in reference 4, Frasch, 1995): specifically, meningococcal A polysaccharide vaccines appear to be both immunogenic and protective in early infancy (2), and may even prime for secondary antibody responses to a subsequent injection (25). Meningococcal C polysaccharide vaccine shows none of these properties. The immunologic basis for these differences remains unknown.

Recently, toddlers who participated in the UCLA study of the Chiron Biocine meningococcal A and C conjugate vaccine were also given a booster injection of unconjugated meningococcal polysaccharide vaccine approximately one year later (1). The group that had been primed with the conjugate vaccine showed evidence of induction of memory B cells to <u>both</u> the serogroup A and C polysaccharides, as evidenced by very high serum bactericidal booster antibody responses. These results are in contrast to those of the Gambian infants given this conjugate vaccine in whom evidence of induction of memory B cells was limited to the serogroup C polysaccharide, and not the serogroup A polysaccharide (see above) (24). Further studies of immunologic priming induced by meningococcal A conjugate vaccines at different ages and in different populations are needed to clarify this discrepancy.

Table 2. Effect of priming with meningococcal A and C oligosaccharide-CRM conjugate vaccine on serum bactericidal antibody responses of Gambian toddlers boosted with unconjugated meningococcal polysaccharide vaccine*

	Geometric Mean Bactericidal Titer (Reciprocal) 10–14 Days Post-Booster		
	Priming Vaccine		
Meningococcal Bactericidal Antibody	None (N = 34)	Polysaccharide Vaccine (N = 17)	Conjugate Vaccine (N = 15)
Anti-A	338	1783	549
Anti-C	239	26	4390

*Toddlers in the Gambia were boosted with a dose of unconjugated meningococcal polysaccharide vaccine (Menpovax A plus C, Biocine, Siena, Italy) at a mean age of 19.7 months. The subjects had either been previously vaccinated at 3 and 6 months of age with unconjugated meningococcal polysaccharide, or at 2 and 6 months of age with meningococcal A and C oligosaccharide-CRM conjugate vaccine (Twumasi, et al.) (20). A group of control unprimed toddlers from the same study area were vaccinated for the first time. Data shown are bactericidal titers measured in sera obtained 10 to 14 days after the booster vaccination (adapted from Leach A, et al., J Infect Dis 1996, in press) (24).

Meningococcal B polysaccharide-protein conjugate vaccines. Investigational polysaccharide-protein conjugate vaccines also have been prepared for prevention of disease caused by serogroup B organisms (1,2,3,15). In general these conjugates are much less immunogenic than those prepared with serogroup A or C polysaccharides. However, one promising immunogenic meningococcal B vaccine candidate is a conjugate in which the polysaccharide component has been modified by substitution of N-propionyl groups for N-acetyl groups (NPr-meningococcal B polysaccharide) (28). To date, there are no published data from trials in humans with this type of conjugate. However, it will be a difficult task to prove that such vaccines are safe in humans because, in mice, data from our laboratory suggest that NPr-meningococcal B polysaccharide-protein conjugate vaccines induce anti-NPr-meningococcal B polysaccharide antibodies that cross-react with native NAc-meningococcal B polysaccharide and also appear to have autoantibody activity (Bartoloni A, et al., unpublished data). Whether or not the use of alternative substitutions, such as N-butanoyl, will truly permit an antibody response that is functional, entirely pathogen-specific, and not cross-reactive with host antigens, remains to be ascertained (1).

Conclusions and the future. Meningococcal A and C conjugate vaccines hold enormous promise for providing solid long-term protection to infant age groups that are currently poorly protected by licensed unconjugated meningococcal polysaccharide vaccines. Further, "third-generation" conjugate vaccines are under development in which the acquisition of immunity is accelerated and immunogenicity is enhanced by administration of the conjugate vaccine with novel adjuvants (1). The use of adjuvants suitable for humans holds promise for decreasing the number of doses of conjugate vaccine required for induction of immunity in infants. This approach also may be useful for enhancing or maintaining immunogenicity of future multicomponent conjugate vaccines. For prevention of group B meningococcal disease, adjuvanted polysaccharide-protein conjugate vaccines also may prove to be immunogenic and protective. However, alternative approaches may be needed to avoid the safety concerns of inducing anticapsular antibodies with autoantibody reactivity.

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Protective epitope of N-propionylated group B meningococcal polysaccharide.

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Neisseria meningitidis is a human pathogen of worldwide significance. Although group B *N. meningitidis* is the most pathogenic serogroup its capsular polysaccharide is precluded from the current polysaccharide vaccine due to its poor immunogenicity (1). Furthermore this problem, which is attributed to structural mimicry between the group B meningococcal polysaccharide (GBMP) and human tissue antigens (2), cannot be satisfactorily overcome by coupling the GBMP to protein carriers (3). Currently there is no vaccine against group B meningococcal meningitis and most efforts to develop an effective vaccine have focused on sub-capsular components such as outer membrane proteins and lipopolysaccharides. Problems have been encountered in the development of these vaccines not the least of which is the intrinsic antigenic diversity exhibited by the components (4). Because the GBMP is a conserved antigenic structure on group B meningococci, a polysaccharide-based vaccine would be the vaccine of choice, provided one could overcome its poor immunogenicity.

One simple way to achieve this goal is to use a synthetic vaccine composed of the N-propionylated (NPr) form of the GBMP, which when conjugated to tetanus toxoid (TT), induces in mice high titer antibodies that are bactericidal for all group B meningococci (5). The NPr-GBMP-TT produced two distinct populations of antibodies, one of which (minor population) cross-reacted with the GBMP. Of significance to the development of a vaccine was that the major population of antibodies did not cross-react with the GBMP and yet contained all the bactericidal activity towards group B meningococci (6). In addition the induction of GBMP cross-reactive antibodies could also be reduced by adjuvant manipulation. Thus the NPr-GBMP must mimic a unique epitope on group B meningococci (6).

In order to further define this epitope a series of mAb's of the IgG isotype were produced in BalbC mice using an (NeuPr)~35-TT conjugate vaccine. Most of the mAb's which were only minimally cross-reactive with the GBMP, recognized an extended helical form of the NPr GBMP. However, unlike GBMP-specific antibodies, which only recognize extended helical epitopes on the GBMP (2), a few were able to recognize short (random coil) segments of the NPr GBMP. Because of the paucity of clones specific for these short epitopes, additional mAb's with this specificity were generated using an (NeuPr)4-TT conjugate. Two important conclusions can be drawn from the properties of the above mAb's. The first is that while antibodies of the IgG1 isotype are not bactericidal, they confer good passive protection in mice challenged with live group B meningococci.The second is that regardless of isotype only mAb's specific for the extended helical form of the NPr GBMP are protective as defined by either passive protection experiments or

bactericidal activity. Therefore one can draw the intriguing conclusion that whereas the serologically distinct extended helical epitopes of both the GBMP and the NPr GBMP co-exist in the capsular layer of group B meningococci and *E. coli* K1, only the former are present in purified α2-8-polysialic acid.

The presence of extended NPr GBMP-specific epitopes in the capsular layer of the above organisms was substantiated by electron microscopy, using a mAb with this specificity as the binding antibody.In addition using this technique, it was demonstrated that a mAb specific for short NPr GBMP epitopes did not bind to either organism, which is consistent with the lack of protection provided by mAb's with this specificity.

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Preclinical evaluation of a combination vaccine against groups A, B, and C meningococci in both mice and nonhuman primates

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Neisseria meningitidis is the major cause of bacterial meningitis worldwide. Meningococci of serogroups A, B, and C are responsible for approximately 90% of all the reported cases (1, 2). A combination polysaccharide-protein conjugate of each serogroup (A, B, and C) is being developed as a vaccine candidate against meningococcal infections.

For group B, the polysaccharide was chemically modified at the C-5 position of the sialic acid residue wherein the N-acetyl groups are replaced with N-propionyl groups to alter the immune tolerance and provide greater immunogenicity (3, 4). The chemically modified B polysaccharide, as well as the native A and C polysaccharides, were coupled to the carrier protein, a recombinant class 3 porin (rPorB) of group B meningococci (5), by reductive animation (3). The rPorB was chosen as the carrier protein due to its ability to significantly increase the bactericidal activity towards the group B polysaccharide when conjugated (6, manuscript submitted for publication).

The groups A, B, and C conjugates were evaluated individually and in combination in both mice and nonhuman primates (African green monkeys). Immune responses were assessed in terms of polysaccharide-specific IgG (by ELISA) and antibody-dependent complement-mediated bactericidal activity.

In mice, the combination vaccine is highly immunogenic, eliciting high levels of polysaccharide-specific IgG and bactericidal activity against all three components. Booster effects were also clearly demonstrated after subsequent injections for all components indicating that T-dependency was achieved. No significant interference in immunological responses was observed for the trivalent vaccine formulation when compared with the monovalent vaccine controls.

The responses in nonhuman primates for individual and combination vaccines are being evaluated, but initial results have shown significant bactericidal activity against all 3 serogroups after only one injection of the trivalent formulation.

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Peptide mimic-induced primary human antibody response to the capsular polysaccharide of *Neisseria meningitidis* serogroup C

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Recent trials with a conjugated meningococcal vaccine preparation have failed to show enhanced immunogenicity over the conventional capsular polysaccharide vaccine (1). An alternate approach to convert the T-independent meningococcal vaccine into a T-dependent vaccine is through the use of an anti-idiotypic mimic of the native antigen. We have developed an anti-idiotype-based peptide mimic of the capsular polysaccharide of *Neisseria meningitidis* serogroup C (MCPS). Immunization with this peptide complexed to proteosomes results in a protective anti-MCPS antibody response in Balb/c mice (2). Study of the human immune system has been hampered by the lack of experimental models to generate a primary immune response to T-independent or T-dependent antigens. Mosier *et al.* (3) demonstrated mutant severe combined immunodeficient (SCID) mice could be engrafted with functional human peripheral blood lymphocytes (hu-PBL). A major limitation of the hu-PBL-SCID model has been the failure to demonstrate a primary human immune response (4-8). We hypothesized that the lack of consistent human primary immune response may be attributed to the lack of human cytokines resulting in impaired differentiation and maturation of human lymphoid cells in reconstituted SCID mice. We have developed a reliable system of inducing a human primary antibody response in the reconstituted hu-PBL SCID mouse model (9). This study was undertaken to define the optimal dose and configuration of MCPS peptide mimics.

Three healthy volunteers were leukopheresed. Fifty five SCID mice/volunteer were reconstituted with 10^8 human lymphocytes and immunized with 10 μg MCPS; 10, 25, 50, or 100 μg of the P3 peptide (CARIYYRYDGFAY) complexed to proteosomes; 25 or 50 μg of 3xP3 peptide (IYYRYDIYYRYDIYYRYD) complexed to proteosomes; 25 or 50 μg of 3xYPY peptide (IYYPYDIYYPYDIYYPYD) complexed to proteosomes. The human anti-MCPS response was measured by ELISA. Functional activity was determined by bactericidal assay.

The results of these studies showed that immunization with 50μg of P3 peptide complex or 25 μg of 3xP3 peptide complex resulted in the highest human anti-MCPS antibody titer (21.5 and 21.4 μg/ml respectively). Immunization with the 3xYPY peptide resulted in 10-15 μg/ml human anti-MCPS antibody. Immunization with MCPS (one dose) results in 0.26 μg/ml anti-MCPS at 4 weeks. All antisera with an anti-MCPS titer exceeding 1 μg/ml proved to be functional in bactericidal assay. These data indicate that an anti-Id based peptide mimic of MCPS induces a protective, T-dependent antibody response in humans.

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Murine monoclonal antibodies to an N-propionylated meningococcal B polysaccharide exhibit heterogeneity with respect to cross-reactivity with N-acetylated meningococcal B polysaccharide and autoreactivity to host polysialyated glycoproteins

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The poor immunogenicity of the *Neisseria meningitidis* group B (MenB) polysaccharide capsule (PS), a homopolymer of α 2-8 sialic acid, represents a major challenge in developing an effective polysaccharide-based vaccine to prevent MenB disease. The poor immunogenicity of MenB PS has been attributed to immunologic tolerance induced from exposure to host polysialyated glycoproteins (e.g., neural cell adhesion molecules, termed N-CAMs). Substitution of N-propionyl (NPr) for N-acetyl (NAc) groups on the MenB PS, and conjugation of the resulting NPr MenB PS to a protein carrier, has been reported to result in a conjugate vaccine that is immunogenic in experimental animals and capable of eliciting protective antibodies that activate complement-mediated bactericidal activity (1). However, little is known about the cross-reactivity of anti-NPr MenB PS antibodies with NAc MenB PS, or autoreactivity of these antibodies. To address these questions, we raised a panel of 28 murine anti-NPr MenB PS antibodies. After partial purification of the Mabs from tissue culture supernatants by ammonium sulfate fractionation and exhaustive dialysis, the Mabs were characterized for isotype, cross-reactivity with NAc MenB PS by ELISA, and autoreactivity with a neuroblastoma cell line (CHP-134), which has been reported to express long chain α 2-8 linked polysialic acid (2). Of the 28 Mabs , one was IgM and the remaining 27 were IgG (three IgG1, three IgG2a, thirteen IgG2b and eight IgG3). Fourteen of the 28 antibodies (50%) cross-reacted with NAc MenB PS as demonstrated by direct binding to NAc MenB PS in a solid phase ELISA format. The specificity of this cross-reactivity was confirmed by inhibition of binding with soluble NAc MenB PS. The remaining 14 Mabs showed no cross-reactivity with NAc MenB PS when tested by ELISA at antibody concentrations up to 25 μg/ml. In preliminary studies, complement-mediated bactericidal activity was detected among Mabs that cross-reacted with NAc MenB PS, and those that did not. However, the Mabs that cross-reacted with NAc MenB PS appeared to have the highest bactericidal activity (i.e., lowest concentrations needed to activate 50% killing [BC_{50}]). Analysis of autoreactivity of the Mabs was performed using the CHP-134 human neuroblastoma (NB) cell line with and without neuraminidase (sialidase) treatment as a specificity control. Binding to this cell line was detected with several of the Mabs. Alternative approaches for measuring autoantibody activity are being used to confirm the pattern of reactivity. In conclusion, the murine anti-NPr MenB PS monoclonal antibodies described here are heterogeneous with respect to cross-reactivity with NAc MenB PS, their ability to bind to the NB cell line, and their ability to elicit complement-

mediated bactericidal activity. Within the panel of Mabs there are examples of anti-NPr MenB PS Mabs that are bactericidal but do not cross-react with NAc MenB PS and do not show binding to the NB cell line. Presumably such antibodies could protect the host against the pathogen and pose no risk of eliciting autoimmune disease. However, many of the anti-NPr MenB PS antibodies cross-reacted with native NAc MenB PS and also showed strong binding to the NB cell line. Although there is no evidence that the ability of an antibody to bind to host tissue will necessarily result in autoimmune disease, it will be a difficult task to prove that a conjugate vaccine that elicits such antibodies is safe to use in humans.

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Immunogenicity of a meningococcal serogroup A and C conjugate vaccine in UK infants.

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Currently approximately 1500 cases of meningococcal infections are notified each year in England and Wales with serogroup C disease accounting for about one third of infections (1). Serogroup C vaccines have been developed from capsular polysaccharide but, unconjugated, these vaccines do not protect those under two years of age (2). Similar experience with the *Haemophilus influenzae* type b (Hib) native polysaccharide led to the development of the Hib conjugate vaccines, which have demonstrated enhanced immunogenicity in young infants and the capacity to induce immunological memory (3). Initial trials addressing the safety and immunogenicity in adults of a serogroup A and C conjugate vaccine demonstrated significant rises in antibody levels to both A and C polysaccharide and bactericidal antibody titers to serogroup C meningococci (4).

The most important role for an effective meningococcal conjugate vaccine is the protection of infants and children. Studies in infants in the Gambia with a serogroup A and C polysaccharide-conjugate vaccine have demonstrated high levels of serogroup C antibodies although the bactericidal activities of these antibodies were not measured (5).

In this study the immunogenicity of a serogroup A and C meningococcal polysaccharide-CRM_{197} conjugate vaccine was evaluated in 58 infants who received three doses at two, three and four months of age. Sera were tested for antibodies to the serogroup A and C capsular polysaccharide by enzyme-linked immunosorbent assay (ELISA) and bactericidal assays, against two serogroup C strains, using standardized protocols (6,7).

The pre-vaccination total immunoglobulin geometric mean titers (GMT) to anti-A and C polysaccharide antibodies were respectively 2.8 and 0.6 μg/ml rising to 21.5 and 38.5 μg/ml one month after the third dose and falling to 3.1 and 2.2 μg/ml by 14 months of age. Pre-vaccination serum bactericidal titers against two serogroup C meningococci were <1/4 in 49 out of 52 infants, rising to a GMT of 1/3082 one month post third dose and falling by 14 months of age to a GMT of 1/10. Thus this meningococcal conjugate vaccine proved to be immunogenic, inducing high levels of anti-C polysaccharide antibodies which were bactericidal in young infants. This is the first report of a significant antibody response to a meningococcal polysaccharide-protein conjugate vaccine in non-African infants and the first report of an effective bactericidal response in infants.

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Evaluation of the innocuity of a group B meningococcal polysaccharide conjugate in hyperimmunized, pregnant cynomolgus monkeys and their offspring

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Introduction. The poor immunogenicity of group B meningococcal polysaccharide has been suggested to result from its structural similarity with carbohydrate groups borne by the mammalian glycoprotein N-CAM (1). During feto-embryonic and early postnatal development, NCAM exhibits long polysialic acid (PSA) chains [α2-8 $(NeuAc)_n$ with n>8] which are recognized by B polysaccharide-specific antibodies whereas adult forms, with shorter chains, are negative (2). Several studies have shown that the expression of PSA on NCAM found, in particular, on neuroectodermally and mesodermally derived cells, modulates cell-cell interactions occuring during vertebrate organogenesis (3). Thus, it was hypothesized that pregnancy could be hindered or fetal development perturbated by antibodies cross-reacting with α 2-8 $(NeuAc)_n$ long chains. The conjugate we prepared for vaccine purpose is composed of the capsular polysaccharide of group B meningococcus, chemically modified (N-propionylated) and covalently linked to tetanus toxoid. The main objective of this study was to assess, in cynomolgus monkeys, whether hyperimmunization with the conjugate, resulting in high levels of maternal antibodies specific for N-propionylated B polysaccharide (B N-Pr), would affect gestation and/or the morphology and behavior of the offspring.

Study design. The study involved 39 adult female cynomolgus monkeys. One group of 24 was hyperimmunized (before mating) with the conjugate administered with Freund's complete adjuvant (FCA) and with booster injections of the same antigen with Freund's incomplete adjuvant (FIA) and then (after mating) with aluminum hydroxide (Al). One control group of 15 received the adjuvants. The study included several parts: reproduction study itself, characterization of the antibodies induced in females and transmitted to their offspring, behavioral tests on newborns, immunohistochemical and histological examinations on fetal tissues and histological studies of stillborns.

Results. The presence of antibodies specific for the vaccine antigen was monitored in the sera of females: IgG and IgM B N-Pr polysaccharide specific antibody titers were very high and sustained throughout the study (more than one year). Of the 11 hyperimmunized mothers getting full gestation, 8 were among those having the highest titers. Induced antibodies were bactericidal to group B meningococcus. Their ability to recognize polysialylated structures was also studied. Induced IgG recognized unmodified B polysaccharide far less than B N-Pr (about 1% cross-reactivity); the sera of

3 hyperimmunized females among the 11 getting a full gestation reacted strongly with PSA-NCAM structures in two highly sensitive *in vitro* tests.

The reproduction study showed no significant difference between the 2 groups of females in terms of fecundation, abortion and stillbirth rates:

fecundation rate:	75% for hyperimmunized group versus 87% for control group
abortion rate:	61% for hyperimmunized group versus 62% for control group
stillbirth rate:	43% for hyperimmunized group versus 67% for control group.

The analysis of antibodies specific to the polysaccharides, in the sera from fetuses and newborns, demonstrated that a large proportion of maternal IgG had been transmitted to them *in utero*. These passively acquired antibodies were only slightly bactericidal. Their levels decreased to reach a titer near the ELISA detection threshold, at 3 months of age. Few sera of infants obtained near delivery, recognized PSA-NCAM and only to a small extent.

The study conducted on 6 infants (2 from control mothers and 4 from hyperimmunized mothers), from 10 days of age until 6 months of age, included morphometric observations and neurobehavioral tests (4) to evaluate the neurologic development of these sucklings: neither constitutive abnormality nor behavioral difference were observed between infants born to control females and to hyperimmunized females.

Immunohistochemical investigations were conducted on tissues from 5 fetuses taken by caesarian about one month before the end of gestation, 4 of which were born to hyperimmunized females. Histological examinations provided additional data concerning these 5 fetuses and also involved the 8 stillborns, 3 of which were born to hyperimmunized mothers: no maternal antibodies linked to the polysialylated structures of fetal organs were found on immunohistochemical investigations, and histological examinations of fetuses and stillborns evidenced no particular lesions.

Conclusion. Within the limits of the number of cases observed in the present study and of the sensitivity of the methods used, it can be concluded that a high amount of antibodies specific for N-propionylated B polysaccharide, present during all the gestation in cynomolgus monkeys and transmitted to the fetuses had no harmful consequences on the development of the organs and nervous system of fetuses and sucklings.

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Safety and immunogenicity of an N-propionylated group B meningococcal polysaccharide conjugate vaccine in adult volunteers

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Introduction. Meningococcus is the second cause of bacterial meningitis after *H. influenzae* type b, serogroups B and C being predominant in industrialized countries. Although meningococcal groups A, C, Y and W135 capsular polysaccharide vaccines have been shown to be safe and effective, attempts to develop polysaccharide-based vaccines to prevent group B infections have remained unsuccessful. It has been shown that a chemically modified group B meningococcal polysaccharide conjugate is able to induce polysaccharide specific IgG and protection in mice (1,2). Thus, we developed a conjugate vaccine using the N-propionylated B polysaccharide (B N-Pr), the tetanus toxoid as carrier and Aluminum hydroxide (Al) as adjuvant.

Phase I study design. The safety and immunogenicity of the vaccine were evaluated in healthy male volunteers (age ranging from 19 to 27 years). Four escalating doses (1, 5, 25 and 50 µg of polysaccharide) were tested and injected intramuscularly into 3 individuals for 1 and for 5 µg and 5 individuals for 25 and for 50 µg. Volunteers received 3 injections at 4 weeks intervals. Sera were obtained before the first and one month after each injection. Safety studies involved the observation of local and systemic reactions and the evaluation of binding of the serum antibodies to α2,8-linked polysialic mammalian structures (polysialylated form of N-CAM named PSA-NCAM). The immunogenicity study included the determination of the amount and the functionality of the induced antibodies.

Results.

Local reactions observed were dose-related but not more severe than those usually produced by Al-adsorbed vaccines. No systemic reactions were recorded. No binding of serum antibodies (IgG and IgM) to purified PSA-NCAM or to tumor cells expressing PSA-NCAM was demonstrated. The serological analyses showed that all preimmune sera (16/16) contained IgM specific for B N-Pr and B (B PS) polysaccharides but no specific IgG.

Immunization with the conjugate elicited : a) an increase of the preexisting IgM titers; these titers sustained at least for one month after the last injection (mean seroconversion rate = 19 for B N-Pr and = 3 for B PS); b) B N-Pr specific IgG with both a dose and a

booster effect (induced IgG were mostly from IgG_1 subclass); c) no B polysaccharide specific IgG; d) an increase of anti-tetanus titers (mean seroconversion rate = 7, for the 2 highest dosages)

Functional activities of pre- and post-immunization sera were investigated. Bactericidal assays performed with human complement did not demonstrate any specific bactericidal activity whatever the day of blood sampling. However, high preimmune bactericidal titers were observed in the presence of baby rabbit complement, but immunization did not induce a significant increase of these titers. Purification of antibodies showed that IgM were responsible for most of this activity and that induced IgG were far less effective.

The following experiments were also performed on preimmune and post 3 sera from the 2 highest dosage groups: a) opsonophagocytosis using human PMN and human complement; b) Passive protection test in infant rats. No functional activity of the induced antibodies could be determined through these tests. However, the ability of post 3 antibodies to bind to group B meningococcus was demonstrated. On average, 100 % of B PS specific IgM, 83 % of B N-Pr PS specific IgM and 23 % of B N-Pr specific IgG bound to the bacteria.

Conclusion. These findings indicate that the conjugate is safe and immunogenic in human adults and able to induce an increase of preexisting B PS specific IgM titers and B N-Pr specific IgM and IgG.

Four hypotheses can be proposed to explain the apparent lack of function of induced antibodies: a) presence of high preimmune bactericidal titers (with baby rabbit complement), b) induction of too low levels of antibodies by the vaccine, c) lack of sensitivity of functional tests, and d) restriction of the functional tests used, to some aspects of the immune response.

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Immunological activity of serogroup B meningococcal vaccine from natural complex of capsular polysaccharide and outer membrane proteins

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Antibody response of adult volunteers given a natural complex of capsular polysaccharide and outer membrane protein vaccine was studied by bactericidal assay (1). The vaccine used in these studies was prepared from *Neisseria meningitidis* group B serotype 2b strain 125 (B:2b:P1.2) (2), and contained 50 μg of group B polysaccharide and 57.7 μg of outer membrane protein per 0.5 ml dose when lyophilized vaccine was reconstituted with either aluminum hydroxide gel (concentration of AH 4.6 mg/ml) or with 0.9% NaCl.

The vaccine corresponded to WHO requirements (3) concerning sterility, pyrogenicity, and general toxicity. Volunteers at the age of 18-20 years were included in the study. Blood specimens were obtained from all volunteers before vaccination and four weeks after the second immunization. Only local reactions without systemic reactions were registered.

Bactericidal activity of sera was studied against three group B strains; B:2b:P1.2, B:2a:P1.2, and B:15:P1.7. Vaccine administered with and without AH induced bactericidal antibodies to all three strains. High antibody levels were detected after the second immunization when the AH gel was used. Eighty percent of individuals showed a four-fold or greater increase in bactericidal antibody titers to the homologous strain and 60% to the heterologous type 15 strain. The antibody response of adult volunteers who received the vaccine without adjuvant was less. Only 40% of individuals showed four-fold or greater increases in antibody titers to the homologous strain and 20% to the heterologous strain.

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Conjugate and Polysaccharide Vaccines, Poster 74

Cost-effectiveness analysis for routine immunization with a quadrivalent meningococcal polysaccharide (A,C,Y,W-135)- protein conjugate vaccine in the United States

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Background. Meningococcal disease is a leading cause of meningitis and septicemia among children less than five years old in the United States. The currently available meningococcal polysaccharide vaccine (A,C,Y,W-135) is poorly immunogenic in infants and young children making it unsuitable for use in routine infant immunization programs for the control of endemic meningococcal disease. The recent success in development of polysaccharide-protein conjugate vaccines against *Haemophilus influenzae* type b (Hib) disease has prompted development of vaccines using similar technology for *Neisseria meningitidis*. We evaluated the potential cost-effectiveness (C-E) of a quadrivalent meningococcal polysaccharide-protein conjugate vaccine (MenConj) (serogroups A, C, Y, W-135) when used as routine infant immunization in the United States.

Methods. We developed a C-E decision model, using the societal perspective, that compares the costs and benefits from routine infant immunization to the costs and benefits from the current situation where no routine infant immunization exists. Key estimates for meningococcal disease incidence, vaccination program, and costs associated with meningococcal disease are summarized below.
*Meningococcal disease incidence due to serogroups A, C, Y, W-135-- estimated birth cohort of 3,979,000 with a cumulative incidence between ages 6-59 months of 8.9 cases/100,000 [average incidence from CDC active surveillance in the United States between 1989-1995], a 8% case-fatality rate, and a 12% sequelae rate (1).
*Vaccination program-- 89% vaccination coverage, 90% vaccine efficacy, 4 doses of MenConj vaccine administered at ages 2, 4, 6, and 12-15 months of age in the same syringe as Hib conjugate vaccine, a vaccine cost of $4.76 per dose (the current public sector price of Hib conjugate vaccine), moderate and severe adverse reaction rates equivalent to those used for C-E models of Hib vaccines (0.002 and 0.000017) (2,3) and average costs per moderate and severe adverse reaction of $45 and $1,500, respectively.

*Costs associated with meningococcal disease-- direct costs include hospitalization for all patients ($13,431 per case). Long-term costs of sequelae were calculated with the same annual costs (adjusted to 1995 dollars) and duration used by the Institute of Medicine for a similar analysis of Hib vaccines (4). The discounted present value of lifetime costs per sequelae were $44,187 for a hearing deficit, $110,467 for a learning deficit, and $864,980 for an institutionalized patient with severe retardation. Indirect costs were calculated using estimates for lifetime productivity losses due to death or severe retardation ($964,490).

The costs to individuals and to health departments for chemoprophylaxis of contacts in response to sporadic cases of meningococcal disease and mass vaccination campaigns to control meningococcal disease outbreaks are not included.

Univariate sensitivity analyses (analyses where the estimate of one model parameter is varied from that used in the C-E model, with all other parameters remaining the same) were conducted on vaccine cost ($4.76 v. $2.80), number of doses in the vaccination regimen (4 v. 3), and disease incidence (8.91/100,000 v. 14.18/100,000).

We calculated three commonly used measures of the cost-effectiveness of vaccination: the cost per case averted, the cost per death averted, and the cost per life-year saved. Life-years lost were calculated by subtracting the average age of death from the average life expectancy. All costs and benefits were calculated in 1995 dollars, and future costs and benefits (including life-years saved) were discounted at a rate of 3%.

Results. In the C-E model, where no routine meningococcal vaccination exists, an estimated 355 cases, 28 deaths, and 843 years of life are lost annually due to meningococcal disease potentially preventable by a quadrivalent MenConj vaccine among children 6-59 months old, at a total estimated cost of $48.1 million. Routine infant immunization would be anticipated to prevent 284 cases, 23 deaths, and 675 years of life lost, annually. At a cost of $4.76/dose of MenConj vaccine, the costs per case averted, per death averted, and per life year saved are $98,146; $909,303; and $41,279 respectively. At a vaccine cost 2.80$/dose, the vaccine program results in net savings. If the same vaccine effectiveness can be accomplished with 3 doses, instead of 4 doses, of vaccine, the cost per case averted decreases by 60% to $38,787 per case averted. If the most recent estimate of disease incidence is used (14.18/100,000) [Source: CDC active surveillance in 1995], rather than the average of 7 years surveillance, the cost per case averted decreases by 85% to $9,419 per case averted.

Discussion. Meningococcal disease is a substantial economic and disease burden in the United States. The estimates of vaccine C-E from this analysis are likely to be conservative underestimates of the true C-E because the substantial costs to health departments in response to meningococcal clusters are not included . The C-E of MenConj vaccine in this analysis depends on some important assumptions. First, it must be administered in the same syringe with Hib conjugate vaccine (or other appropriate vaccine), thereby eliminating costs for additional visits or equipment to store and administer the vaccine. Second, we assumed that the vaccine will provide 90% protection for at least 4.5 years. Vaccine cost and the number of doses required to successfully immunize an infant strongly influence the C-E of routine MenConj immunization, as does the estimated disease incidence. Vaccine manufacturers can help optimize the C-E of MenConj vaccine by efforts to provide MenConj vaccine at cost equal to or less than that of Hib conjugate vaccine and in a formulation that allows it to be administered in the same syringe as Hib conjugate vaccine (or other appropriate vaccines). Herd immunity through decreased carriage of meningococci similar to what has been observed with use of the Hib conjugate vaccines may also improve the C-E of vaccination. While use of a quadrivalent MenConj vaccine against serogroups A, C, Y, W-135 in routine infant

immunization programs is likely to have a substantial impact on endemic meningococcal disease, an effective serogroup B meningococcal vaccine appropriate for use in infants is needed for comprehensive control of endemic meningococcal disease.

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Bivalent A/C meningococcal conjugate vaccine in toddlers: persistence of antibodies and response to a polysaccharide vaccine booster

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We evaluated the immunologic memory elicited by a bivalent A/C meningococcal oligosaccharide conjugate vaccine (MOCV) (Chiron Biocine) compared to the licensed quadrivalent meningococcal polysaccharide vaccine (MPV). In an earlier study,1 ninety 18-month-olds had been randomized to 3 groups and given 2 doses (2 months apart) of 1) MPV, 2) 5.5 μg MOCV, or 3) 11 μg MOCV. Following two doses, geometric mean antibody levels against group C meningococcus were twice as high in MOCV recipients as in MPV recipients, but antibody levels against group A were not significantly different. Serum bactericidal assays showed striking differences between the conjugate and polysaccharide vaccine groups, as MOCV induced much higher titers of bactericidal antibody against both serogroups. We concluded that the immune response induced by the meningococcal conjugate vaccine was qualitatively different from that induced by the polysaccharide vaccine, and that the antibodies it elicited provide greater functional activity. In this follow-up study, serum specimens were obtained from 25 children in each group 1 year after the 2nd dose. A booster dose of MPV was offered to all children, and 11-14 subjects in each group accepted. Another serum specimen was obtained 1 month after the booster.

Group A meningococcal ELISA antibodies. Geometric mean antibody titers (GMT) were 22.7 μg/ml and 21.2 μg/ml in MOCV and MPV recipients, respectively, after the 2 dose primary series ($p = 0.7$). Antibody levels fell markedly in the year following vaccination, and did not differ significantly between MOCV and MPV recipients (4.5 μg/ml vs. 6.7 μg/ml; $p = 0.26$). Following the booster dose of polysaccharide vaccine, antibody levels increased in all children and the GMT was 3-fold higher in conjugate vaccine recipients (78.0 μg/ml vs. 22.2 μg/ml; $p < 0.006$). In contrast to children who had been given MPV as their primary series, in children initially given MOCV the GMT was significantly higher after the polysaccharide booster than it was after the 2 dose primary series ($p < 0.0001$).

Group C meningococcal ELISA antibodies. After the initial 2 vaccine doses, the GMT was significantly higher in MOCV recipients compared with MPV recipients (16.7 μg/ml vs. 8.3 μg/ml; $p < 0.001$) However, antibody levels against group C declined sharply in the year after vaccination, and did not differ significantly between groups (1.5 μg/ml vs. 1.4 μg/ml; $p = 0.94$). After the polysaccharide booster, antibody levels increased in all vaccinees, but the GMT was more than 5-fold higher in children given MOCV compared with those given MPV (29.9 μg/ml vs. 5.3 μg/ml; $p < 0.0001$). Furthermore, unlike

children initially given MPV, MOCV recipients had higher antibody levels after the booster than they did after their primary series ($p < 0.004$).

Group A serum bactericidal activity. After the primary vaccine series, the geometric mean serum bactericidal titer (SBT) was significantly higher in conjugate vaccine recipients (755.6 vs. 37.6; $p < 0.0001$). One year later, serum bactericidal activity did not differ significantly between MOCV and MPV recipients (13.5 vs. 7.1; $p = 0.22$). Whereas all conjugate vaccine recipients had serum bactericidal titers ¤ 128 after their primary vaccinations, 38% had no detectable bactericidal activity a year later. However, after the polysaccharide booster, the geometric mean SBT was about 16-fold higher in conjugate vaccine recipients compared with polysaccharide vaccine recipients (1673.1 vs. 107.6; $p < 0.0001$) After the booster, all children initially given MOCV had detectable serum bactericidal activity and 92% had titers ¤ 1024, whereas 17% of children initially given MPV had no detectable serum bactericidal activity.

Group C serum bactericidal activity. After the primary vaccine series, the geometric mean SBT was significantly higher in MOCV recipients compared with MPV recipients (3197.9 vs. 11.4; $p < 0.0001$) Serum bactericidal activity fell sharply in the following year. Although the geometric mean SBT remained significantly higher in conjugate vaccine recipients (101.6 vs. 4.5; $p < 0.0001$), 21% of children initially given MOCV no longer had detectable serum bactericidal activity. After the polysaccharide booster, the geometric mean SBT was almost 1000-fold higher in children given MOCV compared with those given MPV (6502.1 vs. 7.1; $p < 0.0001$). All children given the conjugate vaccine had bactericidal titers ¤ 1024, whereas 83% of polysaccharide vaccine recipients had no detectable serum bactericidal activity.

As has been shown with *Haemophilus influenzae* type b2 and pneumococcal3 conjugate vaccines, this meningococcal conjugate vaccine primes for memory antibody responses. Compared with the licensed polysaccharide vaccine, the antibodies it elicits have much greater functional activity against both serogroup A and serogroup C meningococcal polysaccharides. It offers the potential of providing durable protection against these important pathogens.

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Analytical methods for the quality control of *Neisseria meningitidis* C polysaccharide.

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Introduction. The problem of conformational stability control is an important factor. A detailed analysis of conformational changes requires the use of exact, precise and sensitive techniques (1). Using HPLC and NMR methods gives a very interesting information about molecular size and primary structure of polysaccharide C and more accurate than others methods. Physical parameters had been reported, such as molecular weight, radius of gyration, diffusion coefficient, viscosity, light scattering for group C meningococcal (2). In this paper, we evaluated some methods for the quality control and stability studies.

Materials and methods. Serogroup C meningococcal polysaccharide was purified from *Neisseria meningitidis* and was stored at - 70°C.
Chromatography: The samples employed were dissolved in distilled water. Molecular size was determined by gel filtration on Sepharose 4B (Pharmacia Fine Chemicals) using 0.2 M ammonium acetate (3) and by HPLC on TSK G-3000 column (4).

Viscosimetry: The polysaccharides (1 mg N-acetylneuraminic acid/ ml) were used to measure the rehology properties when they are stored at some temperatures. Reduced viscosity was performed on Ubbelohde viscometer.
Optical rotatory dispersion. Optical rotations were measured by Polartronic universal polarimeter with halogens lamps and sodium filter.
NMR spectroscopy: Samples were dried in vacuum over P_2O_5 and then dissolved in deuterium oxide in a 5 mm NMR tube. Spectra were obtained at 250 MHz on a Bruker spectrometer (5).

D.S.C.: A Mettler differential scanning calorimeter was used, cooling rate 5°K. min^{-1}, hating rate 5°K. min.

Results. Molecular weight determination of polysaccharide on HPLC has a fitting precision (variation coefficient below 2 %). The differences with chromatography on Sepharose 4B increased 40 %. The viscosity to different polysaccharide lots (stored at - 70°C) was 3.43 ± 0.54 ml . mg^{-1}. The value of specific optical rotation indicated - 5.32 ± 1.3°. g^{-1} . cm^{-2}. The irreversible conformational changes of polysaccharide produced by temperature to determine changes in the physical parameters. NMR spectra for the type C polysaccharides from different lots showed a similar pattern at Jones and Currie's

spectra[5]. The most important region is containing methyl resonance from O-acetyl groups. Calorimetric measurements gave a glass transition temperature of approximately -12°C and crystallization temperature was nearly -35°C.

Discussion. The dependence diffusion coefficients of molecule's viscosity impose difficulties in partition coefficients values. HPLC would partially resolve the problems quicker and with more accuracy. Some conformational events in subgroup C polysaccharide would be characterized by optical rotation and viscosity studies. The similar NMR spectra between production lots shown homogeneity in purification processes and a good stability at - 70°C. In the thermograms obtained, it is evident that a crystallized state can protect polysaccharide against degradation, in the stored condition.

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Long-term follow-up of late complement component deficient patients vaccinated with meningococcal polysaccharide vaccine: Antibody persistence and efficacy of vaccination.

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Of 45 Russian patients with late complement component deficiency (LCCD) who experienced one-to-five meningococcal infections, thirty-three were immunized with meningococcal polysaccharide vaccine (A+C+W135+Y) and followed for one to five years. Their immune status was normal before vaccination (1). The pre-vaccination levels of antibodies to A, C, W135 and Y polysaccharides were slightly, but not significantly, higher in the group of LCCD patients, than in the control group of 33 vaccinees without complement deficiency and meningococcal infections.

Total and immunoglobulin class specific concentration of antibodies to meningococcal capsular polysaccharides in sera of LCCD patients increased significantly one month after vaccination and remained stable for 1 year. Total Ig levels to A, C, W135 and Y were increased 5, 15, 15 and 16 times, respectively. The specific antibody declined in the next 2-3 years, but remained at least two fold above the concentration in preimmunization sera. The proportion of specific IgA to IgA+IgM+IgG was about 20% before vaccination, and either remained stable (to group A polysaccharide) or decreased (to other polysaccharides) after vaccination. Thus vaccination maintained or increased the prevalence of Ig classes, having complement-activating and opsonophagocytic functions. The kinetic and quality of antibody response did not differ in LCCD vaccinees and control group (2).

Revaccination of 12 patients 3 years after the first dose restored the total antibody concentrations to those observed one year after the first vaccination. The increase was mainly caused by specific IgG, which consisted 80-90% of total specific Ig after revaccination. All patients developed a significant antibody response to all polysaccharides after revaccination, whereas after the first dose one fifth of the patients had only a weak response to some of the polysaccharides.

Six new episodes of meningococcal infection in four patients developed in the group of 33 vaccinees; one of these cases occurred after revaccination. Six episodes in six patients developed in the same time in the group of 12 non-vaccinated LCCD persons. Survival analysis demonstrated that the risk to contract meningococcal disease decreased significantly for vaccinees (0.04 episodes /individual /year) in comparison to non-vaccinees (0.15).The interval between consecutive infections was prolonged from 3.6 years in the non-vaccinated group to more than 6 years ($p < 0.02$, Kaplan-Meier test) in

the vaccinated group. Data from a historical control (same patients followed for ten years before vaccination) was comparable to the later data of non-vaccinated LCCD persons; the interval between the consecutive infections was 10 years and the risk of disease was 0.15 episodes /individual /year in both groups.

Two LCCD vaccinees experienced two episodes of disease after vaccination. This statistically hardly probable event ($p < 0.07$) indicated that some individual properties might have caused the vaccination failure in these cases. However, no differences in immune response were found between the LCCD vaccinees, who were ($N = 4$) and were not ($N = 29$) newly infected.

Unfortunately no clinical isolates of meningococci were obtained from ten newly infected patients; the clinical diagnosis was confirmed by microscopy in 7 cases and PCR in 5 cases. No significant increase of antibody level to group A, C, W135 and Y polysaccharides was detected after five new episodes of meningococcal disease in vaccinees suggesting that post-vaccination infections were caused by group B meningococci. After one episode the level of antibody to group C polysaccharide was increased from a relatively low level of 4.8 mg/l to 72 mg/l, suggesting group C infection. The antibody concentrations after the disease of non-vaccinees were not studied. In 1980-1990 Russian LCCD patients were infected by meningococci of group A (3 isolates), B (3 isolates), C (1 isolates) and unidentified serogroup (3 isolates).

In conclusion, vaccination with of A+C+W+Y polysaccharide vaccine seems to decrease the risk for meningococcal infection in LCCD patients even in a situation when group B meningococci are most frequent serogroup in general (3). This protection might be caused by an increased killing capacity of neutrophils (4).

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NMR analysis of meningococcus type A and C polysaccharide antigens: patterns of *O*-acetylation

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Biocine SpA has developed conjugate vaccines against meningococcal serotypes A and C micro-organisms. The production of these glycoconjugate vaccines all involve generation of the suitable oligosaccharides by fragmentation of the capsular polysaccharide. This procedure and the following steps of oligosaccharide activation and subsequent conjugation to the carrier protein (CRM_{197}) can be monitored by colorimetric and other traditional methods of analysis. However, recent advances in analytical technology permit the structure of these complex immunogens and precursors to be evaluated by physicochemical methods, particularly by NMR analysis. As a starting point in our characterization studies, we have examined the type A and C polysaccharide antigens and will present our data here. The ^{1}H, ^{13}C and ^{31}P 1D spectra provide fingerprints which can be used to show the identity and purity of different polysaccharide lots. The unambiguous assignment of these resonances and hence confirmation of the molecular structures follows from the use of 2D NMR homonuclear and heteronuclear correlation spectroscopy (1).

The MenC polysaccharide consists of a simple homopolymer of 2,9-α-linked sialic acid, however, the NMR spectra are complicated due to the presence of O-acetyl groups (2). The O-acetyl ester groups are alkali labile but can migrate spontaneously under physiological conditions. Most studies of O-acetyl migration have been performed by NMR analysis applied to simple systems such as the sialic acid monomer (3). The data show that acetyl groups at O-7 and O-8 migrate spontaneously to O-9. $T_{1/2}$ for the O-7 acetyl migration to O-9 is 4-8h at physiological pH and temperature, whereas that from O-8 to O-9 is too rapid for measurement.

For the MenC polysaccharide, elucidation of this spectral complexity is hampered by the fact that acetyl migration occurs in solution and thus may change during the actual NMR analysis. Not surprisingly, most of the proton and carbon NMR data published are for the de-*O*-acetylated polysaccharide (4-6). A ^{13}C study of the native polysaccharide with assignments based on inspection, showed that O-acetylation is at O-7 and/or O-8 (4). We have confirmed this from our detailed 2D NMR examination of our MenC lots. We were able to make assignments of the major ^{1}H and ^{13}C resonances of the different spin systems and so are able to interpret the spectra of our batches as well as those published. The amount and position of O-acetylation can be determined most easily from the intensity of the different acetyl signals and recording the spectra with time shows that acetyl migration occurs from O-8 to O-7. This reaches an equilibrium which appears to be 50% complete after 7 days at room temperature (7). We suspect that the acetyl group

is initially solely on O-8 and begins to migrate to the more stable position O-7 during isolation of the antigen and continues when the polysaccharide is in solution. This means that the spectrum obtained depends on the history and preparation of the actual sample, as well as the conditions under which the spectrum is recorded. Different acetylation is manifested by signals near δ5 due to H7 and H8 as well as different H3 and CH_3CON signals so that spectra can appear different although they are still of the MenC polysaccharide. This accounts for the large spectral differences observed between preparations of the same antigen from different manufacturers (2). It is not yet known how acetylation in different *positions* affects immunogenicity, as most investigations of the relationship between O-acetylation and immunogenicity have been restricted to the presence or absence of O-acetyl groups (8, 9).

In the case of the MenA polysaccharide, full proton, carbon and phosphorus assignments were made by use of 2D homo- and heteronuclear NMR experiments. The PS was reported to be →6)-α-ManNAc-(1-OPO_2 → containing approximately 70% O-Ac at O-3 (10). These assignments were made by use of 1D ^{13}C NMR, but our 2D NMR experiments clearly reveal the presence of at least 3 spin systems: unacetylated (29%), Ac on O-3 (66%) as well as on O-4 (5%). The TOCSY diagram showed that the mannosyl H2 resonance is very useful for the "fingerprinting" of the different spin systems. With this knowledge, it is possible to interpret the spectra published for other manufacturer's vaccines (2); this reveals acetylation on O-3 as well as O-4.

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An improved method for meningococcus C polysaccharide purification

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During the past decade vaccines of *Neisseria meningitidis* group A, C, W135 and Z have been developed. These vaccines are composed of purified polysaccharides which are the main component of bacterial capsule. *N. meningitidis* capsular polysaccharide purification method currently in use was first described in 1969 by Gotschlich [1]. By this method, polysaccharide together with protein, nucleic acids and LPS contaminants are precipitated with 0.1% Cetavlon followed by resuspension in 1M $CaCl_2$. Nucleic acids are eliminated by precipitation in 25% ethyl alcohol, proteins are extracted by 45% phenol and LPS are pelleted by ultracentrifugation at 100,000g for 4 hrs. This process has two inconvenient steps for large scale production: phenol is a very corrosive reagent and therefore contaminant protein elimination by phenol extraction should be substituted and ultracentrifugation step is expensive for large scale production since many ultracentrifuges are needed.

In the method established at Instituto Butantan for group C polysaccharide purification, the initial steps were made as already described [1] and two steps of this procedure have been modified: removal of protein and LPS.

Contaminant protein was removed by proteinase digestion using a mixture of three proteinases: proteinase K, nagarse and trypsin. After 25% of ethyl alcohol precipitation and nucleic acid removal by centrifugation, the supernatant was precipitated with ethyl alcohol to 80%. The precipitated PS was resuspended in 20mM Tris-HCl buffer pH 8.5.This solution was incubated overnight at room temperature with 5 mg of each proteinases (for 40L of fermentor), and this treatment was repeated for further 4 hours.

LPS forms a high molecular weight complex which is efficiently pelleted by ultracentrifugation [1]. Detergents such deoxycholate (DOC) are able to disaggregate the complexed LPS to a low molecular weight monomers.[2]. Tangential ultrafiltration on hollow fiber 100 kDa cutoff in buffer containing DOC was used instead ultracentrifugation. Extensive diafiltration on the 100 kDa cutoff hollow fiber (AMICON) in Tris-HCl buffer containing 0.5% DOC was able to eliminate LPS as well as low molecular weight protein resulted by proteinases action. The solution was washed in hollow fiber with five separate volumes of 20 mM of Tris-HCl buffer pH 8.5 containing 0.5% DOC, five separate volumes of the same buffer without DOC and three separate volumes of water.

The resulted purified polysaccharide has around of 2% protein and 1.2% nucleic acid. The polysaccharide molecular weigh determined in Sepharose 4B column showed a K_D

around 0.3 and it passed in the pyrogen test in rabbit according to WHO. The polysaccharide recovery using this process was around 50%.

This process is easily suitable for scale-up due to its easiness. The great advantage of the hollow fiber use is the fact that large volumes for instance, a scale up of 40 to 400 L fermentation can be processed at the same time and by the same way.

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Efficacy of *Neisseria meningitidis* serogroup A/C polysaccharide vaccine among children in Mongolia

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Background. During winter 1993-94, an increased number of meningitis cases were reported to the Mongolian Ministry of Health (MOH); cultures of blood and cerebrospinal fluid revealed *Neisseria meningitidis* serogroup A. Eight strains were subtyped by multilocus enzyme electrophoresis and found to belong to the III-1 clonal complex; strains within this complex have caused recent epidemics in the Middle East and Africa (1). From January-April 1994, a total of 1,754 meningitis cases resulting in 122 deaths were reported to the MOH; the country-wide attack rate was 80 case/100,000 population. The capital city of Ulaan Baatar reported 1070 cases during January-April 1994 and had an attack rate of 179/100,000, the highest among Mongolia's provinces. In response, the MOH organized a campaign to vaccinate children 2-18 years of age in Ulaan Baatar with A/C meningococcal vaccine (France, Pasteur-Merieux) in November 1994.

Methods. During June 1995 we conducted a retrospective case-control study to estimate vaccine efficacy and identify risk factors for disease. Case-patients $\leq$ 18 years old who had positive sterile-site cultures and a systematically selected group of other meningitis case-patients were identified for enrollment. Three controls matched by neighborhood and age were recruited for each case-patient. We collected information on exposures and vaccine history using a standardized questionnaire and reviewed written records to confirm vaccination status. To estimate the impact of the vaccination campaign, we compared cases of meningitis reported to the MOH from January-April 1995 to those received during the same period in 1994.

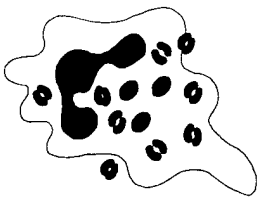
Results. In all, 85 cases and 255 controls were enrolled (32 case-patients < 2 years of age and 53 cases 2-18 years); 13 case-patients (15.3%) had culture-confirmed *N. meningitidis* serogroup A infections. We identified written confirmation of vaccination for 92% of cases and controls who reported receiving the vaccine. Using logistic regression, estimated vaccine efficacy for those 2-18 years of age was 91.9% (95% CI 76.3-97.2%). Among participants 2-4 years of age, estimated vaccine efficacy was 92.6% (95% CI 62.5-98.5%). Analysis limited only to those with written confirmation of vaccination showed the same point estimates of vaccine efficacy. In addition to vaccine efficacy, factors independently associated with disease included maternal educational level of secondary school or less for those 2-18 years of age (OR 4.0, 95% CI 1.3-11.8); living in Ulaan Baatar for at least 2 years reduced risk of disease (OR 0.4, 95% CI 0.1-1.1, p = .08). No significant risk factors for disease were identified among

study participants < 2 years old. The incidence of meningitis in Ulaan Baatar decreased to 74 cases/100,000 during January-April 1995. The most dramatic decline occurred among those of vaccination age, where the incidence decreased 74% from 359 cases/100,000 in 1994 to 92 cases/100,000 in 1995. In contrast, the incidence of disease increase by 10.2% and 31.1%, respectively, among those <2 years and >18 years in Ulaan Baatar. Assuming a similar rate increase would have occurred among those 2-18 years old without immunization, an estimated 555-730 cases were prevented by the vaccination campaign. Outside the capital, 17 of 21 provinces had higher meningitis attack rates in 1995 compared to 1994.

Conclusions. The results indicate that the *N. meningitidis* serogroup A polysaccharide vaccine is highly effective, even among those 2-4 years of age. This finding confirms apparent efficacy reported in earlier clinical trials among children (2,3). The estimated vaccine efficacy of 92% is similar to that previously found among military recruits (4). In response to the increasing incidence of disease in provinces outside the capital in 1995 and the high attack rate among infants, the MOH conducted a countrywide immunization campaign in Fall 1995 for children 2-18 years old and for infants between 6 months and 2 years of age in Ulaan Baatar.

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Pathogenesis

Pathogenesis, Plenary Review

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

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

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

Carrier isolates are often acapsulate but capsulate bacteria are also found in the NP (1). LPS of carrier strains also tends to be structurally different, often asialylated. Other differences may include the expression of distinct porins (class 1 / 2 / 3) which have been implicated in impairment of host cell functions (5). Putative toxic factors (e.g. RTX-like proteins) have been reported in some meningococcal strains (6). These are environmentally regulated and their expression could also increase pathogenic potential of a strain. Meningococcal phase variable opacity proteins (Opc, Opa) and pili are

usually present in isolates from the throat. They are also widely expressed in case isolates (2, 7). Pili and Opa proteins are structurally heterogeneous and which structural features are selected at various sites of colonization or dissemination are not defined.

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

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

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

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

internalized bacteria may also depend on the numbers of bacteria engulfed. To this end, it would be important to know if there is overt multiplication of bacteria in the nasopharynx prior to dissemination.

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

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

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

on the infant rat model of haemophilus bacteraemia and meningitis have shown that bacteremia may arise as a result of survival of a single organism in the blood stream (16).

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

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

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

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

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

Interactions via the outer-membrane protein Opc: molecular mechanisms and identification of host cell receptors. Opc, a basic protein appears to have the capacity to bind to multiple extracellular matrix (ECM) components and serum proteins (30) giving the organism the potential to interact with several different integrins by bridging via their respective ECM or soluble ligands. Indeed, studies using cultured human endothelial cells have shown that interactions of Opc-expressing meningococci with the apical surface of polarized host cells require serum-derived RGD-containing proteins. The integrin $\alpha v\beta 3$ (the vitronectin receptor, VNR) appears to be the major receptor involved in serum dependent apical interactions of meningococci (31).
Opc-mediated invasion of cultured epithelial and endothelial cells can be inhibited by monoclonal antibodies against Opc which appears to be the major requirement on bacteria for this interaction. However, the cloned Opc protein does not confer invasive property to the host *E. coli* even though the protein is surface expressed and is immunologically similar to that of meningococci. This suggests that additional bacterial factors may be required in host cell interactions mediated by Opc. It is also possible that the level of Opc expressed by *E. coli* is not optimum since efficient interactions of meningococci via Opc require the protein to be expressed at a high density on bacterial surface (29). Further factors may be involved in the interactions via the VNR. In analogy with the complement receptor CR3, which has been shown to interact simultaneously with C3bi-coated particles and with microbial glycolipids at distinct sites (32), VNR may require multiple ligand engagement. Indeed the vitronectin receptor also exhibits binding sites for ganglioside GD2 (33). Gangliosides and LPS share structural similarities in that both are amphipathic with strongly anionic hydrophilic groups and some manner of LPS interaction with the vitronectin receptor may be an additional factor required. This is at present a speculation and there is no evidence to support this hypothesis.

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

The role of Opa proteins. In contrast to gonococci that may encode up to 12 distinct Opa proteins, a single meningococcal strain encodes fewer (< 4) Opa proteins. Studies on a serogroup A strain C751, have shown that, of the three Opa proteins expressed by this strain, OpaB and OpaD are effective in epithelial adhesion and invasion whereas OpaA is

ineffective (10). A receptor for a gonococcal Opa protein on some epithelial cells has been described (34). Whether OpaB and OpaD of C751 also engage this receptor is not known at present. However, on certain epithelial cells and PMN, another receptor may be targeted by meningococcal Opa proteins (see below).

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

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

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Pathogenesis

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

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

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

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

Because the studies of Schneider and co-workers (4) and that of Cannon (Abstract, this meeting) have suggested that an intact Galb1-4GlcNAcb1-3Galb1-4Glc LOS structure is important for human infection, we investigated the possibility that the asialoglycoprotein receptor was present on human urethral epithelial cells. We have shown that the asialoglycoprotein receptor present on human hepatocytes recognizes and binds these terminal sugars of the gonococcal LOS molecule. In HepG2 cells, this receptor-ligand interaction is important for invasion. Previously, it was also found that this receptor is upregulated in HepG2 cells upon exposure to gonococcus (3). Our studies using polyclonal antiserum and *in situ* hybridization indicate the presence of ASGP-R in urethral epithelial cells both from infected patient exudates and primary tissue culture . Polyclonal antibodies to the ASGP-R used in confocal microscopy indicated an association between the gonococcus and this receptor. The level of receptors detected with the antibody increased with increasing times of gonococcal exposure from 0 to 4 to 24 hours. *In situ* hybridization using a fluorescent labeled probe specific to the ASGP-R supported this data; i.e., a significant increase in message compared to the uninfected control was observed.

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

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

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Pathogenesis

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

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

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

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

Our model for gonococcal invasion of HecIB cells is as follows: When gonococci contact HecIB cells they induce a new adhesin in response to the presence of the lutropin receptor; gonococcal binding to the lutropin receptor mediated by this new adhesin induces uptake by the host cell. Once the gonococci have entered the HecIB cells, the plasmid encoded genes are induced, allowing intracellular survival and/or escape from the vacuole.

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Pathogenesis

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

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

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

expression pattern for any particular proteoglycan. Hence, the invasion-associated Opa protein appears to use alternative proteoglycan receptors to mediate attachment and uptake. Considering the high conservation of the intracellular domains of syndecans, the interaction of gonococci with different syndecan receptors may still lead to the generation of a common intracellular signal, which might be involved in organizing the cytoskeletal rearrangements associated with cellular invasion.

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

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

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Pathogenesis

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

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

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

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

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

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Pathogenesis

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

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

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

Pathogenesis

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

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

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

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

E. coli expressing an Opa protein on their surface adhere to and invade ME-180 cells in culture (5). This indicates that Opa mediates *E. coli(opa)* invasion. However, there is considerable homology between the C-termini of gonococcal Rmp and *E. coli* OmpA (6).

An alternative interpretation of the *E. coli(opa)* invasion results is that Opa brings *E. coli* in close proximity to the host cell, and that OmpA mediates invasion. With this in mind, we transformed an *E. coli* lacking OmpA (BREΔ51), and its parent strain (MC4100), with plasmid pDS002 containing *opaP* from gonococcal strain F62SF. We performed adhesion and invasion assays with these transformants. *E. coli* OmpA mutant BREΔ51(OpaP) and its parent MC4100(OpaP) both adhere to and invade ME-180 cells to similar degrees -- Adhesion: MC4100(OpaP) 10.6/cell; BREΔ51(OpaP) 9.8/cell. Invasion: MC4100(OpaP) 0.51/cell; BREΔ51(OpaP) 0.34/cell. Thus, OmpA is not involved in *E. coli(opa)* invasion of ME-180 epithelial cells.

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

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

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

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

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

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

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

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

Based on these observations, we speculated that the component responsible for PMN and pEXI interaction might be CGM1a. A stably transfected CGM1a HeLa cell line (HeLa-CGM1a) was used to test this hypothesis, and the HeLa cell line transfected only with the vectors (HeLa-Neo) was employed as a control. These HeLa cell lines were kindly

provided by Dr. Fritz Grunert, Institute for Immunobiology, Albert-Ludwigs University, Freiburg, Germany. We showed there was no adherence of pGEM to both cell lines, but pEXA adhered to both cell lines. pEXI attached to HeLa-CGM1a cells only. OpaA protein-mediated adherence could be blocked by soluble heparin in both cell lines, but the adherence of pEXI to HeLa-CGM1 was not influenced by heparin. Furthermore, the interaction of pEXI to HeLa-CGM1a cells was blocked by anti CGM1 monoclonal antibody. When we studied the phagocytic ability of HeLa-CGM1a, pGEM (Opa$^-$) could not adhere to, or enter the HeLa-CGM1a. In contrast, HeLa-CGM1a bound and strongly engulfed pEXI (Opal$^+$). Almost 30% of HeLa cell associated bacteria were gentamicin resistant. This result was confirmed by electron microscopy, which showed evidence for a classic phagocytic process; bacteria first attached to the cell surface and this was followed by appearance of phagocytic processes and membrane fusion. These results demonstrate that the non-phagocytic HeLa cells when expressing the CGM1a antigens mimic the functions of PMN; they bind and phagocytose Opal$^+$ bacteria.

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Role of the gonococcal cryptic plasmid in epithelial cell invasion

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

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

To investigate this phenomenon, a derivative of the cryptic plasmid, pLES96, was created. Due to the putative overlap of the cryptic plasmid's two divergent promoters and termini, there was no naturally-occurring location in which to ligate a selection cassette which did not interfere with transcription. To rectify this, the termini of both transcripts were replicated via PCR and a copy of the P*tac cat* cassette was ligated between them. This allowed pLES96 to be replicated and transcribed as a wild-type cryptic plasmid yet also furnished a selection marker. Strain F62 was transformed with pLES96 and passaged on chloramphenicol plates to ensure complete replacement of the wild-type cryptic plasmid and this was confirmed by PCR. F62(pLES96) had its new invasion index established and it was found to be indistinguishable from the parent strain's index, indicating that pLES96 functioned in a similar manner to the wild-type cryptic plasmid. Finally, RUN (Rochester University *Neisseria*) 5288, a plasmidless isolate, had its unique invasion index established and was then transformed with

pLES96, creating an isogenic strain with and without the plasmid. The invasion index for RUN 5288(pLES96) was found to be approximately five-fold higher than RUN 5288, confirming a role for the cryptic plasmid in human epithelial cell invasion.

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

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

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Internalization of *Neisseria gonorrhoeae* by Chinese Hamster Ovary cells

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

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Neisseria meningitidis toxicity for cultured human endothelial cells requires soluble CD14

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

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

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

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

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

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

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

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

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

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

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

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

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

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

this region is measured in square microns. This region of interest template is superimposed on the corresponding fluorescein-stained bacterial image. Fluorescent objects are distinguished from background by choosing a grayscale "threshold" on a scale of 0 to 255. Total cell-associated bacterial areas are measured by including any threshold-defined fluorescent objects that are inside or touching the template. In a second measurement, intracellular bacteria are quantitated by including only those bacteria that are inside but not touching the template. Extracellular attached bacteria are represented by the difference between these two measurements. An invasion ratio (IR) for each region of interest is calculated as "area of invasive bacteria/ total area occupied by cell-associated bacteria." One must assume that bacterial attachment is a precursor to any alleged invasion-triggering by Por. EC which express Por alone show little interaction with the epithelium, presumably because Por conveys no attachment advantage (unpublished observation). We compared EC expressing Por only (IR = 0.4265), to the following experimental groups: Opa alone (IR = 0.3271), 3F11 LOS alone (IR = 0.5344), Opa plus Por (IR = 0.616), 3F11 LOS plus Por (IR = 0.5166). The IR for the Opa plus Por group was significantly higher than the Opa alone group. Thus, Por caused a higher proportion of cell-associated bacteria to be intracellular or invasive ($p = 0.0012$). In addition, the area of attached bacteria corrected for the area of epithelium measured was significantly higher for the Opa-producing variants compared to 3F11 variants ($p < 0.002$). 3F11 variants showed a relatively low overall level of attachment or invasion in absolute terms, although the invasion ratio was intermediate. These data show that Opa is an attachment and invasion factor for GC in the FTOC and suggests that Por is an invasion factor when the bacteria first have a means to attach to the epithelium.

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

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

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

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

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

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

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

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Gonococcal entry into Chang conjunctiva epithelial cells

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

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Pathogenesis, Poster 90

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

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

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

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

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Pathogenesis, Poster 91

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

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

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

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

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Pathogenesis, Poster 92

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

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

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

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

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

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Pathogenesis, Poster 93

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

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

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Pathogenesis, Poster 94

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

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

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

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

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Pathogenesis, Poster 95

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

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

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

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

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

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

process of being purified and consequently the exact characteristics and its targeting potential can be defined.

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

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

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

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

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

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

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

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

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

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Pathogenesis, Poster 98

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

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

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

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

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

heterozygous haplotypes were found in the affected subjects indicating that the two molecular defects in the C7 genes will also differ.

Thus the terminal complement deficiencies in Ireland are heterogeneous in the components affected and probably in the genetic defects within the genes. Although there are reports of mild disease in some terminal complement deficient individuals (6) the index case in the C8 deficient family and a C7 deficient member of one of the families had suffered extremely severe disease in which they nearly lost their lives. Thus in complement deficient individuals, as well as sufficient individuals, there is a wide spectrum of disease severity although there may be more mild cases in complement deficient individuals. The differences in disease severity may depend on the *N..meningitidis* strains (7) as much as on differences in host factors in deficient subjects.. Counseling and prophylaxis against meningococcal disease is very important in these patients and they remain at risk even at times when there is a low incidence of disease in the population at large.

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Pathogenesis, Poster 99

The problem of septic shock in patients with meningococcal disease

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

dependent endothelial cell damage might be one of the specific mechanism of MOF development in patients with meningococcal disease. The secondary activation of DIC at the areas of endothelial damage leads to microcircular disturbances and skin lesion. Our data suggested that the high level of endotoxin and complement consumption were the obligatory conditions for MOF development in patients with meningococcal disease. TNF serum levels were elevated in different patients with shock, but not correlated with MOF development and fatal outcome. So anti-TNF strategy would not be promising for the treatment of patients with meningococcal septic shock.

Pathogenesis, Poster 100

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

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

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

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

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

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

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Pathogenesis, Poster 101

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

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

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

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

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

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Pathogenesis, Poster 102

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

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

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

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

To determine if Opa binds PK in vivo, rabbit antiserum was raised against recombinant human PK subtype M2 and used to determine if PK surrounds *N. gonorrhoeae* within host cells. Opa-expressing gonococci were allowed to invade ME-180 human cervical epithelial cells for 4 hours, the cells were fixed and probed with anti-PK antiserum followed by fluoresceinated anti-rabbit antibody, and visualized via confocal fluorescent

microscopy. These in vivo studies revealed that intracellular, but not extracellular, gonococci bind PK.

Gonococci are thought to use only three carbon sources - glucose, pyruvate, and lactate (7). Intracellularly, there is little available glucose, since glucose is present mainly as glucose-6-phosphate. Pyruvate, on the other hand, can be continuously produced intracellularly. It appears that intracellular gonococci bind active PK, and use it to supply themselves with pyruvate. Additionally, this interaction may enable intracellular gonococci to interact with the T3 metabolic pathway.

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

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Pathogenesis, Poster 103

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

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

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

A putative adhesin induced in the Inv^+ GC results in enhanced binding to Hec1B cells, a human endometrial cell line, both in terms of rate and final level of adherence, exceeding Inv^- GC adherence by two-fold. Addition of hCG simultaneously with GC in adherence assays leads to a dose-dependent decrease in the adherence of both Inv^+ and Inv^- GC. Although the magnitude of the effect is different, the relative loss of binding is equivalent for both phenotypes. Competitive binding assays showed that Inv^+ GC interact with Hec1B targets via a unique mechanism that is not blocked by addition of

100x excess of Inv⁻ GC. Addition of hCG to this competitive assay eliminates the enhanced binding of Inv⁺ GC to the Hec1B targets. This implies that the Inv⁺ -specific adherence to Hec1B cells is mediated by the lutropin receptor (common receptor for hCG and luteinizing hormone). Addition of hCG to invasion assays decreases only Inv⁺ GC uptake, with minimal effect on Inv⁻ GC invasion. These data suggest that the access to the lutropin receptor is critical for gonococcal adherence that leads to subsequent uptake of Inv⁺ GC. Overnight exposure of either Hec1B induction monolayers or invasion targets to hCG, conditions known to stimulate down-regulation of the lutropin receptor (5), affects gonococcal invasion in two ways. First is the loss of the ability of treated Hec1B cells to convert GC to the Inv⁺ phenotype. The second is a decrease in the susceptibility of hCG treated Hec1B targets to invasion by previously induced Inv⁺ GC.

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

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Pathogenesis, Poster 104

High erythromycin prescribing during an outbreak of meningococcal disease

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

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Pathogenesis, Poster 105

Respiratory syncytial virus infection and meningococcal disease

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

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Pathogenesis, Poster 106

Gc susceptibility to heparin-like compounds

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

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

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

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

Opa$^+$ Gc typically show enhanced positive charge whose magnitude varies according to which Opa is expressed. Those Opa proteins conferring greatest positivity have the greatest net excesses of cationic amino acids (arginine, lysine) in their surface-exposed domains and have highest (most alkaline) pI's. PorIA Gc are also more positively

charged than Gc with PorIB and PorIA has a higher pI than PorIB. Taken all together, these observations suggest that polysulfated compounds interact electrostatically with arginine/lysine-rich surface-exposed portions of Opa and Por polypeptides. We suspect that polysulfated compounds cause conformational alterations of the outer membrane proteins *in situ*, analogous to those seen in apolipoprotein E after its interaction with heparin (2). Such conformation changes likely induce distort outer membrane geometry such that vesiculation (blebbing) ensues (3). In this way, the expression of both PorIA and certain Opa proteins "destabilizes" the outer membrane of Gc when they are exposed to polysulfated compounds. Understanding the apparent relationships among p36, Opa phenotype, and PAVAS susceptibility awaits elucidation of p36's structure and function.

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Pathogenesis, Poster 107

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

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

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

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

specifically with Opa proteins of meningococci and the N-terminal domain is sufficient for this interaction.

CD66 comprises a large number of related CEA receptors, normally implicated in inter-cellular interactions via homotypic or heterotypic binding which may involve the terminal domain/s (3). These molecules are expressed on numerous cells, including epithelial, endothelial and myeloid cells (2,3). The surface expression of these receptors may be regulated by inflammatory cytokines (6). Targeting of these receptors by meningococcal Opa proteins suggests that factors such as prior viral or other infections, during which inflammatory cytokines are upregulated and which may increase the surface expression of CD66 molecules, may determine host susceptibility to meningococcal infection.

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Pathogenesis, Poster 108

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

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

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

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

molecules are a target for meningococcal Opa proteins on both PMN and certain epithelial cells.

In a dot blot overlay experiment using human mucosal and disease isolates (including 50 strains each of gonococci and meningococci), we examined the interactions of soluble CD66a-Fc. This study provided a striking demonstration of the specific adherence of the N-terminal domain of CD66a to> 95% of Opa-expressing pathogenic *Neisseriae*. All isolates that did not produce Opa did not bind CD66a-Fc. None of the strains adhered to CD34-Fc. No commensal *Neisseriae* (14 strains) or other human commensals or pathogens (including *E. coli*, *Pseudomonas* and *Haemophilus*: a total of 16 strains) adhered significantly to CD66a-Fc. These studies show that CD66a is a target for a conserved epitope present on the majority of Opa proteins of gonococci and meningococci.

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

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Pathogenesis, Poster 109

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

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

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

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

Roles of distinct Opa proteins and Opc of strain C751. In order to examine the effects of distinct Opa proteins compared with Opc, we used a panel of defined acapsulate variants derived from strain C751 which expressed one of three distinct Opa proteins or Opc (5). The highest CL response was induced by bacteria expressing Opa proteins B and D, and a slightly lower response induced by Opa A-expressing variants. Opc, previously shown to be the most effective protein in increasing interactions with monocytes (6) was the least effective protein in inducing CL responses in PMN. Bacteria expressing undetectable levels of opacity proteins failed to interact with PMN. Results from a phagocytic killing assay correlated with the CL assay. There was no reduction in viability of Opa^-, Opc^- bacteria and all capsulate organisms were resistant to phagocytic killing.

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

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

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

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

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

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

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Pathogenesis, Poster 111

Rapid detection of blood contamination in the cerebr pinal fluid of infant rats by Sangur™test strips

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

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

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

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

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

adherence may be sustained by defensins, through interaction with surface structures on the eukaryotic cells other than the Opc receptor, whereas for internalization of meningococci by Chang epithelial cells, this host cell receptor appears to be required.

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

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

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Superoxide dismutases (SODs) are metalloenzymes involved in the dismutation of the highly reactive superoxide free radical (O_2^-) to hydrogen peroxide and molecular oxygen (1). The removal of O_2^- effectively aborts further reactions including the formation of the highly toxic hydroxyl radical, which once generated would readily attack proteins, lipids, carbohydrates and nucleic acids.

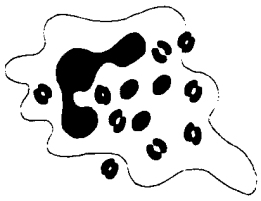
Two groups of SOD have been identified in bacteria; those containing Fe or Mn, which have very similar primary structures and generally protect against endogenously generated O_2^-, and more recently, enzymes with Cu and Zn as their cofactors. [Cu, Zn]-SOD (encoded by *sodC*), once considered a solely eukaryotic protein, has now been identified in a considerable number of micro-organisms, including some important human and animal pathogens. Localization studies have revealed [Cu, Zn]-SOD to be situated in the periplasm (2,3,4,5) and a role in defense against a periplasmically or exogenously located source of O_2^- has been proposed (3,6). This might operate during the respiratory burst of polymorphonuclear leukocytes (PMNLs) - a first line defense against invading micro-organisms.

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

Our results so far do not suggest that bacterial periplasmic [Cu, Zn]-SOD is crucial for protection of meningococci against killing by phagocytic cells. However, a contribution earlier in the interaction between host cell and microbe, perhaps during phagocytosis, has not been ruled out, and this is being examined further.

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Genetics

Genetics, Plenary Review

The Neisserial genome: Importance and where do we stand?

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In 1995, Fleischman et al. (1) demonstrated that the complete nucleotide sequence of a procaryotic genome (*Haemophilus influenzae* Rd) could be assembled from randomly sheared and cloned genomic DNA, without the benefit of prior restriction site mapping and cosmid cloning. This was a landmark event, as it signaled that automated methods for DNA sequence acquisition and analysis have matured into robust, highly efficient technologies that promise to revolutionize the biological sciences. Indeed, the genomic sequence of several other procaryotes (including *Mycoplasma genitalium* (2), *Escherichia coli*, and others) have or will be completed in the near future. These studies will enhance our understanding of procaryotic molecular biological processes, and provide new avenues for gene discovery and comparative genetics. From a practical standpoint, procaryotic genome sequencing will enhance our ability to understand processes occurring during the pathogenesis of infectious disease. It is highly probable that these studies will provide new approaches for drug discovery, a necessity of increasing importance as microbial antibiotic resistance threatens the ability of the biomedical community to treat bacterial infections. Our own procaryotic genome sequencing efforts are currently centered on the obligate human pathogen Neisseria gonorrhoeae, with other projects either planned or beginning shortly. The purpose of this review is to describe the organization of the gonococcal genome sequencing project, placing this project in the context of the current understanding of Neisserial genome organization and population genetics.

Correlated physical and genetic maps of the *Neisseriae*. Studies were first presented in 1991 by Dempsey et al. (3) and Bihlmaier et al. (4) that determined the physical/genetic maps of the genomes of gonococcal strains FA1090 and MS11, respectively. The organization of the 2.2 Mb genomes of FA1090 and MS11 are remarkably similar, with the locations of most mapped genetic markers being the same, within the limits of resolution of the gel electrophoresis techniques available. A further study by Dempsey et al. placed an additional 28 markers on the FA1090 map (5). By analogy with other gram-negative bacteria, Dempsey et al. suggested that the origin of replication of the gonococcal chromosome may be located near *gyr*B, at 12 o'clock on the FA1090 map. Loci affecting the synthesis and assembly of pili (*pil*C, *pil*D, *pil*F, *pil*T and *reg*G) were not found to be adjacent to the gene encoding the major pilin subunit (*pil*E), as in some other bacterial species. Similarly, genes of the iron regulon (including *tbp*AB, *fur*, *frp*B, *fbp*ABC and *lbp*A) did not appear to be clustered, but rather scattered about the gonococcal chromosome. Recently, Dempsey et al. (6) determined the

physical /genetic organization of *N. meningitidis* Z2491, and compared this to strains FA1090 and MS11. A similar study was earlier performed by Bautsch (7) on strain B1940, although no genetic markers were placed on the physical map of that genome. The meningococcal genome size is, within experimental limits, essentially identical to the 2.2 Mb gonococcal genome, and genetic markers were found in the meningococcus to be in the same approximate location. This may not be surprising, considering the high degree of similarity between the two organisms (8, 9). However, in an approximately 500 kb region of DNA of the meningococcal chromosome, Dempsey et al. found considerable differences between the Z2491 genome and that of gonococcal strain FA1090, suggesting complex translocations and/or inversions between the two strains. These differences occurred in a region thought to be near the terminus of replication, as in the inversion-susceptible region of the enteric terminus.

Neisserial population genetics. Population genetic studies indicate that many bacterial populations are clonal (10). For certain pathogens, specific clonal lineages are uniquely capable of producing disease in a specific host, while other clonal lineages appear relatively avirulent. Thus, strains of the *Bordetella bronchiseptica* ET1 lineage appear uniquely capable of causing respiratory tract infections in pigs, but do not routinely infect other mammalian hosts (11, 12). *Bordetella pertussis* is an exclusively human pathogen, despite a genetic relationship with *B. bronchiseptica* that is so close that Musser et al. concluded that these organisms are biovars of a single species. Similarly, *Salmonella* biovars are differentiated into clones with particular disease characteristics, including host specificity (13-17). Thus, one might expect that certain clonal gonococcal lineages would be specialized for causing specific disease syndromes, suchas disseminated gonococcal infection (DGI) or pelvic inflammatory disease (PID). If this were true, selecting a single gonococcal strain for genome sequencing could miss important information. However, the population structure of *N. gonorrhoeae* is panmictic (18, 19). That is, genetic recombination occurs at such a high frequency that the population is essentially randomized; no single determinant or combination of determinants has been specially selected for virulence fitness. For our purposes, this is extremely important: any strain of *N. gonorrhoeae* will be appropriate for genome sequence analysis (provided that other practical criteria are met; see below); analysis of a single strain should identify all important virulence determinants.

Sequencing the gonococcal genome. Strain selection. From the viewpoint of a student of microbial pathogenesis, the *H. influenzae* genome sequence database presents several concerns, despite the singular importance of that sequencing project. As noted by Fleischmann et al. (1): "The nonpathogenic *H. influenzae* strain Rd varies significantly from the pathogenic serotype b strains. Many of the differences between these two strains appear in factors affecting infectivity" (emphasis added). Strain Rd is a derivative of a wild-type *H. influenzae* strain first isolated by Alexander and Leidy (20) in the early 1950s from the throat of a healthy child. Initially, then, the organism was not a disease isolate. Further, the wild-type strain was a serotype d organism, which is only rarely associated with invasive human disease (21). The organism was then passed in vitro to isolate a nonencapsulated variant, yielding strain Rd. Although strain Rd has been extremely useful for in vitro studies (22-24), nonselective in vitro passage may have

resulted in loss of information important for *in vivo* growth and survival (i.e., pathogenesis). Indeed, Fleischmann et al. noted sixty potential frameshift mutations in their sequence, by comparison to entries in peptide databases. Many of these probably resulted from nonselective in vitro passage for almost 50 years. An important difference between the strain Rd sequence and virulent serotype b organisms is the complete deletion of the capsular biosynthesis cluster from strain Rd. Importantly, if this region had not already been sequenced from a pathogenic serotype b strain, Fleischmann et al. would not have known that it was missing from their own sequence! How many other silent, extremely important gaps lurk within the Rd database? This is obviously unknowable; such mutations may diminish this database's usefulness for studies in microbial pathogenesis. (Not to belabor the point, as we recognize the pioneering efforts of Venter and colleagues. It is not so much that we know what is wrong with the *H. influenzae* database. Rather, our concern is that we have no way of knowing what may be missing.) Thus, a primary concern for sequencing the *N. gonorrhoeae* genome is the strain that selected for the sequencing protocol.

With the exception of a few model procaryotes (such as *E. coli*), one of the primary reasons to sequence the genome of a pathogenic procaryote is that these organisms cause human disease; this fact dictates many of our prejudices. Thus, for a strain to be selected for sequencing, we require that at least two conditions be met: 1) The strain must be representative of those organisms isolated from human disease, relying on population genetic and epidemiological data. As noted above, the gonococcus has a panmictic population structure, suggesting that all members of this species have essentially the same disease potential. This greatly simplifies the strain selection problem. 2) The genome of the strain in question must, insofar as it can be determined, be representative of that organism as it existed in the disease state. Thus, the organism must be a low-passage isolate obtained directly from an infection, or at least must retain infectivity in humans and/or relevant animal models. There are currently two gonococcal strains that, to our knowledge, meet this second criterion: strains FA1090 and MS11. Despite long-term in vitro cultivation, each strain still causes disease in human volunteers (25-27). It is important to note two limitations of these in vivo experiments. i) These assays are all performed in male volunteers, as experiments with female subjects is not considered ethical. ii) Experiments in male volunteers are terminated at the onset of inflammation, generally within 48-96 hours. Strictly speaking, we only know that strains FA1090 and MS11 will participate in the early steps of inflammatory disease in males. Later stages of disease, or asymptomatic infection, are not examined in these experiments. These two caveats aside, strains FA1090 and MS11 are well suited for genomic sequencing; we have arbitrarily chosen strain FA1090.

Methods of procedure. The sequencing project is divided into two distinct phases.
1. Primary sequence. Here, the genome is randomly sheared, pUC18 plasmid clones produced, and sequence obtained from the end of each insert using M13 forward and reverse primers and standard dideoxy sequencing technology. In this phase, we will randomly collect sequence, seeking to reach 5-fold coverage of the genome. According to the Smith-Waterman algorithm, assembly of genome sequence representing five-fold coverage should theoretically yields > 99% of the genome (1). Briefly, gonococcal DNA

will be randomly fragmented by shearing in a nebulizer (28), which is more efficient than other fragmentation methods. DNA fragments of 800-1000 bp are gel purified, end-repaired, and re-purified using Sephacryl S-500 and phenol extraction. The DNA fragments are ligated into the SmaI site of pUC18, and transformed into *E. coli* XL1-Blue MR. This host strain contains the *mcr*A, *mcr*CB and *mrr* mutations (29), avoiding problems due to the extreme methylation state of gonococcal DNA (30). White colonies are selected, plasmid purified, and cycle sequencing reactions performed using fluorescent-labeled M13 forward and reverse primers. Template isolation employs an automated procedure using the Beckman Biomek 2000 workstation. The four reaction mixtures for each clone are pooled, ethanol precipitated, suspended in loading buffer, boiled, and loaded on a 6% polyacrylamide gel mounted in an ABI 377 sequencer. At present, assembling the sequencing reactions is done manually, but the Roe laboratory is working to automate this process, to remove this labor-intensive and error-prone manual step. After electrophoresis, the raw information is transferred to a Power Macintosh and reduced to usable sequence data. This analyzed data is then transferred to a Sun SPARCstation 5 where the UNIX script OU-OTTO automatically removes vector sequence and enters the data into one of three sequence assembly databases. (OU-OTTO is a derivative of the original OTTO script obtained from the C. elegans group at Washington University, modified for optimal handling of genomic data by the Roe laboratory). The three sequence assembly databases are generated by: 1) CAP2, from Dr. Huang at the Michigan Technical Institute, 2) FAK2 from Dr. Gene Myers at Arizona, and 3) the Phred/Phrap programs obtained from Dr. Phil Green at the University of Washington. Both the CAP2 and FAK2 programs have been modified by the Roe laboratory to create a final sequence assembly database read by either the Staden XGAP or GAP4 programs. Alternatively, the output from Phred and Phrap is assembled into a database read by the CONSED program (also from Dr. Green's laboratory). In any case, the assembled sequence is manually proofread by reviewing the on-line fluorescent tracings from within XGAP, GAP4 or CONSED, prior to proceeding to the final closure and finishing stages.

2. Sequence closure. Continuing to obtain sequence by random shotgun methods beyond about 5-fold coverage is not cost- or labor-effective, and here we will switch to the second "finishing" or closure phase. Closure strategies will be indicated after manual proofreading of the primary contigs obtained by shotgun sequencing. Problem regions will be identified and sequencing protocols altered (synthesizing new primers close to the problem region, altering polyacrylamide concentration in the resolving gel, or employing dye-terminators). Gaps in the sequence due to "unclonable" regions will be bridged by synthesizing PCR primers that flank gap regions, PCR amplification and direct sequencing of the amplified gap segment. Sequencing the amplicon will be done directly, using this fragment as template, by fragmentation into very short pieces (ca.100 bp), or by walking along the fragment. Using PCR conditions described by Cheng et al. (31), up to 23-42 kb can be amplified for closure purposes. We anticipate that, as found by Fleischmann et al. (1), closure will require the use of a l library. This library will be used for single-pass end sequencing, to order the lambda clones on the genomic sequence, and to resolve gaps in the primary sequence. Finally, the lambda library will be deposited with the ATCC, for availability to the *Neisseria* community. The final

contiguous sequence then must be annotated prior to database submission, aided by the FA1090 physical/genetic map. The assembled contigs will be imported into an ACEDB database for storage of sequence data and results of subseq t analyses. Briefly, a contig will first be analyzed by three programs (Blast X, XGrail and GeneFinder) that together will identify ORFs that have similarities in the databases. Potential ORFs without known homologues in the databases will be examined for typical gonococcal codon preference to determine whether these regions represent potential genes. Putative regulatory elements will be searched for by inspecting DNA sequence for consensus sequence elements (promoters, etc.). A rigorous analysis of the gonococcal genomic sequence will require more than simply determining the nucleotide sequence of the genome, and reporting the results of a computer analysis of this sequence. It is important to interpret the sequence data in the larger context of the pathobiological and clinical significance of the organism. We have therefore assembled the Gonococcal Genome Consultants Group (GGCG), bringing together extensive expertise in clinical medicine, microbial pathogenesis and genetics, epidemiology and public health. The GGCG includes MA Apicella, M.D., JG Cannon, Ph.D, SA Morse, MSPH, Ph.D., RF Rest, Ph.D., and PF Sparling, M.D.

Future studies. As of this writing, we are in the primary sequence phase; we have approximately 2 Mb of raw sequence, tentatively assembled into over 1000 contigs of about 600 bp to over 10 kb. At our current rate, we should be well into the closure phase by the end of calendar year 1996. Once the gonococcal sequence is complete, our plans include the following:

1. Comparisons with other human mucosal pathogens. The genomes of several other obligate human mucosal pathogens have been or are being sequenced, including *H. influenzae*, *M. genitalium*, *Helicobacter pylori*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. The ecological niches that these organisms occupy have significant similarity which will be most certainly reflected in similarities at the genomic level of these organisms. We anticipate that careful comparison of the gonococcal genomic sequence with that of these other organisms will yield useful insight into the similarities and differences between these organisms, and point towards important comparative studies.

2. Whole-genome transcript mapping. Transcriptional control of gene expression is a common mechanism for controlling how bacteria respond to their environment. The FA1090 genomic sequence will provide the information to globally examine transcriptional regulation of the gonococcal genome. This will be done by preparing hybridization filters carrying all of the identified ORFs from the FA1090 genome, similar to the strategy described by Chuang et al (32) for *E. coli*. This collection of filters will then be hybridized with gonococcal cDNAs prepared by reverse transcription (perhaps with associated PCR amplification) of total RNAs isolated from organisms grown in vitro under environmental conditions that are expected to be important for gonococcal pathogenesis in vivo. Similar experiments can be done using cDNAs prepared from organisms recovered from in vitro infection assays, such as the fallopian tube organ culture model. Ultimately, hybridization probes can be prepared from RNAs

obtained from gonococci isolated from experimental or natural infection, to directly examine gonococcal gene expression in vivo. Although some animal models have been proposed for examining gonococcal pathogenesis, none exactly replicates the process of gonococcal infection. Thus, the ability to directly assess gonococcal gene expression in vivo will be extremely important for understanding the intricacies of pathogenesis of this obligate human pathogen.

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Genetics

Chromosome organization in *Neisseria gonorrhoeae* and *N. meningitidis*

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Recent construction of chromosome maps of several strains of *N. gonorrhoeae* and *N. meningitidis* provides a means to assess the effects of frequent recombination and horizontal exchange on genome organization, as well as helping to understand the genetic differences contributing to the type of infection caused by each species. Gonococcal strains FA1090 and MS11 are nearly identical in location of mapped genes (1-3), as are meningococcal strains Z2491 (Group A) and B1940 (Group B) (4, 5). Gene order is similar over much of the chromosome for all 4 strains. However, comparing the maps of FA1090 and Z2491 revealed a region of 400 kb showing complex rearrangements (4). The current maps do not reveal the extent of differences in genome organization between and within the two species. We have characterized these differences, digesting neisserial DNA with restriction enzymes recognizing rare sites and resolving the fragments by CHEF electrophoresis. The most extensive restriction site polymorphisms in 7 gonococcal strains were in the region of the chromosome that is rearranged between strains FA1090 and Z2491, although a detailed comparison of gonococcal strains FA1090 and F62 showed polymorphisms in *Spe*I and *Nhe*I fragments mapping in other regions of the chromosome as well. There were also differences between variants of one strain (*N. gonorrhoeae* MS11mk and MS11ms). Gonococcal strains fell into two groups in terms of location of rRNA genes on the chromosome. For meningococci, rRNA genes were located in the same places for all Group A strains analyzed, whereas Group C strains showed extensive variation. However, Group A strains did show polymorphisms in other restriction sites, even among strains of a single clone (isolates of subgroup IV-1 obtained during an epidemic in the Gambia). Just as there is no single type strain of the gonococcus or the meningococcus, we believe that there is not a single chromosome map that is representative of all strains of the two species.

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Genetics

Characterization of a peptidoglycan transglycosylase from *Neisseria gonorrhoeae*

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Peptidoglycan hydrolysis is involved in three important processes in *N. gonorrhoeae*: autolysis (1), genetic transformation (2), and release of toxic peptidoglycan fragments into the surrounding medium (3). We have identified a gene whose deduced amino acid sequence is homologous to peptidoglycan transglycosylases from bacteriophages. We have constructed mutations in the gene and characterized the mutants with regard to autolysis, transformation, and peptidoglycan turnover.

Our results suggest that the gene encodes an autolysin. Although the mutants are capable of lysis in buffer, they do not suffer the rapid death in stationary phase as is seen in the wild type. The mutants undergo a slow reduction in CFUs in late stationary phase culture, surviving 30 hours longer than the wild type strain.

Since the mutants appear deficient in autolysis, they would be expected to be poor donors in transformation. We are investigating the possibility that DNA release is reduced in the mutants and that transformation in mixed culture is reduced. Other peptidoglycan associated proteins have been shown to be necessary for gonococci to serve as recipients in transformation (2,4). However, experiments have shown that our mutants are as competent as the wild type.

The wild type and mutant strains have identical high rates of peptidoglycan turnover during exponential growth, but upon entry into stationary phase, the mutants' rate of hydrolysis slows. The predicted product of the transglycosylase reaction is the 1,6-anhydro disaccharide tetrapeptide monomer, a molecule identical to the tracheal cytolytic toxin released from *Bordetella pertussis* (5) and the toxic substance released by gonococci which kills cultured fallopian tube cells (6). We are investigating the possibility that the enzyme may be required for the formation or release of the cytotoxin and thus important for the organism's pathogenesis.

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Genetics

Molecular mechanisms of capsule phase variation in group B *Neisseria meningitidis*

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The meningococcal polysaccharide capsules are pivotal virulence factors, which enable the meningococcus to survive during dissemination. However, recent data clearly indicated that the first steps of the infection, i.e. entry and transcytosis of the nasopharyngeal epithelial cells are hindered in the presence of the capsule (1,2). Furthermore, we could demonstrate, that only spontaneous capsule negative variants of an encapsulated meningococcal wildtype strain were able to enter epithelial cells (2). The analysis of these capsule-negative variants of a serogroup B strain (B1940) demonstrated two independent mechanisms of capsule phase variation. In class 1 variants we observed a molecular weight shift from 2 kb to 2.9 kb in one *Eco*RI fragment of the capsule gene locus (*cps*), whereas the class 2 variants exhibited no alterations in the restiction pattern of *cps*. The altered *Eco*RI gene fragment in class 1 variants harbours the *siaA* and *siaB* genes and the 5´ end of the *siaC* gene (3), which encode proteins of the biosynthetic machinery required for α-2,8 linked polysialic acid synthesis. Using PCR and primers which flank each of these genes, we could detect an insertion within the *siaA* gene, which encodes an epimerase catalysing the first step in α-2,8 linked polysialic acid synthesis by converting GlcNAc-6-phosphate to ManNAc, a precursor for NeuNAc synthesis (Frosch, unpublished results). Sequence determination of the enlarged *siaA* gene revealed an insertion at position 587 of the open reading frame, which exhibited characteristics of a transposable genetic element. The size of the insertion sequence element, termed IS*1301*, was 844 bp. The characteristics of this element are described in the accompanying abstract by Hilse *et. al.* Since inactivation of *siaA* causes substrate depriviation for the CMP-NeuNAc synthetase, this defect also resulted in the inability to endogenously sialylate the LOS. Thus, insertion of IS*1301* within *siaA* regulates both, capsule expression and endogenous LOS sialylation.

The biological significance of inactivation of capsular polysaccharide synthesis and of LOS sialylation by IS*1301* depends on its potential to reverse, since expression of the capsular polysaccharide and sialylated LOS are required for survival during dissemination and uncapsulated bacteria have never been observed as disease isolates. To confirm that the capsular polysaccharide can be re-expressed in *siaA*::IS*1301* inactivated meningococci, single colonies were plated and monitored by colony-blotting using mab 735 for capsule expression. This experiment revealed a frequency of 4×10^{-4} for reversion to the encapsulated phenotype.

The defect in the class 2 variants was due to the inactivation of the polysialyltransferase gene, *siaD*. Sequence data obtained by chromosomal sequencing of class 2 capsule negative variants demonstrated a deletion or an insertion of a single cytidine residue within a stretch of seven d(C) residues at position 89 of the *siaD* sequence. The additional insertion of one dC residue resulted in a frame shift and a translational stop behind the $(dC)_7$ box (4). In a subsequent analysis we determined the number of dC residues in several capsule positive phase revertants of capsule negative clones, which spontaneously appear in a frequency of 10^{-3}. In these clones the original $(dC)_7$ box was reconstituted. These observations suggested a translational regulation of *siaD* by a slipped-strand mispairing mechanism.

There is evidence in two respects that capsule phase variation by the latter mechanism is of major biological significance in the pathogenesis of meningococcal disease. (i) *In vitro* invasion experiments using human epithelial cell lines indicated that the vast majority of all intracellular capsule-negative meningococci (85%) are characterized by the frame-shift within the *siaD* gene, whereas the *siaA*::IS*1301* genotype was observed only in 15% of the unencapsulated variants. (ii) We analyzed several meningococcal isolates collected during the outbreak of endemic and epidemic meningococcal disease. In contrast to the analyzed disease isolates meningococci, which were collected from the nasopharynx of carriers or diseased individuals and which were of clonal identity compared to the disease isolates, were unencapsulated due to the *siaD* mutation. These observations suggest that capsule phase variation due to the slipped-strand mispairing within the *siaD* gene is a prerequisite for meningococcal invasion and the outbreak of disease.

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Genetics

Transcriptional control of antibiotic resistance in *Neisseria gonorrhoeae* due to the *mtr* efflux system

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Energy-dependent efflux systems are possessed by a variety of bacteria and these export systems can enhance bacterial resistance to multiple, often structurally diverse antibiotics (1). Efflux pumps have the capacity to capture antibiotics in the periplasm or cytosol and, through a poorly defined mechanism, export these agents through the inner and outer membranes of Gram-negative bacteria or the peptidoglycan-rich cell wall of Gram-positive bacteria (2). Several classes of efflux pumps have been described in recent years but they all contain a cell membrane-associated translocator protein. Some efflux pumps also contain a periplasmic protein that fuses the inner and outer membranes as well as an outer membrane protein that channels the export of antibiotics to the outside environment. The genes encoding efflux pumps can be constitutively expressed or in certain instances, they can be up-regulated by stresses including antibiotics (2).

We recently described (3) the *mtr* (*m*ultiple *t*ransferable *r*esistance) efflux system possessed by *N. gonorrhoeae* and showed that it is remarkably similar to efflux pumps possessed by *E. coli* and *P. aeruginosa* (2). The *mtr* system was first recognized by Maness and Sparling in 1973 (4) in their studies on multiple antibiotic resistance in gonococci that resulted from a single mutation. The gonococcal efflux pump consists of three membrane proteins (MtrC, MtrD and MtrE) and has the ability to export structurally diverse hydrophobic antimicrobial agents (5), including antibacterial fatty acids and bile salts that bathe certain mucosal surfaces. Levels of gonococcal resistance to these hydrophobic compounds correlate with levels of expression of the *mtrCDE* genes (6), which form a single transcriptional unit.

Recently, we found that expression of the *mtrCDE* efflux system in gonococci is regulated at the level of transcription by both *cis*- and *trans*-acting factors associated with the *mtrR* gene, which is positioned upstream and transcriptionally divergent from the *mtrCDE* gene complex. The MtrR protein is a transcriptional repressor (7) that decreases expression of *mtrCDE*. However, expression of *the mtrR* gene requires the presence of a 13 base pair inverted repeat that is between the -10 and -35 regions of its promoter (3). We report herein that the MtrR repressor recognizes the nucleotide sequence within the -10 and -35 region of the promoter utilized by the *mtrCDE* operon. We also determined that mutations that cause single, radical amino acid substitutions within the helix-turn-helix (HTH) motif or in a downstream region of MtrR result in loss of or reduced MtrR-binding. Although the 13 bp inverted repeat sequence lies just upstream of the MtrR-binding site, we found that a single bp deletion in the inverted repeat resulted in altered MtrR-recognition of its binding site. Thus, the 13 bp inverted

repeat can directly modulate transcription of the *mtrRCDE* genes as well as serving a role in MtrR-DNA interactions. We conclude that although tight control of *mtrCDE* gene expression in wild-type strains is present, the action of the controlling elements described herein can be circumvented through mutations that alter repressor activity or promoter utilization. While these studies have a direct impact on how the *mtr* system is regulated, they also help to serve as a model for how expression of gonococcal virulence genes can be regulated at the level of transcription.

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Genetics

Do sexual bacterial have species?

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We have shown that the exchange of alleles of housekeeping genes between strains of *Neisseria gonorrhoeae* is frequent enough to generate a panmictic population structure (1). High levels of chromosomal exchange were also determined for strains of the meningococcus, although the population structure of this pathogen, which we described as 'epidemic', was obscured by the over-representation of certain strains that had recently increased to high frequency. Although high levels of gene exchange can obscure the identification of organisms we have demonstrated that the rate of gene exchange between *Neisseria meningitidis* and *N. gonorrhoeae* is not high enough to blur the species boundary between them (2); in general, sequences of housekeeping genes derived from *N. gonorrhoeae* can be distinguished from *N. meningitidis* because of differences that are apparently fixed in the gonococcal population. We propose that *N. meningitidis* and *N. gonorrhoeae* represent extremely closely related 'sexual' bacterial populations that appear to be genetically isolated in nature and thus conform to the biological species concept (2). We suggest that ecological isolation - of populations that can colonise the genital tract from those that can colonises the nasopharynx - may be an important component of the barrier to gene exchange between these two pathogens. However, the meningococcus is not ecologically separated from other, closely related, commensal *Neisseria* that occupy the naso-pharynx and the exchange of both highly selected genes (i.e. penicillin resistance determinants) and housekeeping genes between commensal *Neisseria* and strains of meningococci has been reported (3, 4). It is not clear, therefore, if strains of *N. meningitidis* form a valid species according to the biological species concept, or even if such a concept can be applied to organisms that are capable of high levels of chromosomal gene exchange and occupy the same niche.

Recently, a numerical taxonomic study of 315 strains of *Neisseria* for 155 phenotypic tests was published by Barrett and Sneath (the Sneath collection, [5]). This study shows that several of the traditional neisserial species are not readily identified by phenotypic traits; strains of *Neisseria elongata,* for example, were distributed in two phenons, one phenon associated with *N. meningitidis* and the other more distantly related. Furthermore, the relationship between some species, such as the placing of strains of *Neisseria cinerea* within the population of *N. meningitidis*, was unusual. To clarify the phylogenetic relationship of the commensal *Neisseria* and to determine if identifiable groups of organisms (species) exist among bacteria that have the potential for the frequent exchange of housekeeping genes we have undertaken a large scale sequencing project of well characterised strains from the Sneath collection. We have determined over 500 bp of the sequence of the *argF* and *recA* genes of a collection of over twenty five commensal strains of *Neisseria*. The strains sequenced include type strains of

Neisseria sicca, *Neisseria subflava*, *Neisseria pharyngis* and *Neisseria perflava*, as well as strains identified as *Neisseria lactamica* (7 strains), *Neisseria polysaccharea* (4 strains), *Neisseria cinerea* (4 strains), *Neisseria elongat-* (4 strains) and *Neisseria mucosa* (3 strains). These sequences complement similar data previously derived for 8 strains of *N. meningitidis*, 9 strains of *N. gonorrhoeae* and single isolates of selected commensal strains (4). The total data set contains sequences from over fifty strains. We have concentrated on strains that are considered to be closely related to *N. meningitidis* and *N. gonorrhoeae*.

The sequences of both *recA* and *argF* produced similar groupings of strains although these groups do not necessarily conform to the traditional neisserial species. This concordance between *recA* and *argF* suggests that recombination at these loci is not common between the groups. However, in some cases the relationships between the groups were not necessarily the same for *recA* and *argF*, suggesting that recombination has, in the past, played a role in the phylogenetic history of these genes. We conclude that it may be possible to identify groups of *Neisseria* that conform to the biological species concept but that the phylogenetic history of these groups may depend on the gene chosen for analysis.

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Genetics

Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The closely related pathogenic *Neisseria* species, *Neisseria meningitidis* and *N. gonorrhoeae*, though very similar at the level of DNA sequence (1, 2) produce markedly different disease. The presently-known virulence factors which distinguish the meningococcus from the gonococcus (capsule, Opc, Frp proteins, and type II pilin) have not been shown to account fully for the differential pathogenesis of meningococcal meningitis, in particular the crossing of the blood-brain barrier. We therefore used a genomic subtraction technique to search for new genes specific to the meningococcus, some of which might code for virulence attributes which could help to elucidate the molecular mechanisms of disease caused by this organism.

We adapted the subtractive technique of representational difference analysis (3) to the isolation of probes for genes present in meningococcus strain Z2491, an epidemic group A strain (4) but absent from gonococcus MS11 (5). The libraries achieved were comprehensive and specific in that they contained sequences corresponding to the presently identified meningococcus-specific genes (capsule, *frp*, rotamase, *opc*) but lacked genes more or less homologous between the two species, for example *ppk*, *pilC1*.

Sequence analysis has shown that a few have some homology with structural genes involved in the virulence of other pathogenic species (haemolysins of *Serratia marcescens* and a ferric-chelate receptor of *Pseudomonas*). However, the large majority have no significant homology to known neisserial or indeed any other sequences, and therefore constitute a bank of previously undiscovered Nm-specific loci. Localization of the Nm-specific genes with respect to the recently-published macro-restriction map of meningococcus Z2491 (6) has revealed that the majority (63%) of the genetic differences between Z2491 and MS11 are clustered in three distinct regions of the chromosome. One of these corresponds to the capsule-related genes, another is restricted to meningococci of serogroup A, whilst one (region 2) of the regions is common to all meningococci tested.

we believe that this technique has wider application as a powerful tool for a rapid and directed analysis of the genetic basis of inter- or intra-specific phenotypic variations between related bacteria. Furthermore among the genes corresponding to the discovered sequences may be some which will help to elucidate the differential pathogenesis of meningococcal meningitis, or provide candidates for new anti-meningococcal vaccines.

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A two-component regulatory system in *Neisseria gonorrhoeae* involved in Opa (P.II) expression.

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In the course of infection pathogenic bacteria encounter numerous different environments within the host. An ability to sense changes in environment, and adapt to them, is of fundamental importance for the bacterium to survive and thrive.

Two-component regulatory systems (2CR's) are recognized as crucial mediators of adaptive responses to environment (1). 2CR's typically consist of an inner-membrane sensor protein which responds to a specific signal to activate a cytoplasmic response protein which mediates response through alteration of gene expression of specific target genes. Sensors, when triggered by signal, autophosphorylate a cytoplasmic residue, usually histidine. The phosphorylated sensor then acts as a kinase, transferring the phosphate to a residue of its cognate response protein (2). The phosphorylated response protein is then able to bind to specific DNA sequences within the promoter region of target genes, causing a change in the level of transcription of these genes that constitutes the response. The phosphorylated response protein may bind to multiple target genes, effecting a global response to a specific signal. (3)

Response regulation in *N. gonorrhoeae* is largely unknown. Many structural genes encoding surface components important for infection have been identified. However, whether these genes are regulated and what possible mechanisms of regulation may exist is unknown. To address this issue, we attempted to identify 2CR's of N. gonorrhoeae as candidates for mediators of N. gonorrhoeae responses to environmental (i.e. human host) signals.

Degenerate oligonucleotide primers, designed to a conserved amino-acid motif in the response proteins of previously characterized 2CR's, were used in a PCR reaction using N. gonorrhoeae strain 1291 as template. A single DNA species of the expected size (322bp) was amplified, cloned and sequenced. The predicted amino-acid sequence of this fragment showed high levels of homology to the corresponding region of other 2CR's. Southern hybridization confirmed that this fragment was amplified from the gonococcal genome. The PCR fragment was used as a probe to identify two contiguous Sau3A fragments of the gonococcal genome that together encode the entire response regulator, and immediately downstream of this, the N-terminal 322 amino-acids of the sensor protein of a 2CR. Flanking regions of this region are probably lethal when cloned at high copy number in *E. coli* as suggested by deletion of the gonococcal DNA from constructs carrying this flanking region.

Comparison of the predicted amino-acid sequence of the putative 2CR to database sequences revealed the highest homology to a 2CR of *S. typhimurium* and *E. coli* (pmrAB and basR/S respectively) involved in LPS phosphorylation.

To investigate the involvement of the gonococcal 2CR in LOS modification, an insertion mutation in the sensor protein encoding gene was constructed. An erythromycin resistance cassette was inserted after the seventh codon of the sensor gene.

The mutant LOS migrated more slowly on SDS-PAGE than wildtype LOS, although this difference was slight. This is perhaps consistent with an alteration in phosphorylation of LOS in which molecular weight differences between mutant and wild-type LOS would be small. Fine detail structural analysis of the mutant LOS is being performed.

Outer membrane protein profiles of the mutant were also analyzed on SDS-PAGE. The mutant does not express a 31Kd protein which is a major component of the wild-type profile. The major outer membrane protein of N. gonorrhoeae in this size range is Opa (P.II). Western blot analysis using MAb 4B12 which recognizes a conserved epitope on all known Opa proteins confirmed that multiple mutants selected after transformation of a 1291 Opa+ express none to markedly reduced amounts of Opa.

The mutant strain is slower to invade confluent monolayers of HepG2 cells when compared to wild-type, as indicated by a ten-fold reduction in cfu's recovered after 30 minutes infection and a four-fold reduction after 4 hours infection. This is consistent with the loss of Opa expression in the mutant. Opa is an important for adhesion of gonococci to host cells and thus loss of Opa expression may be expected to slow the adherence/invasion process (4,5).

In conclusion, we have identified a 2CR in N. gonorrhoeae that is putatively involved in modification of LOS, although the nature of this involvement is at present unknown. The 2CR is also involved in the expression of Opa. Opa expression is subject to phase variation through variation in a repetitive DNA element within the signal sequence of Opa genes (6). Other mechanisms of Opa expression regulation have not been described. 2CR's ultimately mediate effects through alteration of transcription of specific genes. Thus it is likely that the observed effect on Opa expression is at the level of transcription in response to environmental signals.

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A sixteen bp palindrome sequence encompassing the putative ribosomal binding site is conserved in pathogenic *Neisseria rfaC* genes and is involved in the regulation of expression of meningococcal *rfaC*.

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The lipooligosaccharide (LOS) of pathogenic *Neisseria* is an important factor in disease pathogenesis. *Neisseria gonorrhoeae* 1291 and *N. meningitidis* NMB wild type strains express a single LOS that has an Mr of 4.5 kDa and binds monoclonal antibody (MAb) 3F11. We have previously identified the *rfaC* homologues in *N. gonorrhoeae* and *N. meningitidis* that are able to complement the α1,5 LOS heptosyltransferase defect in *Salmonella typhimurium rfaC*630 (SA1377) (1). Sequence analysis of the *rfaC* genes in both *N. gonorrhoeae* and *N. meningitidis* revealed a thirty-two bp A+T-rich region between the putative -35 and -10 elements and the RfaC coding sequences. In addition, a sixteen bp palindrome sequence encompassing the putative ribosomal binding site of *rfaC* was also observed in both *N. gonorrhoeae* and *N. meningitidis*. To investigate whether this palindrome sequence is involved in the regulation of expression of *rfaC*, transcriptional *lacZ* fusions were constructed in *rfaC* and introduced into the chromosome of *N. meningitidis* NMB. The expression of *lacZ* was monitored under a range of environmental conditions to determine the effect, if any on the expression of *rfaC*. These conditions included the growth phase, decreased O2, carbon source, pH and addition of human cell extracts. None of these affected the *lacZ* expression.

To further investigate whether the palindrome sequence is involved in the expression of *rfaC*, site directed mutagenesis was performed to disrupt the palindrome sequence. The length of the palindrome region was unchanged in the mutated sequence. Transcriptional *lacZ* fusions were constructed in *rfaC* with the wild type or the mutated palindrome sequence. These constructs were then introduced into the chromosome of *N. meningitidis*. The expression of *lacZ* was found unchanged in fusions with the intact or mutated palindrome. Consideration was given to the fact that an intact *rfaC* might be necessary for modification of expression through the palindrome. To accomplish this, a single copy of *rfaC* was cloned into a non-essential gene we had previously identified and transformed into the chromosome of *N. meningitidis* containing the reporter fusions with intact or mutated palindromes. Analysis of the new constructs by SDS-PAGE and western blot indicated that the *rfaC* which was incorporated intact into the chromosome was functional and that a full length LOS was produced which bind MAb 3F11. A set of reporter controls was also constructed in which the the non-essential gene was disrupted by a aminoglycoside phosphotransferase gene cassette. SDS-PAGE analysis of these constructs showed the truncated LOS of the original *rfaC* mutant and this LOS did not react with MAb 3F11. The expression of *rfaC* was then analyzed in the presence and absence of wild type *rfaC* in constructs with the palindrome intact and the palindrome altered. Expression of *rfaC* was identical in the constructs containing an intact *rfaC*

with the palindrome intact, and in the two construct without *rfaC* with the palindrome either intact or mutated. The level of *lacZ* expression in the construct with *rfaC* intact but with the palindrome altered was four to seven times higher than that with the wild type palindrome throughout all phases of its growth. Analysis of glucokinase activity indicated that there was no significant change in the activity of this enzyme in any of the constructs. This result suggests that the 16 bp palindrome sequencec between the promoter and the ATG start codon is involved in suppression of expression of *rfaC* and that RfaC may be involved in some way in the regulation of its own expression.

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Identification and characterization of *gly1*, a PilA-regu ed locus in *Neisseria gonorrhoeae*

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PilA and PilB comprise a two-component regulatory system that controls pilin expression in *N. gonorrhoeae.* We have identified a second locus, *gly1*, that is also controlled by this system.

The *gly1* locus was initially isolated from a *N. gonorrhoeae* gene bank as a clone with hemolytic activity when expressed in *E. coli.* DNA sequence analysis of this locus revealed the presence of two open reading frames (ORFs), which are likely co-transcribed. ORF1 encodes a polypeptide of 18.5 kDa that contains a signal sequence. N-terminal sequencing of this peptide expressed in *E. coli* showed that the 21 amino acid signal sequence is cleaved yielding a mature protein of 15.7 kDa. This protein was observed in outer membrane fractions as well as in culture supernatants of overnight cultures of these strains. ORF2 encodes a protein of 31.8 kDa with homology to no known genes. Both ORFs are required for hemolytic activity.

To demonstrate regulation by PilAPilB, pNG4-26 ($pilA^+pilB^+$) was introduced into an *E. coli* strain also containing *gly1*. Strains containing pNG4-26 had a significantly larger zones of hemolysis compared to otherwise isogenic strains lacking *pilA* and *pilB*. Analysis of a P*gly1-lacZ* transcriptional fusion in *E. coli* showed that $pilA^+pilB^+$ expressing strains had 1.5-fold more b-galactosidase activity than strains lacking *pilA* and *pilB*. This demonstrates that PilAPilB control of *gly1* is at the level of transcription.

DNA-binding by PilA *in vitro* was demonstrated by gel retardation analysis using purified PilA. Sequences 5Õ to ORF1 were bound by PilA in this assay, whereas sequences within or 3Õ of either ORF were not. Deletion analysis to localize the PilA-binding site within this fragment indicated that two regions are required for PilA binding to *gly1* DNA. This region spans from -60 to -383 with respect to the start of transcription (as determined by primer extension analysis). That two regions of the DNA are required for PilA binding is similar to our previous observations with PilA and the *pilE* promoter, which we suspect may involve DNA looping.

Deletion of the *gly1* locus in *N. gonorrhoeae* strain MS11 resulted in viable gonococci, indicating that these genes are not essential. These deletion mutants were analyzed for their interactions with various epithelial cell lines in tissue culture. The results of these experiments showed that adhesion to and invasion of three different cell lines was unaffected by deletion of the *gly1* locus. Interestingly, non-piliated *gly1* mutants were

shown to cross polarized T_{84} monolayers more slowly than the otherwise isogenic non-piliated parent. These results could suggest a role for one or both of the *gly1* polypeptides in trafficking of *N. gonorrhoeae* through epithelial cells.

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A phase-variable type III restriction-modification system in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae possesses a number of Type II restriction/ modification systems (R/M) but characteristically expresses fewer restriction enzyme activities than corresponding methyltransferases (1). Methyltransferase specificities have been identified for S.NgoI (PuGCGCPy), S.NgoII (GGCC), S.NgoIII (CCGCGG), S.NgoIV (GCCGGC), S.NgoV (GGNNCC), S.NgoVI (GATC), S.NgoVII (GC[C/G]GC), S.NgoVIII (TCACC) (1, 2 and references therein) and characterization by Piekarowicz et. al. (3) of purified methyltransferase which recognizes the sequence $GTAN_5CTC$ (S.NgoIX). Restriction enzymes have been detected which correspond to the sequences for NgoI, II, III, IV, and IX in various strains.

We have identified the genes encoding a Type III restriction modification system (reviewed in 4) present in all strains of *N. gonorrhoeae* tested. Sequence analysis of the *ngo*X locus from strain MS11 indicate the predicted protein for the restriction enzyme (NgoX.R) is 59% identical to the restriction enzyme of the P1 system. The modification enzyme (NgoX.M) is predicted to be 38% identical to the modification enzyme of the P1 system.

The postulated 5' region of the *ngo*X.M gene is unusual in that the predicted start codon is followed by a series of direct pentameric repeats reminiscent of the signal-peptide encoding region of neisserial *opa* genes, responsible for regulating expression of *opa* genes by "phase variation". The *ngo*X.M gene of strain MS11 contains eight repeat elements (an out-of-frame configuration) while strain FA228 contains twelve repeats elements (an in-frame configuration). Mutations constructed in *Escherichia coli* and returned to the gonococcal chromosome by allelic replacement indicate that the *ngo*X.M gene is expressed in strain FA228 but not in strain MS11 but the gene is phase-variable from both the on and off configurations. Phase variation can not be detected in the viable cell population in wild-type strains. Purified heterodimeric NgoX enzyme is active against DNA purified from strains MS11mk (no expression of NgoX.M) and FA228 *ngo*X.M::*lac*Z but not strain FA228 (expresses NgoX.M).

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Detection of single-strand DNA during transformation of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae is naturally competent for DNA transformation; with several factors being required. Pili are essential for DNA uptake into a DNaseI-resistant state, although the mechanism of uptake is not understood. Following uptake, ComA, ComL, and Tpc are thought to be involved in the transfer of DNA to the cytoplasm (1). During uptake, transforming plasmid DNA (pDNA) is converted to linear, double-stranded (ds) molecules (2). RecA-mediated recombination with either a homologous chromosomal locus or linear plasmid completes the transformation process (3). Although RecA is required for transformation, single-strand (ss) DNaseI-resistant DNA, presumed to be the substrate for RecA, has not been previously detected (1,2). In an effort to detect ss pDNA following uptake by *N. gonorrhoeae,* we have re-examined the nature of DNaseI-resistant DNA during transformation.

N. gonorrhoeae MS11 (P^+) or *N. gonorrhoeae* MS11*dud* (P^-) were suspended in broth medium containing one of two isogenic plasmids isolated from *Escherichia coli* DH10. The isogenic plasmids pRML115 and pRML110 differed with respect to the presence or absence, respectively, of the gonococcal DNA uptake sequence (DUS). Following addition of DNaseI (0.5U/ml) to degrade extracellular pDNA, the cells were washed twice and total nucleic acid isolated. The DNaseI-resistant DNA was then analyzed by native blotting to detect ssDNA and by Southern blotting using a radiolabeled probe specific to the pDNA. In control experiments, DNase-resistant pDNA was not detected when either MS11 wild-type (P^+) was incubated with pRML110 (DUS^-) or when MS11*dud* (P^-) was incubated with pRML115 (DUS^+). In contrast, *N. gonorrhoeae* MS11 (P^+) sequestered pRML115 (DUS^+) in a DNaseI-resistant state and both ds linear and ds circular forms were detected by Southern blotting. In addition, native blotting revealed the presence of ss pDNA that corresponded to the linear form of the plasmid based on electrophoretic migration in agarose. Treatment of DNaseI-resistant DNA with S1 nuclease diminished the amount of ssDNA detected by native blotting compared to untreated DNA. Experiments designed to assess the stability of DNaseI-resistant DNA over time indicated that both the ds and ss forms of the pDNA were stable up to 4 hours after uptake.

These preliminary results suggest that ss pDNA is formed during the transformation of *N. gonorrhoeae*, although much ds pDNA is also present. Similar experiments using pDNA isolated from *N. gonorrhoeae* MS11 are being conducted to determine if the ss pDNA is formed specifically by the activity of restriction endonucleases. The ss pDNA

detected by native blotting may represent recombinogenic molecules involved in the productive transformation of *N. gonorrhoeae*.

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PilA is unlikely to be an activator of *pilE* transcript[illegible] in *Neisseria gonorrhoeae*.

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Upstream of the gonococcal pilin subunit gene (*pilE*), is a potentially functional σ^{54} promoter, with an overlapping σ^{70} promoter. The predicted transcription start points (tsp) for these two promoters are within 3 nucleotides, thus making resolution on the basis of primer extension extremely difficult. However, following site-directed mutagenesis of the promoter sequences, we have clearly shown that in *N. gonorrhoeae*, grown under normal *in vitro* conditions, *pilE* is transcribed from the σ^{70} promoter (1). When a P*pilE::cat* fusion is cloned and expressed in *Escherichia coli*, the host σ^{54} -RNA polymerase holoenzyme (σ^{54} -RNAP) competes with the σ^{70}-RNAP for binding to the respective promoter sequences, resulting in relatively low levels of CAT (2). Site-directed mutagenesis of the σ^{54} promoter, or expression of the P*pilE::cat* in an *rpoN* mutant, results in a 30-fold increase in CAT levels (1).

Early studies on the regulation of the gonococcal *pilE* gene (3) were performed with the aim of identifying genes encoding putative transcriptional regulators, using a P*pilE::cat* transcriptional fusion, cloned into an *E. coli* vector as a reporter. A gonococcal library was screened for clones capable of boosting CAT levels. However, this reporter construct contained both the overlapping promoters, thus favouring detection of indirect activation through the inhibition of RpoN binding, via a variety of possible mechanisms. Consequently, two divergently transcribed genes, designated *pilA* and *pilB*, the products of which were believed to activate and/or repress the transcription of *pilE*, were identified (3). Subsequently, despite the demonstration of significant similarity between PilA and the *E. coli* proteins FtsY and Ffh (4,5,6), components of a system involved in the targeting of nascent secretory proteins to the membrane (7), it was reported that *pilA* and *pilB* encoded a two component regulatory system, with PilA functioning as the activator of the *pilE* σ^{54} promoter (4,5). On the basis that gonococci do not use the σ^{54} promoter for transcription of *pilE* during growth *in vitro*, this conclusion seems unlikely.

Purified PilA protein has been shown to bind to a DNA fragment containing the *pilE* promoter (8). In particular, the region from -125 to -161, with respect to the tsp, was shown to be essential for PilA binding *in vitro*. However, we have shown that transcription of *pilE* in the gonococcus is independent of this region. Therefore the potential role of PilA as an essential regulator of the σ^{70} promoter is also unlikely.

We believe that *pilA* does not in fact encode a transcriptional activator of the gonococcal *pilE* gene, but is the gonococcal *ftsY* homologue and has a role in protein translocation.

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Regulation of DNA repair in *Neisseria gonorrhoeae*

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Recombination is indirectly related to virulence in *Neisseria gonorrhoeae* since this process allows phase and antigenic variation of cell surface components (1). Antigenic variation in pilin involves recombination between variant pilin gene sequences and depends on the presence of a functional *recA* gene (2,3). The *Escherichia coli* RecA protein is a multifunctional enzyme having roles in homologous recombination, DNA repair and control over expression of a set of co-regulated genes termed the SOS regulon (4). Many of the genes that comprise this regulon encode enzymes involved in DNA repair or the restoration of DNA replication. The DNA repair capacities of *N. gonorrhoeae* have not been well characterised, however, it is known that the gonococcus possesses an excision repair system (5). The fact that genes in this system are part of the SOS regulon in *E. coli* prompted this investigation into the regulation of genes involved in DNA repair in *N. gonorrhoeae*. Northern (RNA-DNA) dot blot hybridisation was used to investigate potential DNA damage-mediated induction of the gonococcal *recA*, *uvrA* and *uvrB* genes. In contrast to the situation in *E. coli*, transcription of these genes in *N. gonorrhoeae* was not induced in response to treatment with methyl methanesulphonate (MMS) or UV light. These data indicated that the gonococcus does not possess an SOS-like system that is induced in response to DNA damage. Furthermore, the lack of induction of the gonococcal *recA* gene during the heat shock response suggested that the *recA* gene is not regulated by thermal stress in *N. gonorrhoeae*.

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The gonococcal *rsp* gene appears to have evolved from a two component regulatory system that controls type IV piliation in another species.

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Upstream of the type IV pilin gene *pilE* of *Neisseria gonorrhoeae* is a consensus sigma-54 promoter and an associated upstream activator sequence (UAS) (1). Sigma-54 promoters require the alternative sigma factor, RpoN and an activator protein to function. The activator protein binds to the UAS, which is characteristically located approximately 100 bp upstream of the promoter (2). The type IV pilin gene, *pilA* of *Pseudomonas aeruginosa* is also preceded by a sigma-54 promoter (3). The activator of this gene has been identified as PilR which is part of a two component regulatory system with the sensor protein PilS (4,5). The potential UAS of the *pilE* upstream region was noted, based on its similarity to the sequence required for PilR binding (6), upstream of the *P. aeruginosa pilA* gene. The identified sigma-54 promoter is not involved in *pilE* transcription when gonococci are grown *in vitro* (7). However, it has been shown to be fully functional in *P. aeruginosa*. This transcription is dependent on the presence of the *P. aeruginosa* activator PilR, and the potential UAS of the *pilE* upstream region, implying PilR can bind to gonococcal sequences. On the basis of these results and the conservation of sequences similar to sigma-54 promoters and corresponding upstream sites, it was decided to search for *pilR* like sequences in *N. gonorrhoeae* MS11, with the intention of identifying a regulator of piliation in this bacteria. A fragment that hybridized to a probe derived from the *pilR* gene was cloned and sequenced, and a 1.7 kb open reading frame denoted *rsp* was identified. The derived amino acid sequence of Rsp is similar to regions of both PilS and pillar of *P. aeruginosa*. Homologues of *rsp* appear to be present in *N. meningitidis*, and non-pathogenic *Neisseria*. In each of these, this gene is located downstream of the previously characterised *parC* gene, encoding a topoisomerase subunit (8). Transcriptional analysis indicates *rsp* and *parC* are co-transcribed genes. Expression of *rsp* impairs the growth of piliated *P. aeruginosa* strains. Rsp expression does not result in activation, but rather partial repression of transcription from the gonococcal *pilE* and the *P. aeruginosa pilA* promoters.

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Identification and characterization of PilP, a lipoprotein essential for type IV pilus biogenesis in *Neisseria gonorrhoeae*

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Expression of type IV pili appears to play a critical role in the colonization of the human host by *Neisseria gonorrhoeae* (Gc) (1) and contributes to a number of distinct phenotypes, including competence for natural transformation (2), autoagglutination (3), and adherence to epithelial tissue (4). Previous studies have shown that PilQ, a member of the GspD/PulD/pIV protein family, forms a multimeric complex in the outer membrane, and is essential for pilus biogenesis (7). The function of the PilQ multimer is as yet undetermined, however it is speculated to form a channel through which pili are extruded, or perhaps act as a platform for extracellular assembly of pili.

DNA sequencing of the region upstream of *pilQ* revealed the presence of four open reading frames (ORFs) which display significant sequence homology and similar organization to the pilM-P gene cluster of *Pseudomonas aeruginosa* (8) and *P. syringae* (9). Furthermore, an ORF upstream of *pilM* was identified which showed homology to the penicillin binding protein, PonA. This finding was also consistent with the organization of Pseudomonas spp.

Gonococcal mutants bearing transposon insertions in *pilO* and *pilP* were non-piliated and failed to express pilus-associated phenotypes. As predicted by the presence of a consensus lipoprotein signal sequence, [3H]-palmitic acid labeled PilP was detected in both *E. coli* and Gc. The piliation defects in the mutants could not be ascribed to polarity on distal *pilQ* expression as shown by direct measurement of PilQ antigen in those backgrounds and the use of a novel technique to create tandem duplications in the Gc genome. Both the PilP- and PilQ- mutants shed PilC, a protein which facilitates pilus assembly and is implicated in epithelial cell adherence (10). Combined with the finding that levels of multimeric PilQ were greatly reduced in PilP- mutants, the results suggest that PilP is required for PilQ function and that PilQ and PilC may interact during the terminal stages of pilus biogenesis.

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Expression of capsular polysaccharide in *Neisseria meningitidis*: Comparison of biosynthetic and transport genetic loci responsible for serogroup A, B, C, Y and W-135 capsules

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Meningococci of serogroups A, B, C, Y, and W-135 are leading worldwide causes of meningitis and septicemia in otherwise healthy individuals. Dramatic increases in serogroup B and C meningococcal disease have occurred in parts of the United States between 1991-1995 (1,2), emphasizing the need for improved prevention strategies. One of the most important virulence determinants elaborated by the meningococcus is extracellular capsule. Capsules of serogroups B, C, Y and W-135 *N. meningitidis* are composed of polysialic acid or sialic acid linked to glucose or galactose, while serogroup A capsule contains N-acetyl-D-mannosamine. We have studied the biosynthetic and membrane transport gene clusters of Regions A and C of the meningococcal *cps* capsule gene complex (3). Region A encodes four biosynthetic genes (*synX,B,C,D*) and Region C encodes four genes (*ctrA,B,C,D*) necessary for membrane transport of capsule. Transcription of *synX-D* and *ctrA-D* appears to be divergently initiated from promoters present within a 134 bp intergenic region that separates Regions A and C. PCR and Southern DNA hybridization studies demonstrated DNA homology between the sialic acid capsule producing serogroups B, C, Y, and W-135 in *ctrA*, in the intergenic region and in *synX, B,* and *C*. However, nucleotide sequencing outward from the 3¢-end of *synC* demonstrated that the sequence downstream of *synC* was unique between sialic acid producing serogroups B, C and Y/W-135. Sequence diverged at the last codon of *synC* or directly following the *synC* ORF. In contrast, serogroup A *N. meningitidis* did not show homology with *synX-D* or the intergenic region, but did contain a *ctrA* homologue as defined by PCR and Southern analysis. These results show the close genetic relationship of biosynthesis and transport genes of the sialic acid capsule-producing meningococcal serogroups B, C, Y, and W-135, which appear to differ only at the capsular sialyltransferase. However, the capsule biosynthesis region of serogroup A meningococcal strains is clearly different.

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Integration host factor is required for efficient transcription of *pilE* in *Neisseria gonorrhoeae*

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Integration host factor (IHF) is a heterodimeric DNA binding protein found in gram-negative bacteria. IHF binds DNA in a sequence specific manner that usually results in bending of the target DNA molecule. IHF is pleiotropic with respect to function. Biological phenomena influenced by IHF include transcriptional regulation, plasmid partitioning, DNA replication, and genetic recombination (reviewed 1).

We purified IHF to homogeneity from *N. gonorrhoeae* using Fast Performance Liquid Chromatography (FPLC). Gel retardation assays demonstrated specific binding of gonococcal IHF to the *pilE* promoter region. The IHF binding site was defined by DNaseI footprint analysis and mapped proximal to the three previously identified *pilE* promoters; deletion of the putative IHF binding site negated retardation of *pilE* promoter DNA fragments. Binding of IHF to *pilE* promoter DNA was confirmed by Kleinschmidt Electron Microscopy, where binding of the protein induced bending of the promoter DNA fragment.

Isogenic *N. gonorrhoeae* strains were constructed that contained either a wild type *pilE* locus or a deleted *pilE* locus where the promoter IHF binding site was removed. Primer extension analysis of *pilE* and Northern blotting of total gonococcal RNA indicated that in the absence of the IHF binding site transcription was reduced approximately tenfold. A recombinant assay was developed whereby the *N. gonorrhoeae ihf* genes were expressed in *E. coli* in conjunction with *pilE* on a compatible plasmid. Again, efficient transcription required the presence of gonococcal IHF. However, a surprising finding was a translational defect in pilin expression in the absence of IHF.

Overall, these data indicate that efficient transcription of *pilE* requires binding of IHF to a site within the *pilE* promoter region. The mode of action of IHF appears to be the induction of a static bend in the promoter DNA that possibly allows the correct alignment of a transcriptional regulator with its cognate promoter.

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IS*1301*, a novel IS element of *Neisseria meningitidis*: Si' pecific insertion and variable distribution in genetically related strains

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Reversible insertional inactivation of the *siaA* gene by transposition of IS*1301* leads to phase variation of capsular polysaccharide in serogroup B *Neisseria meningitidis* (1). We determined the target site specificity of this element by cloning and sequencing the insertion sites of 12 identical IS*1301* copies found in *N. meningitidis* strain B1940 (2). A target consensus core of 5'-AYTAG-3' was identified, but the presence of additional features around the target sites may indicate that other factors like DNA secondary structure are involved in target recognition; these features include extended palindromic symmetry, stem-loop formation, and the high incidence of A/T-tracts. The left inverted repeat of an IS*1016*-like element common in *N. meningitidis* acts as a hot-spot for insertion and one IS combination was located upstream of the *frpC* gene. By sequence analysis we were able to place IS*1301* into the IS*5* subgroup within the IS*4* family of elements.

IS*1301* did not occur in *N. gonorrhoeae* or in apathogenic Neisseriae. 28.4% of 341 *N. meningitidis* strains from different global sources contained IS*1301*. It was found in serogroups A, B, C, E, W135, X, Y, and among nongroupable isolates. MEE (multilocus enzyme electrophoresis) data were available for 223 of the meningococcal strains. The collection included serogroup A strains from 5 subgroups and serogroup B and C isolates from clusters A4, the ET-5 complex, and the ET-37 complex, as well as other bacteria representative of the genetic diversity of these serogroups (3, 4).

Among serogroup A strains, only subgroup VI strains isolated in the German Democratic Republic and two new ETs contained IS*1301*. In cluster A4, all IS*1301*-containing strains were serogroup B. 27% of strains of the ET-37 complex, 14% of the A4 cluster and 7% of strains of the ET-5 complex possessed IS*1301*. IS*1301* was found in 22 of 32 strains of cluster A3 (69%), which contains many serogroup Y and X strains, and other bacteria from the whole spectrum of genetic diversity also contained IS*1301*. These results indicate, that IS*1301* was probably imported by horizontal genetic exchange after speciation of *N. meningitidis* and has been spreading by horizontal genetic exchange.

Southern blot hybridizations with 23 meningococcal strains revealed an average of 10 (range 2-18) copies of IS*1301* per strain. The band patterns differed among strains belonging to identical or related ETs indicating that transposition events are more frequent than changes in housekeeping enzymes. Four isolates from one healthy carrier

of the same ET differed in both capsular polysaccharide and presence of IS*1301*. Three consecutive isolates were nongroupable and did not carry IS*1301*, while the fourth isolate carried the IS element and expressed a serogroup W capsular polysaccharide. However, in a few cases, closely related strains presented a similar restriction pattern. These results suggest that testing for presence of IS*1301* and its restriction pattern might be useful for fine epidemiology of related carrier strains that are indistinguishable by other criteria.

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Promoter strength influences phase variation of Neisserial *opa* genes

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The *opa* multigene family of *Neisseria gonorrhoeae* MS11mk encodes eleven related outer-membrane proteins which phase vary *in vitro* and *in vivo*. Illegitimate recombination within direct pentameric DNA repeats, encoding the signal-peptide region of pre-Opas, leads to switches in expression states (1). Despite the conserved nature of the variation mechanism, expression of specific genes is favored in switching from an Opa- to an Opa$^+$ population in vitro and in vivo (2). The genes which are highly expressed differ from the rest of the family with respect to promoter structure, based on sequence comparisons between the *opa* genes of strain MS11mk (3).

We have analyzed transcription of the *opa* gene family of *N. gonorrhoeae* MS11mk, focussing on the different promoters found among the eleven genes to determine whether increased levels of expression are associated with increased phase variation rates. Primer extension and Northern blotting were used to assess the levels of transcription of three representative *opa* genes (*opa*A, B and C) in "on" and "off" states. Full length *opa* mRNA was detected primarily in strains expressing the homologous gene. Truncated *opa* mRNA was constitutively expressed from all *opa* genes regardless of their expression state. Quantitative comparisons of *opa* mRNA in *N. gonorrhoeae* were complicated by the simultaneous expression of all eleven genes and the cross-reactivity of mRNA probes.

Expression levels from the individual promoters were therefore assessed by creating transcriptional and translational *lacZ* fusions to each of the representative *opa* promoters, lacking the DNA repeats responsible for variation. The expression levels were compared to the phase variation rates of translational *opa::pho*A fusions containing the same promoters in addition to the corresponding coding repeat regions. A strong correlation was found between expression levels from the different promoters and the variation rates at which "on" variants appeared from an "off" population (i.e. *opa*A > *opa*B > *opa*C). These results provide an explanation for the favored expression of specific Opa proteins and indicate that expression of *opa* genes may be regulated at the level of transcription.

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Analysis of the recombination producing pilin antigenic variation using insertions in silent and expressed pilin loci

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Gonococcal pilus phase and antigenic variation support immune evasion (1,2) and alter receptor binding specificities (3,4). Antigenic and some phase variants are generated when variant DNA from one of several silent pilin genes replaces sequences in an expressed pilin gene (5,6). These RecA-dependent recombination events (7) are usually non-reciprocal. The original purpose of the study was to devise an assay to quantitate antigenic variation. To this end, we constructed a Gc strain derived from MS11 containing a promoterless *cat* gene (*'cat*) in the conserved *cys2* region of silent pilin copy3 of the *pilS*1 locus. Presence of an inducible *recA* allele, *recA6* (Seifert, in preparation) in the chloramphenicol sensitive (Cm^S) assay strain, BHA*cat1*, allowed control of when antigenic variation could and could not occur. The plan was to measure recombination of *'cat* into the expressed pilin gene (*pilE*), by selecting chloramphenicol resistant (Cm^r) recombinants after RecA induction.

Surprisingly, the simple recombination of *'cat* into *pilE* was not observed among Cm^r variants, thus BHAcat1 could not be used to quantitate the rate of antigenic variation. However, study of the Cm^r variants provided great insight into the mechanism of antigenic variation. Each Cm^r variant contained a new hybrid *pilE/pilS1::cat* locus while both starting loci, *pilS1::cat* and *pilE*, usually remained intact. Among the Cm^r variants, three classes of hybrid loci were defined based on the size and composition of each hybrid locus. Class I hybrid loci were created by recombination between *pilE* and *pilS1* copy3::*cat*. Class II hybrid loci were created by recombination between *pilE* and *pilS1* copy4, and class III hybrid loci by recombination between *pilE* and *pilS1* copy5. The presence of *'cat* in the *cys2* of copy3 during recombination between *pilE* and copy3::*cat* did not appear to affect the frequency or location of recombination producing class I hybrid loci compared to the other two classes. Further analysis of the hybrid loci revealed that they could be created directly through transformation or through intracellular recombination. Each class of hybrid locus was mapped and found to occur in the 10 kb region between *pilS1::cat* and *pilE*. This 10 kb region was duplicated during the formation of each hybrid locus such that each hybrid locus was flanked by 10 kb of directly repeated gonococcal DNA.

It was possible that the inability to detect recombination of *'cat* into *pilE* was due to the interruption of the *cys2* sequence since this sequence, conserved at the nucleotide level, may play a role in pilin copy recombination. To test whether an intact *cys2* sequence was required for recombination of *'cat* into *pilE*, strain BHA*cat2* was constructed by inserting *'cat* into *pilS1* copy3 at the hypervariable region (HV) which lies immediately

upstream of *cys2*. Again, simple recombination of *'cat* into *pilE* was not detected. Instead, class II and class III hybrid loci were detected in Cm^r variants, but class I hybrid loci were never observed. This data suggested that at least a portion of *pilS cys2* must be available for interaction with *pilE cys2* in order for recombination between the two copies to occur. These two *'cat* studies demonstrated that the recombination producing hybrid loci was dependent upon pilin sequences and occurred regardless of whether *'cat* was present in, or absent from, the silent copy undergoing recombination with *pilE*. The data strongly suggest that the hybrid loci represented intermediates in the pathway that leads to antigenic variation. The first step, duplication and recombination between *pilE* and *pilS1*, produced a hybrid *pilE/pilS* copy which typically would resolve at an intact *pilE*. Failure of the hybrid intermediate to resolve at *pilE* allowed a low frequency integration of the hybrid intermediate, detected by selection. The duplication of the 10 kb region between *pilS1*::*cat* and *pilE* suggested that a circular intermediate was created during the formation of each *pilE/pilS1* hybrid locus.

Studies using the small 10 bp *Not*I linker in place of *'cat* revealed that both the size of heterologous sequence and its position in a *pilS* copy affect recombination of the heterologous sequence into *pilE*. Preliminary studies of *'cat* and *Not*I linker insertions in *pilE* showed that recombination of *pilS* sequence to replace a heterologous sequence in *pilE* behaves differently than recombination of heterologous sequence from *pilS* into *pilE*. Further studies using insertion in *pilS* and *pilE* will help to define the mechanisms of antigenic variation, the sequences required for antigenic variation, and the limitations imposed during recombination of heterologous sequences.

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Regulation of the prepilin-peptidase *pilD* gene of *Neisseria gonorrhoeae* and *meningitidis*

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The *pilD* gene of *Neisseria gonorrhoeae* encodes a type IV prepilin-peptidase (1, 2). This gene and the surrounding genes show strong homology with a family of genes implicated in pilus biogenesis in*Pseudomonas aeruginosa* and *Klebsiella oxitoca (3)*. We analyse the regulation of this locus and investigate the hypothesis of a role of *pilA/pilB*, a pleiotropic regulatory system first characterized in the pilin gene regulation (4, 5). We show that a partially purified extract of PilA is able to retardate the migration of a DNA fragment overlapping the pilD promoter region of *Neisseria gonorrhoeae*. The size of the major transcript of the *pilD* gene corresponds to potential promoter with a consensus -12- 24 sequence precedently proposed (1)
We have constructed a *pilD-lacZ* fusion which was introduced in wild type, *pilA*, and *pilB* mutants strains of *Neisseria meningitidis*. The results obtained indicated *that pilA and pilB* are probably implicated in the regulation of this locus.

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Genetics, Poster 129

Characterization of an IPTG-inducible *pilE* strain: relationships between *pilE* transcription, piliation and DNA transformation in *Neisseria gonorrhoeae*

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The gonococcal pilus, a member of the type IV family of pili, is important in both adhesion to eukaryotic cells and DNA transformation. Pili have been suggested to play an important role in the initiation of disease by providing the initial attachment of the bacterial cell to human mucosal tissue (1). The piliation state also correlates with efficiency of DNA transformation; piliated gonococci (Gc) transform at a rate several logs higher than non-piliated Gc (2). In addition to *pilE*, other genes involved with pilus assembly, namely *pilC* (3) and *pilT* (4), are required for full transformation competence. Biogenesis of type IV pili is thought to involve transport of pilin protein to the inner or outer membrane prior to assembly of pili (5). The relationship between assembly of gonococcal pili, piliation state, and DNA transformation efficiency is the focus of the present study.

We have constructed a derivative of MS11-C9 (Δ*pilE1*) in which the *lac IOP* regulatory sequences control *pilE* transcription. In this strain, levels of pilin mRNA and protein correlated directly with levels of IPTG in the growth medium, although wild-type levels of expression were never attained. Transmission electron micrographs demonstrated that the number of full-length pili per bacterial cell directly correlated with the induction level, and that at low levels of induction, single long pili were observed. This result suggests that a threshold level of pilin accumulates in localized pools prior to assembly of pilin monomer into pili. The transformation studies showed that transformation efficiency also directly correlated with the level of *pilE* transcription. However, the transformation efficiency plateaued at an intermediate level of pilin protein expression and never reached the wild-type level. These data support the hypothesis that assembly of pilin into pili is critical for efficient DNA transformation competence.

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Genetics, Poster 130

Molecular characterization of the pyruvate dehydroger gene cluster of *Neisseria meningitidis*.

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The pyruvate dehydrogenase (PDH) complex is a multisubunit enzyme which catalyzes the oxidative decarboxylation of pyruvate, yielding acetyl-CoA and CO_2 as end products with the simultaneous reduction of NAD^+ (1). In *Escherichia coli* PDH is formed by the product of three clustered genes, *aceE* (pyruvate dehydrogenase), *aceF* (dihydrolipoyl transacetylase) and *lpd* (lipoamide dehydrogenase), whose sequence and transcriptional organization have been well studied (1).

Our group has previously characterized the neisserial *lpdA* gene, coding for a lipoamide dehydrogenase which has an N-terminal lipoyl-binding domain similar to those present in dihydrolipoyl transacetylases (2,3,4). During the screening and sequence analysis of a λgt11 meningococcal genomic library with a rabbit antiserum to an unrelated antigen of *N. meningitidis*, we identified a clone bearing 2 truncated open reading frames (ORF) equivalent by sequence homology to the C-terminal third and most of the bacterial *aceE* and *aceF* genes, respectively. Using a combination of restriction analysis, Southern blotting and sequencing of the λEMBL3 clone from which the *lpdA* gene was cloned, these ORF were shown to be linked to the *lpdA* gene in the order *aceE-aceF-lpdA*, forming a putative PDH gene cluster. In addition, a fourth ORF was located in the intergenic *aceF-lpdA* region, with no homologous counterparts in the available sequence databases. This ORF is most probably not translated, since it lacks a ribosome binding site and does not match the meningococcal codon usage pattern.

To further characterize this complex, meningococcal *aceE* and *aceF* insertional mutants were prepared and analyzed by Northern blotting concurrently with an *lpdA* deletion mutant, with the goal of dissecting its transcriptional organization. Our data suggest that the transcription of the whole gene cluster is driven from the same promoter upstream *aceE*, terminating either after *aceF* or *lpdA* and thus generating 2 mRNA species 5 and 7 kb long. This sharply contrasts with the situation in *E. coli*, where one promoter coordinately transcribes the 3 genes into one mRNA molecule and *lpd* is further transcribed from a separate promoter (5).

Such differences in the differential regulation of *aceE-aceF* vs. *lpd* probably reflect divergent biochemical requirements for Lpd in these two organisms, and may help shed light on the biological function of LpdA.

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Isolation of mutants deficient in pilin antigenic variat

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The gonococcal (Gc) pilus is subject to antigenic variation by unidirectional, *recA* dependent homologous recombination from silent to expressed pilin loci (1). Located at the 3' end of all Gc pilin loci is the *Sma/Cla* repeat, deletion of which results in decreased antigenic variation (2). Several distinct *Sma/Cla* binding activities have been observed (3). We hypothesize that antigenic variation depends on gene products involved in general recombination processes, such as RecA, as well as those involved in specific recombination processes, such as *Sma/Cla* DNA binding proteins.

We have adapted Shuttle Mutagenesis (4) to insert mini-transposons throughout the Gc genome in a random fashion. A size-restricted plasmid library of strain FA1090 was mutated with the erythromycin resistant mini-transposon mTnEGNS. Pools of randomly mutated plasmid DNA were used to transform FA1090 *recA*6. This strain contains *recA* under the control of an IPTG-inducible promoter, thus controlling the process of antigenic variation in response to IPTG. The resulting erythromycin resistant mutants were screened for the ability to undergo antigenic variation using a colony-based PCR assay. Mutants found to be antigenic variation deficient (AVD) by this PCR analysis have been classified on the ability to transform exogenous DNA and survive UV exposure. We are characterizing the roles of the mutated genes in general recombination, DNA repair, and pilin antigenic variation.

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Genetic analysis of the *ton*B, *exb*B, and *exb*D operon in *Neisseria gonorrhoeae*

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The acquisition of iron from transferrin (TF) and lactoferrin (LF) by *Neisseria gonorrhoeae* and *Neisseria meningitidis* requires the interaction of these glycoproteins with specific outer membrane (OM) receptors. The TF receptor of *N. gonorrhoeae* and *N. meningitidis* is comprised of two proteins, Tbp1 and Tbp2 (1). Sequence analysis of Tbp1 revealed homology with several TonB-dependent OM receptors including FepA, FecA, IutA, FhuA, and FhuE (1); a similar homology has been observed for the neisserial LF-binding protein LbpA (2). These results suggested that a TonB analog must exist in pathogenic *Neisseria* species. The acquisition of TF-bound iron was shown to be an energy-dependent process (3). The energy-coupled transport of iron complexes across the OM of many gram negative bacteria following their interaction with specific OM receptors has been shown to require a complex of proteins consisting of TonB, ExbB, and ExbD.

In order to identify the neisserial TonB, an *Escherichia coli* heme-requiring mutant expressing the meningococcal HmbR protein was screened with a *N. meningitidis* cosmid library (4). Several colonies were isolated that were able to utilize hemoglobin as both a porphyrin and iron source. Nucleotide sequence of one of the clones revealed homology with *exb*D. Oligonucleotides were synthesized and used to amplify the nearly identical gene from *N. gonorrhoeae* strain F62. Sequence analysis of the region upstream of *exb*D revealed the presence of sequences with homology to *exb*B and *ton*B. The putative gonococcal *exb*D sequence was found to encode for a protein of 145 amino acids; the DNA sequence exhibited 98% homology with that of *N. meningitidis* and 57% homology with the *E. coli* homologue. There is a 6bp intervening sequence between the start of *exb*D and the end of *exb*B. The putative gonococcal *exb*B sequence was found to encode for a protein of 221 amino acids; the DNA sequence exhibited 98% and 55% homology with the meningococcal and *E. coli* homologues, respectively. The putative gonococcal and meningococcal *ton*B sequences terminated 65bp upstream of *exb*B and exhibited considerable divergence from other reported *ton*B genes as well as from each other. The two proline-rich regions (Lys-Pro and Glu-Pro) commonly observed in other TonB proteins are reversed in the neisserial homologue. The organization of the gonococcal and meningococcal genes is unique in that they are arranged in an operon in the order of

*ton*B, *exb*B, and *exb*D. In many other bacteria, *ton*B exists as a single transcriptional unit located at a distance from *exb*B and *exb*D. Or, as in *Pseudomonas putida* (5) and *Haemophilus influenzae* (6), *ton*B follows *exb*B and *exb*D.

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Primary structure of the *rpoB* gene of *Neisseria meningitidis*, coding for the beta subunit of RNA polymerase, which is organized within an operon and description of a new attenuator-like sequence.

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The *rpoB* gene codes for the second largest (beta) subunit of DNA directed RNA polymerase (EC 2.7.7.6). Rifampin (syn. rifampicin), an antibiotic drug, is known to act on the beta-subunit, resulting in the inhibition of elongation (1). Recently the mechanism conferring resistance against this drug could be demonstrated for *Neisseria meningitidis* (2, 3). A single point mutation in a specific region of the gene is responsible. As meningococci represent a bacterial species which is known to be of panmictic population structure, another mechanism for acquiring resistance against rifampin could be the horizontal transfer of at least parts of the *rpoB* gene, either inter- or intraspecific. To recognize those events the knowledge of the primary structure of the gene is of interest.

Here the nucleotide sequence and the deduced amino acid sequence for the *rpoB* gene from a rifampin sensitive reference strain (BNCV-strain) is presented together with information on the organization of the gene in an operon.

From a gene bank (kindly provided by E.C. Gotschlich, New York), constructed using DNA from the so called BNCV strain, four overlapping clones had been captured. A 624 bp PCR generated probe which was characterized extensively (4) was used to screen the library. Analysis of the four clones yielded sequence data about a ribosomal protein gene, *rpoB* and *rpoC*, which codes for the largest subunit of RNA polymerase. The nucleotide sequence of *rpoB* from the BNCV strain is 4170 bp in length (EMBL/GenBank/DDBJ Ac.No. Z54353), being with its 1389 codons somewhat longer than homologous genes from other bacterial species. Amplification of the *rpoB* gene from a set of meningococcal strains and from four further *Neisseria* species indicated that the length of the subunit is conserved throughout the genus. Analysis of the base composition revealed a G/C content of about 49.66 mol%. The percent identity (amino acids) to the homologous gene from *Escherichia coli* (SwissProt Ac.No. P00575) is about 62.37 %. The deduced amino acid sequence was used to construct a phylogenetic tree together with *rpoB* sequences from a set of gram negative and gram positive bacteria as well as common tobacco as outgroup. Clustering of the neisserial gene is within the gram negative bacteria, as expected.

Like in some species of *Enterobacteriaceae* (5) the *rpoB* gene from *N. meningitidis* seems to be located within an operon, consisting of four ribosomal protein genes, *rpoB* and *rpoC*. In *N. meningitidis*, this organization is strengthened by the lack of a promoter sequence or a ribosomal binding site for both the *rpoB* and *rpoC* gene. Further evidence

for an operon structure but with differential expression of *rpoB* and *rpoC* in relation to the ribosomal genes gives a hypothetical attenuator like sequence.

Characterized by a large inverted repeat this hypothetical attenuator is able to build a stemloop. It is of completely different sequence than the attenuator described for *E. coli* (6). In *N. meningitidis* the following sequence is found (the inverted repeats are underlined):

TGTTTACATTTATTTGCTTAGTTTTTATCAAATCATTGCAAATAAATGTAAAAC
A.

This hypothetical attenuator is located in the intergenic region between the *rplL* gene, coding for one of the ribosomal proteins and *rpoB*, thus dividing the operon in two functional subunits. It could lead to a more quantitative expression of the four ribosomal proteins and to a lower expression of both polymerase subunits, *rpoB* and *rpoC*. A differential expression will allow to keep the amount of ap 20000 ribosomes vs ap. 6000 RNA polymerase molecules per cell (Bautz, pers. comm.).

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Genetic transfer between Neisseriae: simulation in microcosm.

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The horizontal gene transfer between commensal and pathogenic Neisseriae has been proposed to be the mechanism by which these later species have acquired altered portions of the *penA* gene that encodes for the PBP2 (Penicillin-Binding-Protein 2) (1,2). These changes in the protein have resulted in a moderately penicillin resistant phenotype in the meningococci, that have been isolated in Spain during the last few years (3). As these isolates did not show an clonal origin (4), this hypothesis could become actual.

Little is known about the possibility of the gene transfer in the nature and about its simulation in the laboratory. Works on soil and water microcosms have been performed (5) but few ones have been done in simulated human environments. We have design a simple microcosm, formed by an agar layer and liquid medium, that mimics the upper human respiratory tract with a co-cultivation of naturally resistant and commensal strain, *Neisseria polysaccharea* with a MIC of penicillin G of 0.4 µg/ml and a sensitive strain of *N. meningitidis* (MIC=0.025 µg/ml).

In a first step of transformation, we obtained transformants with a MIC of 0.1 µg/ml in all stages of growth and a MIC of 0.3 µg/ml in a second step. There was an increase of the transformation efficiency in the exponential phase of growth respect to the lag and stationary ones. This increase was probably due to the cell autolysis that characterizes Neisseriae (6), and the release of the DNA to the medium, because no competence factors have been described in Gram-negative bacteria (7). Surprisingly, when purified DNA from donor strain was added to the medium in a saturating concentration to transform meningococcus, there was a lesser efficiency than when the two strains were co-cultivated.

When we compared the efficiencies between a static microcosm or one that is shaken, we found greater values in the first way of cultivation than in the second; perhaps the good conditions of a shaking culture inhibited the cell autolysis mentioned above.

The presence of DNase into the medium, simulating those secreted by the variety of microorganisms that colonizes the human throat, decreased the transfer of the DNA between both strains but did not inhibit it in its totality. On the other hand, when purified DNA was used in a normal culture, no transformants were obtained when DNase was added. We proposed that a direct contact cell to cell could explain these results and, although conjugation has been described as the mechanism that could occur more frequently in the environment because the DNA is protected against the action of free nucleases (8), transformation is then possible too. The DNA was transferred when an

agar layer was present in the culture, protecting it from the action of the enzyme, simulating the possible interaction between the liquid and epithelial surfaces of the oropharynx, as was described in other natural environments, like soils or waters (8).

From these results, we could conclude that the origin of penicillin resistance in the meningococci possibly was the commensal Neisseriae that colonize the naso and oropharynx and, although in stress conditions and in presence of free nucleases released by other microorganisms, an efficient genetic transfer could be done.

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Identification and characterization of *pilU*, a gene whose product modifies pilus-associated phenotypes in *Neisseria gonorrhoeae*

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Expression of type IV fimbriae is correlated with the ability of *Neisseria gonorrhoeae* to colonize the human host (1). Fimbriae are also associated with other phenotypes, including twitching motility (2), competence for natural transformation (3), and autoagglutination (4). Several *N. gonorrhoeae* genes have been recently found that exhibit close similarity to other members of a family of putative nucleotide-binding proteins which are involved in protein secretion and assembly (5,6). Among those gene products, gonococcal PilF protein was most closely related to the pilus assembly protein PilB of *P. aeruginosa* while the product of the gonococcal *pilT* gene is most similar to the PilT protein of *P. aeruginosa* which is involved in pilus-associated twitching motility and sensitivity to phages (7,8). The nucleotide sequence analysis of the gonococcal *pilT* locus revealed the presence of a large open reading frame located 0.2 kb downstream of the *pilT* gene. This ORF encodes a 408-amino acid protein which shows 33% identity with the gonococcal PilT protein and 45% identity with PilU protein in *P. aeruginosa* (8). A 46 kDa protein corresponding to this ORF, was detected in both *E. coli* and *N. gonorrhoeae*, and the gene was denoted *pilU*. Gonococcal PilU$^-$ mutants did not autoagglutinate in a pilus-specific manner although electron-microscopic examination showed that the defect in autoagglutination was not due to a decrease in a pilus expression. Unlike PilT$^-$ mutants, the PilU$^-$ mutants were fully competent for DNA transformation as compared to the wild type strain. In contrast to what was observed in *P. aeruginosa* (8), gonococcal PilU$^-$ mutants were capable of twitching, indicating that PilU protein is not required for this type of motility in *N. gonorrhoeae*. Furthermore, binding to human epithelial cells was increased eight fold in PilU$^-$ mutants. This finding was in contrast to the studies in meningococci, showing that autoagglutination appeared to promote binding to epithelial cells (9). There appears to be no absolute correlation between autoagglutination and adherence in gonococci. The results show that PilU promotes pilus autoagglutination, but appears to have an inhibitory effect on pilus-mediated adherence to the epithelial cells.

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Genetic analysis of post translational modifications of meningococcal pilin.

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The pilin protein, PilE, is the major structural component of the meningococcal class I pilus. Natural variation in amino acid sequence of PilE may result in functional modifications, notably differential adhesion to human cell lines (1). Phosphate and sugar groups, which have long been supposed to be associated with the neisserial pilin protein (2), have recently been shown to be covalently attached to the pilins of some strains of *Neisseria meningitidis* and *Neisseria gonorrhoeae* (3,4,5). However, the presence and role of such post translational modifications is unclear and variations in these additional moieties may also affect pilus assembly and/or function. The PilE protein of *Neisseria meningitidis* C311 has been well characterised and is known to be a glycoprotein with multiple substitutions which include α-glycerophosphate attached to Ser_{93} (3,4). Mutants have been created in this strain to analyse the role of such modifications in pilus structure and function.

C311 pilin is post-translationally modified with the novel O-linked trisaccharide digalactosyl 2,4-diacetamido-2,4,6-trideoxhexose. Mutants defective in *galE* were constructed that contain an unaltered *pilE* sequence, but which lack the terminal di-gal moiety whilst retaining the unusual diacetamido sugar. Adhesion assays and electron microscopy showed no difference between GalE mutants and their parental strains, indicating that di-gal has no discernible effect on pilus assembly or adhesion *in vitro*. However, the effects of the di-gal modification *in vivo*, or on other unknown pilus functions, or the possible effects of the diacetamido-2,4,6-trideoxhexose alone cannot be ruled out by these studies.

Site-directed mutagenesis was used to remove sites of possible O-linked modifications targeting the highly conserved serine residues at positions 63, 69 and 93 of mature C311 pilin. Pilin genes were constructed that are identical to the parental *pilE* locus except for base substitutions at the relevant codons. These genes were introduced by transformation into the high adhesion variant C311 #16 to replace the parental *pilE* locus. The *pilE* genes of these transformants were amplified by PCR and sequenced to confirm that only the desired mutated *pilE* sequence was present. Stable meningococcal clones containing alterations only at positions 69 or 93 were easily isolated. However, meningococcal transformants containing alterations to codon Ser_{63} of *pilE* frequently reverted back to producing pilin containing the original amino acid at this position, with simultaneous possibly compensating alterations further downstream, particularly to Ser_{79}. This suggests that the Ser_{63} is critical to pilin function and/or assembly. Ser_{63} has been shown to be glycosylated with a disaccharide in gonococcal pilin (5). Our

results suggest that this residue may be essential to the structural integrity and for functional properties of class I meningococcal pilin. Phenotypic studies on pilus structure and adhesive function with these mutant *pilE* loci will be described.

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Tetrameric repeats in *Neisseria*

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Short, repetitive DNA motifs are implicated in reversible on/off switching (phase-variation) of several genes in the pathogenic *Neisseria*. Examples include homopolymeric tracts in the promoters of *opc* (1) and *pilC* (2), as well as in the coding region of the glycosyl-transferases *lgtA* and *lgtC* (3, 4). Similarly, phase variation of Opa proteins is influenced by variation in number of the repeat unit 5'-CTCTT-3' within the promoter.

Recently, a locus has been described in Nm serogroup B strain MC58 containing four copies of the tetramer 5'-GCAA-3', (6) now designated *nmrep4* and we have recently demonstrated the presence of three further loci containing the motif 5'-$(GCAA)_n$-3' (7).

One of these loci, *nmrep2*, has been isolated from a λ library and sequence analysis has revealed the presence of the motif 5'-$(GCAA)_8$-3'. There is 59% similarity between the DNA sequences of *nmrep2* and *nmrep4*. In common with *nmrep4*, *nmrep2* has no obvious translational start site, but the predicted amino acid sequence downstream of the tetramers of *nmrep2* shows homology to the virulence determinants Icsa of *Shigella flexneri* and Aida-1 of *Escherichia coli*, with 48% and 46% similarity respectively.

We have examined a number of isolates of *N. meningitidis* for the presence of *nmrep2* and *nmrep4*. PCR amplification and direct sequencing indicated that *nmrep2* is conserved in 29/29 serogroup B strains and 20/20 strains of other serogroups. The majority (29/29 and 16/20 respectively) contain eight copies of the tetramer. Similarly, *nmrep4* appears to be widely conserved in these strains. However there is greater variation between strains of the number of repeat units at this locus (5'-$(GCAA)_1$-3' to 5'-$(GCAA)_{12}$-3'), and we were unable to amplify *nmrep2* from several of the serogroup B strains.

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Characterization of cell division gene homologues *ftsZ*, *ftsE* and *ftsX* in *Neisseria gonorrhoeae* strain CH811

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We have identified cell division gene homologues *ftsZ*, *ftsE* and *ftsX* in *Neisseria gonorrhoeae* strain CH811. In many bacteria, FtsZ plays a critical role in cell division and aggregates to form a circumferential ring on the inner surface of the cytoplasmic membrane in *Escherichia coli* (1). The gonococcal *ftsZ* gene was amplified from the chromosome using a combination of PCR and inverse PCR strategies. The translated gonococcal *ftsZ* sequence shares 40% to 50% similarity with FtsZ from various bacterial species. The GTP-binding motif common to all other FtsZ proteins (2) is conserved in the gonococcal FtsZ. In vitro transcription/translation of the gonococcal *ftsZ* gene produced a protein of the expected size. The gonococcal FtsZ protein was identified by Western blot analysis of cell extracts of Neisseria species with a polyclonal antiserum to the *E. coli* FtsZ. The gonococcal *ftsZ* gene, without its upstream region, was cloned downstream of an inducible lac promoter in pTAg (R&D Systems) and expression of the gonococcal *ftsZ* in *E. coli* led to filamentation. In *E. coli*, *ftsE* and *ftsX* encode cell division proteins which may be constituents of the septalsome (3). The gonococcal *ftsX* gene was originally isolated from a genomic library in pBluescript KS+ (Stratagene). A partial *ftsE* gene was identified immediately upstream of *ftsX* and the remainder of *ftsE* was subsequently amplified from the chromosome by inverse PCR. The gonococcal *ftsE* and *ftsX* genes are linked and overlap by 4 base pairs. The translated *ftsE* sequence shares approximately 68% similarity with FtsE from *E. coli* and also *Haemophilus influenzae*. As with its homologues, the gonococcal FtsE contains ATP-binding motifs similar to members of the ATP-Binding Cassette (ABC) family (4). The translated *ftsX* sequence shares approximately 50% similarity with FtsX from *E. coli* and also *H. influenzae*. Protein sequence analysis of all known FtsX revealed that it is an integral membrane protein. In vitro transcription/translation experiments produced a protein of the expected size for FtsX. We have also ascertained that as in *E. coli* no promoter lies immediately upstream of *ftsX*. The genes flanking *ftsE* and *ftsX* are not the same as found in *E. coli* or *H. influenzae* indicating a different organization.

Insertion of cat cassettes into the *ftsZ* and *ftsX* genes of *N. gonorrhoeae* strain CH811 indicates that ftsZ may be an essential gene.

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Studies on FA1090 S-Pilin Variants

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Neisseria gonorrhoeae commonly undergoes both pilus phase and antigenic variation. Phase variation occurs via several recombination-dependent and recombination-independent mechanisms. Pilus antigenic variation occurs predominantly by non-reciprocal homologous recombination of silent-copy pilin DNA into the pilin expression locus (1,2). One form of pilin variant results from incorporation of sequence information which creates pilin protein monomers that are excreted as soluble protein, S-pilin (3). S-pilin is an altered form of pilin missing 39 amino acids from the amino terminus in addition to the seven amino acid leader peptide.

We have isolated several strain FA1090 variants based on their colony morphology to characterize S-pilin production. All variants contain *recA6(tetM-lacIOP-recA)* and are phenotypically RecA- until induced with IPTG. Western blot analysis showed both forms of pilin in all variants. Variants with a non-piliated colony morphology were PilC+ as determined by immunoblot analysis. Variants with a piliated colony morphology and visible pili by transmission electron microscopy had more full length pilin than S-pilin as detected by Western Blots. Although many of the piliated colony morphology variants had received semivariable and/or hypervariable sequences from the same silent copy, no direct correlation could be made between primary sequence changes in the semivariable and hypervariable regions of the *pilE* gene and the relative amounts of S-pilin and full length pilin produced. Our studies further substantiate previous studies which have shown that S-pilin variants express a few pili and also produce full length pilin (3). The finding that all variants produce detectable amounts of both S-pilin and full length pilin suggests that S-pilin variation is not a classical form of ON/OFF phase variation. Rather, it represents a spectrum of phenotypes between mostly piliated and mostly S-pilin producing. Rarely is a variant detected that is exclusively piliated or exclusively S-pilin producing.

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Identification of a second homologue of the lysophosphatidic acid acyltransferase in *Neisseria meningitidis* and implications for meningococcal membrane phospholipid biosynthesis

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Lysophosphatidic acid (LPA) acyltransferase is an enzyme intermediate involved in membrane phospholipid biosynthesis in a number of prokaryotes and eukaryotes (1). A meningococcal homologue of LPA acyltransferase (*nlaA* - neisserial LPA acyltransferase) has recently been characterized (2). This study reports the identification of a second meningococcal homologue of LPA acyltransferase and notes its potential role in meningococcal phospholipid biosynthesis.

A meningococcal mutant designated 469, derived by Tn*916* insertional mutagenesis, was isolated on the basis of its severely truncated LOS phenotype. Sequencing of chromosomal DNA flanking the transposon insertion in mutant 469 revealed an open reading frame (*orfB*) with predicted homology (27% identity, 45% similarity over a 239 amino acid region) to the PlsC LPA acyltransferase of *E. coli*. The nucleotide and predicted amino acid sequence of *nlaA* was distinct from *orfA*. Enzymatic assays revealed that mutant 469 exhibited 40-fold less LPA acyltransferase activity than the parental strain, in contrast to the *nlaA* mutant, which demonstrated a three-fold increase in LPA acyltransferase activity (2). Although LPA acyltransferase activity was severely reduced in mutant 469, it did not accumulate significant levels of LPA, as was seen in the *nlaA* mutant (2). These observations suggest that, in addition to *nlaA*, *orfB*, and/or other genes interrupted in mutant 469, are involved in meningococcal membrane phospholipid metabolism.

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Genetics, Poster 141

Regulatory pathways of adhesion in pathogenic *Neisseriae*

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Adherence to eukaryotic cells is essential in the pathogenesis of *Neisseria meningitidis* (Mc) and *N. gonorrrhoeae* (Gc). Pilus-mediated adhesion has been shown to play an essential role in this process. Pilin (PilE) , the pilus major subunit, and two pilus associated proteins, PilC1 and PilC2, are key components in meningococcal adhesiveness. Phase and/or antigenic variation of these molecules are the only identified means by which the pathogenic *Neisseriae* modulates pilus-mediated adhesion.

We are presenting data indicating that a regulatory system coexist with genic variations. Our data suggest that *pilE* promoters are structurally distinct in Gc and Mc, thus being in agreement with different regulatory pathways between the two species.

In Mc, *pilC1* and *pilC2* loci have different roles and distinct regulatory regions (1, 2). These data are in good agreement with the different functions of these proteins in terms of adhesion. Only PilC1 in Mc is ivolved in adhesion (1). *pilC1* promoter has a specific fragment which encompasses a PilA binding domain, thus suggesting that this promoter is controlled by the regulatory protein PilA(2). The acquisition of this region confers to *pilC1* another level of regulation of gene expression compared to *pilC2*. This specific region is analyzed in different meningococcal isolates and its role is studied using site-directed mutagenesis. Moreover, this region seems to be present in other chromosomal loci indicating a common regulatory pathway. A central and pleiotropic regulatory system of genes could allow the pathogenic *Neisseriae* to adapt in a more coordinate and responsive manner to environmental changes.

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A repetitive sequence element cause polymorphism in the PilQ protein of *Neisseria meningitidis*

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Omp-mc was originally characterized as an antigenically conserved, abundant outer membrane protein of *Neisseria gonorrhoeae* (1, 2) and attracted attention as a potential vaccine component (1, 3). Gonococcal mutants expressing defective forms of the protein were found to be non-piliated (4). In line with observations found for related molecules in *Pseudomonas aeruginosa* (5), gonococcal Omp-mc was renamed PilQ (4). The C-terminus of PilQ contains polar residues with a strong probability of assuming an amphipathic b-sheet conformation. This region shares identity with similarly located domains found in members of a large protein family required for translocation of macromolecules across the outer membrane. Accumulating evidence suggests that the molecule functions in pilus biogenesis by serving as a gated channel or as a pore, composed of 10-12 monomers.

Based on its potential to serve as a critical pilus component and protective immunogen in *Neisseria meningitidis*, the gene was cloned and characterized from strain 44/76. Mutants expressing truncated forms of the PilQ protein were constructed and were all devoid of pili and pilus-related phenotypes. Sera from patients recovering from meningococcal disease reacted with the *N. meningitidis* PilQ protein by immunoblotting, whereas acute sera did not. This finding shows that PilQ is expressed *in vivo*.

Sequence analysis of the 5' portion of the *pilQ* gene of strain 44/76 showed the presence of 7 copies of a repetitive element with the motif (CCG)GCAAAACAACAG(GCTGCCGC). The repetitive sequences were located in an area of the gene encoding a structurally and functionally important part of the molecule. This region was studied in more detail in a collection of 52 *N. meningitidis* strains from various geographic origins, including different serogroups and the dominant clone-complexes associated with meningococcal disease. The strains were screened for polymorphism in the size of the PCR fragment of the 5' region of the gene and 21 of these were selected for further analysis by DNA sequencing. Meningococcal strains were found to harbor from 4 to 7 copies of the repetitive element. No association between the number of copies and the serogroup, geographic origin or multilocus genotype of the strains was evident. Strains belonging to the clone ET-5 (6), for example, presented 5, 6 or 7 copies of the element. In most cases, variation in repeat number was also reflected in altered mobility of the molecule in SDS-PAGE.

Although intrastrain repeat variation in meningococcal PilQ has not been formally demonstrated, the variability within clones of the ET-5 complex strongly suggest that intrastrain variation does occur.

The consequences of changes in PilQ repeat number remain unclear and it is difficult to envision that significant changes in antigenicity ensue from the simple gain or loss of repeats. Given its role in pilus biogenesis, changes in PilQ may represent a means of fine tuning assembly to accommodate variability in pilin and PilC expression. In any event, the findings here expand the repertoire of mechanisms by which *N. meningitidis* generates plasticity in biologically important molecules.

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PCRDOP Amplification and Analysis of a 1.8Kb fragment of a potentially novel two-component regulatory system in *Neisseria meningitidis*

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Neisseria meningitidis normally colonises the nasopharynx but during systemic infection meningococci can colonise other sites such as the blood and CSF. The bacteria must be able to sense and adapt to environmental stimuli such as pH, osmotic tension, nutrient availability etc. which may increase survival potential in specific microenvironments encountered. Many of the identified mechanisms for sensing environmental changes involve two-component regulatory systems which may also be an important factor in virulence regulation. Deletion mutants in one such response regulator, *ompR*, has been shown to severely impair virulence of *S. typhi* and *S. typhimurium*. Comparisons of published amino acid sequence data from several global sensory and regulatory proteins revealed conserved regions between the different families. By constructing degenerate oligonucleotide primers to these regions of patch homology we have been able to amplify a 1.8 kb fragment from *N. meningitidis* chromosomal DNA. The PCR product was cloned into the pGEM vector and subsequently sequenced. Comparisons of known response regulator sequences with the sequence obtained showed an amino acid percentage homology range of 32.2% - 49.1% involving seventeen different response regulators and twenty six different organisms. The sensor region showed a range of 23.5% - 30.3% involving just six different sensor proteins from five organisms. The sequence analysis described implies that a potentially novel two-component operon has been amplified. The operon will be mutated and introduced into the wild type strain to produce defined isogenic mutants. These mutants will be used to define the role(s) of this potentially novel response operon in the survival and pathophysiology of *N. meningitidis*.

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Transformation defects in genetically defined pilus mutants of *Neisseria gonorrhoeae*

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In *Neisseria gonorrhoeae*, chromosomal gene transfer appears to occur exclusively by transformation. Analysis of clinical isolates has revealed an exceptionally high rate of horizontal gene exchange at all loci tested (1). These results indicate that natural transformation is not just a laboratory phenomenon, but a significant mechanism for genetic exchange within the host.

Competence for natural transformation is tightly associated with the expression of type IV pili (2). Recently, a number of genes involved in pilus biogenesis have been identified (3,4,5). The products of these genes share identity with gene products involved in transformation in other Gram-negative and Gram-positive species. Conservation of transformation components among species suggests that a common machinery may be involved.

Pilus biogenesis mutants are deficient in natural transformation. The step at which these mutations block transformation has not been determined, but appears to be an early event in transformation, before DNA can be recovered from the periplasm (2). As such, the block appears to be at the level of uptake of DNA into a site inaccessible to DNase I. Other transformation deficient mutants have been described, but the step at which they are affected appears to be subsequent to outer membrane translocation (2,6).

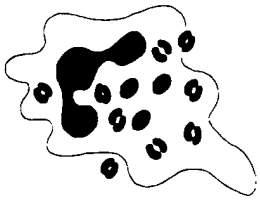
Recovery of transforming DNA from gonococci as well as successful transformation requires a specific 10 base pair sequence to be carried by the transforming DNA (7). We have developed an assay using radiolabeled PCR products to distinguish between the initial pilus-mediated events of transformation, which consist of DNA binding and uptake across the outer membrane. The assay has revealed that the 10 base pair sequence is required for the earliest detectable step in transformation, DNA binding.

Specific non-piliated mutants are defective in DNA binding, although the precise association between pilus expression and binding remains unclear. Either the molecule(s) that engage DNA are physically associated with the pilus filament or alternatively, require pilus biogenesis to achieve their proper localization or functional state. Additionally, PilC and PilT mutants which express pili (2,8), are incapable of binding sequence specific DNA, suggesting that pilus expression is necessary, but not sufficient for DNA binding.

It has been shown that second site suppressor mutants which restore wild type levels of pili in a PilC$^-$ background are defective in DNA uptake (9). Our results show that the PilC mutation alone can account for this defect, and that the defect occurs specifically at the level of DNA binding. However, a defect in PilC expression does not seem to account for the findings in PilT mutants since they bind well to human epithelial cell lines (a correlate of functional PilC expression (10)). In addition to their DNA binding defect, PilT mutants display altered expression of autoagglutination and twitching motility (also pilus associated phenotypes). This argues that the defect in PilT mutants is the result of a qualitative change in pilus filament structure or conformation.

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Epidemiology Typing and Diagnostics

Epidemiology, Typing and Diagnostics, Plenary Review

Population genetics of the pathogenic *Neisseria* and its relevance to molecular epidemiology and typing methods

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The pathogenic *Neisseria* species are naturally transformable, and there is increasing evidence that this mechanism of genetic exchange is important for their evolutionary and population biology. There are several reports of both intra- and inter-species recombinational events in gonococcal and meningococcal genes but the main evidence for frequent recombination comes from population genetics.

In most bacterial species, recombination appears to be less important than mutation in effecting evolutionary change, resulting in populations consisting of largely independent clonal lineages. Clonal populations arise because mechanisms for the exchange of chromosomal genes are rarely used in nature and/or because different lineages rarely meet each other. Clonal populations are usually recognized (often uncritically) by the presence of linkage disequilibrium (non-random association) between alleles in the population, using data derived from multilocus enzyme electrophoresis (MLEE), and by the recovery of indistinguishable isolates that are temporally and geographically unassociated (1). Bacterial populations become increasingly less clonal as the extent of recombination increases, and it has been estimated that they will appear to be non-clonal when a change in an electrophoretic allele is about ten times more likely to occur by recombination rather than mutation (2).

Population structure of meningococci. Isolates of serogroup A meningococci, which cause pandemic disease with very high attack rates, are highly clonal (3,4). The population structure of meningococci of other serogroups (referred to as serogroup B and C meningococci for convenience) is more complex. MLEE studies of serogroup B and C meningococci isolated from invasive disease appear to suggest that these populations are clonal as significant linkage disequilibrium is found (5). However, in most developed countries, for every 100,000 individuals, there will be about 5,000 carriers of serogroup B and C meningococci, but probably only about 5 cases of invasive disease. Sampling of the isolates from invasive disease therefore only samples about 0.1% of the total meningococcal population and can lead to sampling problems that can distort the underlying population structure. Recent studies have suggested that meningococcal populations are basically non-clonal, but that hyper-endemic strains arise at intervals, and become over-represented in populations obtained from invasive isolates (6). The presence of many isolates of the same, or similar, electrophoretic type introduces linkage disequilibrium into these populations, which makes them appear more clonal than they are. Removal of this sampling bias uncovers the non-clonal nature of the population (6). As is expected for a non-clonal population, there is little relationship between serological

markers and electrophoretic type in serogroup B and C meningococci, whereas there is a clear relationship in the clonal serogroup A isolates (5).

It may be thought that focusing on the large fraction of meningococci that are living as harmless "commensals" is inappropriate, and that it is the minority of isolates from invasive disease which should attract all of our attention. However, it is very possible that recombination between isolates from carriers, or between these strains and closely-related commensal *Neisseria* species, produces the particularly favorable combinations of alleles that lead to the unusually virulent or transmissible strains which occasionally arise to cause hyper-endemic disease. It is also very likely that the relatively rapid diversification of the hyper-endemic strains of serogroup B and C meningococci is a consequence of the accumulation of recombinational replacements with chromosomal DNA from carried isolates.

Population structure of gonococci. Two MLEE studies of populations of gonococci have failed to detect linkage disequilibrium, suggesting that the alleles in the population are broken up by frequent recombination (6-8). In support of this view there is little association between gonococcal auxotype, serotype and electrophoretic type (9). Linkage equilibrium also implies that different lineages must meet each other. The presence of mixed infection in a disease like gonorrhea would not be surprising, particularly in the highly sexually active subpopulations (core groups or super-spreaders) that are believe to maintain gonorrhea in the population (10). Even in gonococci the population structure may be complex, since there are many reports in the literature of isolates with similar phenotypes that have loosely been described as clones. In many cases this may be due to the use of typing systems that are not sufficiently discriminatory. However, there are some cases where there is strong support for the existence of gonococcal clones. The best case is that of the arginine-, hypoxanthine- and uracil-requiring isolates (AHU$^-$) which share a number of unusual phenotypic characters (11). We have recently shown that 101 AHU$^-$ gonococci isolated over a 39 year period were of a single electrophoretic type (92%) or differed from this ET at a single locus (12). AHU$^-$ gonococci are clearly a relatively long-lived clone within a basically non-clonal population. How this clone avoids rapid diversification by recombinational exchanges with other gonococci is at present unclear, although it does not appear to be due to a defect in their ability to be transformed. In meningococci, it is possible to argue that serogroup A meningococcal lineages avoid being broken up by recombination because they rarely meet other lineages, as a consequence of their epidemic lifestyle. This type of argument is less easy to apply to gonococci, but possible reasons for the increased stability of AHU$^-$ isolates can be put forward.

Population structure and its relationship to epidemiology. The population biology of meningococci and gonococci is complex, with basically non-clonal populations (excepting serogroup A meningococci and AHU$^-$ gonococci). It might be expected that clones cannot exist within non-clonal populations but, as as described above, this is not strictly true. However, in contrast to highly clonal species such as *Salmonella enterica*, where clones persist virtually unchanged over many decades (e.g. the typhi serovar, or

clone), the clones in non-clonal populations should be ephemeral, since new successful lineages that rise in frequency in the population will be rapidly diversified by recombinational exchanges with other lineages. The rates /hich meningococci diversify appears to vary very considerably. In serogroup A strains the rate is sufficiently low to lead to a clearly clonal population structure. In serogroup B and C meningococci this rate appears to be sufficiently high to lead to a basically non-clonal population. However, certain strains of serogroup B and C appear to diversify more slowly, and give rise to the hyper-endemic transient clones that are a feature of the epidemiology of meningococcal disease (13). The rate of diversification is presumably a function of the rate at which recombinational exchanges with other lineages occur. This could reflect differences in "transformability", but this is not necessarily so, as differences in the frequency and duration of carriage, and the rate of transmission between individuals, will also markedly effect the rate of diversification by reducing contact between lineages (4). Fortunately for epidemiological purposes, even in the non-clonal serogroup B and C meningococci, lineages may still be recognizable over time periods that are significant in human terms (months or years), although extremely short on evolutionary time scales (transient clones).

A knowledge of the population biology of meningococci and gonococci is useful for guiding studies of their molecular epidemiology. Clearly the information that can be obtained, and the typing techniques that have to be used, will be very different for the highly clonal serogroup A meningococci compared to the non-clonal gonococci. In serogroup A meningococci the stability of clones has allowed the well known studies of Achtman and colleagues which have traced the pandemic spread of the major lineages (clones) that cause epidemic meningococcal disease (3). In this case, the stability of the clones, combined with the detection of minor variants, provides the ideal situation for charting the spread of disease from country to country over a period of several decades. In the less clonal serogroup B and C meningococci this type of long term study becomes more difficult since variation accumulates too rapidly, and in gonococci it should be impossible, since the rate of diversification is probably so high that the gonococci isolated in 1996 will be completely different from those in 1986. The rapid diversification of gonococci does not mean that molecular epidemiology is impossible, but just that the questions that can be asked are limited to the spread of isolates within a short time frame. It is very unlikely that gonococci diversify so rapidly that the spread of gonococcal strains over periods of weeks, or months, cannot be followed. Fortunately it is this type of time frame that is of importance in the epidemiology of gonorrhea, e.g. for identifying new strains introduced into a community, or the identification of isolates from sexual contacts.

Population structure and typing methods for meningococci. Is a knowledge of the population structure of the pathogenic *Neisseria* of any help in deciding which types of genes, or gene products, are most suitable for typing isolates for epidemiological studies? As stressed in the previous paragraph, the choice of typing scheme, and the epidemiological questions that can be answered, will be very different if bacterial clones are stable over many decades, or become unrecognizable over a matter of weeks or months, as a consequence of diversification by frequent recombinational exchanges. In

highly clonal bacteria (e.g. the serogroup A meningococci) there is good congruence between trees constructed from MLEE data and from the sequences of house-keeping genes. In some highly clonal species the horizontal transfer of genes specifying the variable cell surface structures used for serological typing is rare, and even serology can mark clones (e.g. in *Salmonella enterica*). In these cases ribotyping, pulsed field gel electrophoresis, PCR with arbitrary primers, or MLEE, should each provide a consistent measure of the relationships between strains, and the choice of characters to use for typing is to some degree arbitrary. The clear advantage of MLEE over the other methods is the ease with which the relationships between isolates can be quantitated.

In the less clonal serogroup B and C meningococci the choice of typing method depends on the question being addressed. Most of the questions relate to the nature of the strains causing meningococcal disease, and their relationship to similar disease-causing isolates recovered within a community, a country, or other countries. As serogroup B and C meningococci should diversify relatively rapidly by the accumulation of recombinational exchanges, the ease with which this can be done should depend on the amount of time since the strains had a common ancestor. Thus, isolates recovered from a localized outbreak within a school, caused by the same strain, will have a very recent common ancestor and can easily be shown to be identical, or very similar, by a number of methods (e.g. pulsed field gel electrophoresis or MLEE, and even serology). However, it becomes more difficult to decide whether these isolates are members of one of the transient clones of serogroup B or C meningococci currently causing hyper-endemic disease by comparing them to reference isolates from other countries, as substantial variation in pulsed field gel patterns, MLEE profile, and serology, may have occurred since the strains had a common ancestor. In this case a more thorough evaluation of the relationship of these isolates to the members of the hyper-endemic clonal complexes, and the overall meningococcal population, using quantitative methods (e.g. MLEE) may be needed. This problem becomes more acute as the time from the origin of the hyper-endemic clone increases, as the clonal complexes currently causing hyper-endemic diseases should continue to diversify by recombinational exchanges (probably at different rates) (13) until the members of these complexes can no longer be distinguished from the background population of meningococci. Unfortunately, new hyper-endemic transient clones will inevitable arise, which will diversify and disappear, to be replaced by further transient clones.

Population structure and typing methods for gonococci. A knowledge of the population structure of gonococci can be used to develop suitable typing systems. If recombination is common in gonococci there should be a vast array of different genotypes within the population. This should mean that almost all randomly selected gonococci will be distinguishable, provided sufficiently high resolution typing systems are used. The commonly used typing systems lack the high degree of resolution required to distinguish the vast array of genotypes expected in a non-clonal population. High resolution typing schemes such as MLEE should be ideal for distinguishing gonococci. Unfortunately, the level of sequence variation in gonococcal house-keeping genes is low, and a large number of enzymes have to be assayed to provide a highly discriminatory method. As much of the interest in gonococcal typing is to recognize isolates from

sexual contacts, or strains newly introduced into a community, it is allowable to use genes for typing in which variation accumulates very rapidly. We have chosen to use a highly variable gene family (the opa genes), that evolves very rapidly (probably by recombination), as the basis of a typing method for the short term epidemiology of gonorrhea. Using these genes it has been possible to devise a typing scheme that appears to distinguish all gonococcal isolates, unless they are from sexual contacts or a short chain of disease transmission (14). This typing method (opa-typing) appears to provide a much higher resolution than other methods and appears to allow us to predict sexual contacts simply by the fact that they share gonococci with identical opa-types.

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Epidemiology, Typing and Diagnostics

Microevolution during epidemic spread of subgroup III serogroup A meningococci

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Subgroup III meningococci caused a pandemic meningitis wave in the 1960s-1970s, which affected China, Scandinavian countries and Brazil (1-3). A second pandemic wave began in China and Nepal in the early 1980's and spread via an epidemic in Mecca during the Haj pilgrimage of 1987 to East, Central and West Africa (4,5). Bacteria were also exported from Mecca to Europe and the USA without causing epidemics. 300 isolates from these diverse sources were analyzed for genetic variation of the *iga* and *opa* sequences and of pulsed-field gel electrophoresis (PFGE) patterns.

iga and some *opa* genes have been transmitted via clonal descent over long periods of time: variable regions of the *iga* gene and the *opaA*, *opaF* and *opaH* alleles were identical in subgroup III and IV-1 bacteria isolated in the 1960's, indicating that they had been inherited from one strain which was the ancestor of both subgroups at a much earlier time. However, during the subgroup III Mecca outbreak, the *iga* gene was replaced through horizontal genetic exchange involving at least 2 kb of DNA by an allele from an unrelated organism. Similarly, *opaH* was replaced through horizontal genetic exchange by the *opaI* allele and concurrently a DNA stretch 4.5 kb upstream of the *opaH* allele was replaced by a homologous DNA stretch containing an *Nhe*I site. The proximity of this DNA stretch to *opaH* suggests that these exchanges reflect recombination with a DNA fragment of >5 kb size. Finally, *opaF* suffered a 1 bp mutation during the Mecca epidemic. The 3 loci, *iga*, *opaF* and *opaH* are widely separated on the genome and must represent 3 independent genetic changes. Indeed, analysis of subgroup III meningococci isolated during and shortly after the Mecca outbreak revealed 4 strains representing the parental genotype, numerous strains representing the new genotype and 2 intermediate recombinants which had acquired the *opaF* mutation and the novel *iga* allele but still retained the region encompassing *opaH*. The ancestral and intermediate genotypes did not spread and were never recovered again among later isolates.

Analysis of bacteria isolated from different countries during both pandemic waves revealed extensive geographically localized genetic variation. A third *iga* allele was found in the early 1960's in China which differed by 1 nucleotide from the 'pre-Mecca' pattern. Numerous strains were found where *opa* alleles had translocated by gene conversion to yield recombinant *opa* loci and a few strains where *opa* alleles had been imported by horizontal genetic exchange or had suffered point mutations. Two additional strains were found among isolates from China in the early 1980's where a sequence variant of the DNA stretch containing the *Nhe*I site had been imported by horizontal genetic exchange. All the variants were localized to individual countries or geographical regions and did not spread extensively. Thus both pandemics were associated with continued genetic variation in individual countries while the basic pattern of these alleles

did not change during extensive spread, except for the Mecca outbreak. The results are interpreted as reflecting repeated sequential bottlenecks which purify epidemic bacteria and occasionally allow clonal replacement (6,7).

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Identification and typing of *Neisseria meningitidis porB* from cerebrospinal fluid using nested PCR and biotin labeled probe hybridization

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The epidemiology of the antigenic characteristics of the class 3 outer membrane protein of *Neisseria meningitidis* has been important both in identification of outbreak strains and for the development of OMP vaccines. Information regarding the genetic basis for antigenic diversity between strains has increased our understanding of traditional serotypes distinctions. We and others have previously described the variable regions of the *por*B gene encoding the class 3 and class 2 proteins and an identification technique using hybridization of oligonucleotide probes to the variable regions of several important serotypes.(1,2,3) We have expanded our panel of probes to include all the major sequence types at each of the variable regions based on the proto-type stains expressing class 3 OMP. Several of these probes have identified variable region homology between non-serotypeable strains and known serotype strains. Of eight Brazilian non-typeable stains tested, five expressed class 3 OMP and 4 of five had at least one VR in common with known serotype strains.

To examine the epidemiology of specific *por*B genotypes among disease causing strains from a population where group B disease is endemic, we are analyzing a series of 100 CSF samples from Brazil using PCR and subsequent VR hybridization. Polymerase chain reaction amplification of *por*A from low copy numbers in cerebrospinal fluid has been described.(4) We have used a similar PCR strategy using outer and nested primers which are external to the VRs to amplify the *por*B gene directly from CSF. Eight CSF samples with known culture results (2 group B and 2 group C *N. meningitidis*, 3 culture negative and 1 *Streptococcus pneumoniae*) were examined in a preliminary study. PCR accurately identified the *N. meningitidis* CSF samples. PCR products from the two group B samples hybridized the VR1-4 probe corresponding to serotype 4 and PCR products from the two group C samples hybridized the class 2 probe.

We have developed a nested PCR amplification of *por*B from cerebrospinal fluid with subsequent hybridization of the PCR products allowing both diagnosis and typing information directly from CSF samples. The epidemiology of individual variable region genotypes shown by this method may aid in the selection of strains for vaccine development. This technique may also be of importance in vaccine trials to analyze culture negative suspected cases (between 1/3 and 1/2 of cases in Brazil) and to asses the ability to protect against some non-serotypeable stains based on individual variable region type.

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Epidemiology, Typing and Diagnostics

Assessment of molecular typing methods for differentiation of *Neisseria gonorrhoeae*

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Over the past forty years, various typing schemes have been devised to characterise isolates of *Neisseria gonorrhoeae*. Traditionally, auxotyping and serotyping have been accepted as the two major phenotypic systems for the typing of gonococci. However, these methods lack the discriminatory power to differentiate many gonococcal isolates (1) and have not examined variation of the entire gonococcal genome. With new developments in genetics, molecular techniques are being increasingly used to differentiate gonococcal isolates (2, 3). Sixty four clinical samples of *N. gonorrhoeae* were isolated from consecutive male patients presenting with acute urethritis at a STD clinic in Durban, South Africa. Isolates were typed using serotyping, macrorestriction and pulsed-field gel electrophoresis (PFGE) and the polymerase chain reaction-restriction fragment length polymorphisms (P -RFLPs) of the *por* gene. Isolates were serotyped, using the Genetic Systems panel (Bayva, Palo Alto), on the basis of their reaction with antibodies to IA or IB Por, the principal protein of the outer membrane of *N. gonorrhoeae* (4). Testing was done in duplicate for each isolate. Isolates were differentiated into 5 IA and 12 IB serovars. There were four major serovars, viz., IA-6, IB-3, IB-1 and IA-3. The IA-6 serovar predominated, constituting 29% of the population. This is a trend peculiar to strains from the African continent (5). The two major PIB strains, IB-3 and IB-1, comprised 19% and 13% of the population, respectively. Overall, 53% of the isolates belonged to PIB serovars. Genomic DNA was prepared by enzymatic and detergent lysis of gonococcal cells embedded in low melting point agarose plugs (2). Plugs were restricted overnight in 150 ml restriction buffer containing 20 U of *Nhe*I, a low-frequency cleavage enzyme that recognises 6 bp sequences containing the tetranucleotide CTAG, an infrequent sequence in most bacterial genomes. Isolates were subjected to PFGE in a contour-clamped homogeneous electric field (CHEF) apparatus - CHEF Mapper System (Biorad) for 26 h with pulse times of 1-25s. Fingerprints consisting of 10-18 fragments (1-485 kb) were obtained and allowed for the differentiation of the entire gonococcal genome. Restriction patterns of isolates with identical serovars showed various degrees of genetic variation ranging from being clonal, to clonally related, or being independent strains which were unrelated. However, the majority of isolates showed multiple similarities between 1-145 kb, suggesting a common ancestry. It has been suggested that serotyping does not adequately discriminate the IB-3 and IB-1 serovars (1). PFGE allows for intra- and inter-serotypic discrimination, especially with regards to the IA-6, IB-3 and IB-1 serovars. Thus, PFGE appears to enhance the discrimination of each isolate, when used in conjunction with serotyping. The *por* gene of each isolate was amplified by the polymerase chain reaction

using specific primers targeted to the *por* gene, viz., ^{84}ATGAAAAAATCCCTGATTGCCC^{105} and ^{1064}TTAGAATTTGTGGCGCAGA^{1046} (6). Amplification of the *por* gene consisted of 35 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 70°C. An initial and final step of 5 min at 94°C and 10 min at 72°C were included. With each of the PIA-expressing isolates, PCR amplimers of approximately 0.9-1.0 kb were obtained, compared to the 1.1 kb fragment obtained from the PIB-expressing isolates. The resulting amplimers were restricted with *Cfo*I, *MspA*1I and *Hpa*II (3,7) and run on 6% non-denaturing polyacrylamide gels to generate fingerprints with 2-3, 3-4 and 2-4 fragments, respectively. Amplification of a specific segment of DNA, the *por* gene, limits the number of restriction fragments obtained, allowing easy and accurate interpretation of fragment patterns. Both PFGE of the entire genome and PCR-RFLP of the *por* gene allow good discrimination of the non-clonal gonococcal isolates and augment the traditionally accepted phenotypic serotyping system.

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Molecular typing of *Neisseria gonorrhoeae* to identify sexual contacts.

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Objective. A molecular typing technique has been developed for use in studying the transmission of gonorrhea in sexual networks. A highly discriminative method that gives unique patterns for unlinked isolates but indistinguishable profiles for isolates from sexual contacts is required. To achieve this degree of discrimination we have examined diversity in the opa gene family.

Method. The opa genes were amplified by PCR, digested by Taq1 to produce multiple fragments and end-labeled with ^{32}P-dCTP. The resultant fragments were separated on a non denaturing polyacrylamide gel and exposed to X-ray film1. RFLPs were compared using GelCompar and the degree of similarity determined by Pearson's correlation coefficient. This method was evaluated using selected strains from a collection of gonococcal isolates which were tested retrospectively and included isolates from known sexual contacts and from consecutive patients not known to be contacts. Isolates are also being tested from two prospective studies of consecutive patients attending St.Mary's Hospital, London and the Royal Hallamshire Hospital, Sheffield. Phenotypic analysis using auxotype/serovar (A/S) classes is also being performed. Detailed epidemiological data is being independently collected.

Results. The method has proved to be highly discriminatory with strains isolated worldwide. Indistinguishable patterns were obtained within all the pairs/groups of isolates from known sexual contacts but were distinct between the clusters.

Analysis of isolates from patients from the prospective studies has shown two different populations. A total of 220 isolates have been analyzed from patients attending the clinic in London between April and September 1995 and appear as a heterogeneous population as identified by both the phenotypic and genotypic parameters tested. Phenotypic analysis showed a total of 20 serovars, 8 auxotypes and 46 auxotype/serovar (A/S) classes with 25% of isolates belonging to A/S class, NR/1B-1. Genotypic analysis found considerable variation in the profiles obtained by opa typing. Data collected from the patients identified only 21 pairs and one triplet of known sexual contacts. Of these, 21 of the 22 had concordant A/S classes and the opa profiles showed a correlation coefficient of >85% between paired isolates which is indicative of a high degree of similarity. The one discordant pair were isolates from two male patients known to be contacts but both of whom were known to have multiple partners. The A/S classes were different (NR/1B-

8 AND NR/IB-7) and the opa profiles were dissimilar. This could suggest the presence of a mixed infection or infection from another contact.

Analysis of the 140 isolates collected from patients in Sheffield between April 1995 and January 1996 has shown a more homogenous population. Phenotypic analysis showed 11 serotypes, 6 auxotypes and 24 A/S classes with 62/140 (44%) belonging to a single A/S class, Arg/IB-3. Genotypic analysis showed that the opa profiles of the Arg/IB-3 isolates were similar (correlation coefficient of >80%) suggesting the presence of a large cluster in this population. The opa profiles of the remaining isolates were largely heterogeneous with a number of pairs and two other smaller clusters.

Conclusion. We have shown opa-typing to be a highly discriminative technique. In a retrospective study it was able to identify isolates from known sexual contacts. Prospective studies have identified diverse gonococcal populations in different cities. The implications for the transmission of gonorrhea in these sites will be discussed.

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The molecular epidemiology of the *por*B gene of *Neiss* : *meningitidis* isolated in England and Wales

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Surface antigens of the meningococcus, such as PorA and PorB, are important as epidemiological markers and potential vaccine components (1,2,3). Monoclonal antibodies that identified meningococcal serotypes and subtypes, introduced in the 1980's greatly improved their characterisation compared with polyclonal reagents (4) but many organisms remained not or only partially characterised for these proteins (5). Nucleotide sequence analyses of *por*A and *por*B have since been used to identify the reasons for this: in addition to completely novel variants there are minor variants of these antigens that do not react predictably with a given monoclonal antibody (6).

With the increasing convenience and availability of automated nucleotide sequence technology it is now becoming feasible to obtain the gene sequences for antigens from a large number of meningococcal case and carrier isolates. In addition to characterising strains, the availability of such comprehensive data for *por*A and *por*B can provide the basis for detailed epidemiological and population genetic analyses which will improve our understanding of the population structure and antigenic diversity of the meningococcus. Nucleotide sequence data has also enabled the development of DNA-based approaches for rapid and accurate *por*B characterisation of meningococcal isolates, providing enhanced disease surveillance by the identification of antigenic variation within dominant serotypes such as serotype 4. The DNA-based approach also allows the rapid identification and characterisation of case isolates with a novel serotype such as serotype 22.

The identification of the meningococcal *por*B gene by a PCR ELISA assay has been modified to enable non-culture confirmation of meningococcal disease in clinical specimens. Non- culture diagnosis has improved case ascertainment and the usefulness of the assay for the diagnosis and confirmation of several institutional outbreaks of meningococcal disease in the UK has been demonstrated.

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Clonal analysis of *Neisseria meningitidis*: Sequence studies on the 16S ribosomal RNA

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Neisseria meningitidis is a major human pathogen and is the commonest cause of bacterial meningitis in children in the UK. Two thirds of all meningitis cases are due to infection with group B meningococci, the remainder being due predominantly to serogroup C (1). Studies on the population genetics of *N. meningitidis* have shown it to have essentially a clonal structure. Serogroup C meningococci are responsible for both sporadic and endemic disease being intermediate between genetically diverse serogroup B and the relatively homogeneous serogroup A. We have applied genotypic typing methods to determine the clonal relationship of recent UK isolates of serogroup C meningococci (2,3). Eleven clones were identified by StuI REA with two of the clones accounting for 64% of strains. In order to further assess the genetic relatedness of the UK serogroup C isolates we have sequenced part of the 16S ribosomal RNA gene and compared the results with those obtained from StuI REA.

Chromosomal DNA from serogroup C isolates was purified and the 5' half of the 16S rRNA gene amplified using primers Po and Pc3mod. The amplicon was purified from excess primers and dNTPs using Centricon C-100 columns. The products were sequenced using Applied Biosystems dye dideoxyterminator cycle sequencing kits on an ABI 373A automated sequencer fitted with a stretch upgrade. Internal primers as well as Po and Pc3mod were used to sequence the fragments on both strands (4). Data was analysed using the Seqed sequence analysis program. Twelve isolates were sequenced and analyzed representing seven of the eleven StuI REA clones plus one serogroup A isolate.

The complete sequence of the 5' 750bp from each isolate was obtained. Comparison of the twelve sequences identified 10 polymorphic sites located throughout the entire 750bp fragment. All sequences from the same StuI REA clone were identical. Some of the StuI REA clones also had identical sequences. StuI REA clones 1,2,3 and 9 had identical 16S rRNA sequences although their StuI REA Dice coefficients varied from 59-74%. Comparison of the sequences from StuI REA clones 1,7,8 and 11 showed them to differ at 4 of the 10 polymorphic sites except clones 7 and 11 which differed at 8 of the 10 sites.

The StuI REA Dice coefficients for clones 1,7,8 and 11 varied between 49-95% but generally less than those having the same 16S rRNA sequence. Isolate A7 differed at 3-7 of the 10 sites when compared to the REA serogroup C clones.

Sequencing of the 16S rRNA from different species has provided an invaluable tool for the simple and rapid discrimination both among and within genera. It is used to study the evolution of bacteria and to determine accurately most phylogenetic relationships up to about the species level (5). During the course of our studies we observed a number of differences within the 16S rRNA genes of *N. meningitidis*. Comparison of the data with our results from StuI REA showed that the differences occurred between clones and not within clones. This suggests that the mutations within the 16S rRNA gene have occurred as these organisms have evolved. The structural constraints on the sequence of the small ribosomal RNA ensures that mutations within the gene are relatively infrequent hence their utility in phylogenetic analysis. Our observation of identical 16S sequences within clones further supports the clonal structure of *N. meningitidis* populations.

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Measurement of antibodies against meningococcal capsular polysaccharides B and C in ELISA: An approach towards an improved surveillance of meningococcal disease.

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Objective. To establish ELISAs specific for meningococcal capsular polysaccharide B and C antibodies and thereby improving the surveillance of serogroup B and C meningococcal disease.

Material. Paired sera from 162 patients with and 101 patients without laboratory evidence of meningococcal disease were used in the evaluation of the tests.

Methods. Microtitre plates were precoated with poly-l-lysine and subsequently coated with purified serogroup B or C polysaccharide (1) or with buffer without polysaccharide as control for non-specific binding (2). Test serum was diluted 1:100 and for each assay an in-house standard reference, a positive and negative quality control serum were employed as controls. The test was performed using peroxidase labelled rabbit anti human IgG and/or IgM antibody and ortho-phenylenediamine as substrate. Absorbance values were read at a wavelength of 490 nm. Each test result was determined as the difference between the optical density (OD) in the antigen well and that of the corresponding control well. The OD test result of each serum sample was transformed to au/ml by the linear portion of the standard reference curve based on a log/log scale. Competitive inhibition by purified B and C polysaccharide was assessed using the same assay principle.

Results. Three ELISAs, anti-B IgM, anti-C IgM and anti-C IgG were established. On the basis of four antibody determinations for each of the three positive quality control sera per week during 10 weeks, the interassay coefficients of variation were estimated to range between 1-19%. The binding of anti-capsular antibodies of the standard reference sera was inhibited 96-99% by preincubation with the homologous polysaccharide.

Sixty out of 76 patients (79%) with culture-confirmed serogroup B disease had high anti-B IgM titres or a significant change in titre; anti-B IgM antibodies waned rapidly in children < 4 years of age; 26 out of 27 patients (96%) with culture-confirmed serogroup C disease had high anti-C IgM and/or IgG antibody titres or a significant change in titre (IgM: 93%; IgG:70%). In patients without meningococcal disease, 19% of children < 4 years of age and 72% of those > 4 years of age had low levels of anti-B IgM antibodies. In contrast, < 10% of these had anti-C IgM and/or IgG antibodies. In 50 out of 59 patients (85%) with culture-negative, but with clinical and serological evidence of meningococcal disease, a definitive serogroup specific diagnosis was established by examination of paired sera in ELISA.

Conclusions. Though the B and C polysaccharides are homopolymers of the same polysialic acid and a cross-reactive epitope exists (3), the methods developed were shown to be powerful tools in discriminating between serogroup B and C disease. A serogroup specific diagnosis may be established in > 80% of culture-negative cases of meningococcal disease. As serogroups B and C meningococci account for practically all cases of meningococcal disease in industrialized countries, the availability of these tests may have implications by improving the surveillance and prevention of the disease.

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Neisseria meningitidis strains that were not serosubtypable in whole-cell ELISA

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Neisseria meningitidis is world-wide a major cause of bacterial meningitis and septicemia. Elevated incidence of meningococcal disease was documented in the province of Québec, Canada, since the beginning of 1991 (1, 2). In an effort to combat this high incidence and its accompanying fatalities, health officials initiated mass immunization programs in the province (3).

Outer membrane proteins (OMPs) of meningococci are of particular interest because they form the basis of serotyping and serosubtyping (4). The use of monoclonal antibodies raised against the class 1 OMP has identified differences in the class 1 OMP and this forms the basis of serosubtyping. Outbreaks of meningococcal disease have been linked with the emergence of specific meningococcal subtypes in Brazil, Chile and England. Proper identification of subtypes is thus essential in epidemiological analyses of meningococcal disease. The currently used protocol for routine serosubtyping relies exclusively on ELISAs using whole-cells and mAbs against the class 1 OMP (5). However, this procedure fails to yield serosubtype information in some strains. Reasons suggested for this failure include masking of epitopes on the cell surface (6) and the presence of novel variable regions for which no mAbs are currently available (7).

Isolated from all patients hospitalized with invasive meningococcal disease in Québec (1993-1994) were 174 meningococcal strains. Of these, 48 (28%) were non-serosubtypable in ELISAs using whole-cells (M. Lorange, Laboratoire de Santé Publique du Québec). These 48 strains were characterized by OMP profiles and ELISAs using outer membrane vesicles. Forty of the 48 strains expressed class 1 OMP indicating that the inability to assign a serosubtype was not due to the absence of the class 1 OMP. 17% (8/48) of the strains did not make the class 1 OMP. In these strains, inability to assign a serosubtype is due to absence of the class 1 OMP on the cell surface. The frequency of isolation of class 1 OMP-deficient strains was thus 5% (8/174). This percentage is significant when considering the design of vaccines that are based on the class 1 OMP: 5% of meningococcal strains would not be targets for such vaccines. Fifteen of the 40 strains that expressed the class 1 OMP were serosubtypable in ELISAs using OMVs. Thus, ELISA using OMVs improves the serosubtyping information that is obtained for epidemiological analyses. However, not all strains will be subtyped using these two procedures. For complete subtyping information, molecular techniques have been proposed (7).

To determine whether the eight strains that do not express the class 1 OMP contained the *porA* gene, we used *porA*-specific primers in PCR. The predicted 1.1 kbp fragment was

obtained in 6 of the 8 strains. Thus, in these 6 strains, lack of expression was not due to deletion of the gene. From the 2 class 1 OMP non-producing strains for which no amplification products were obtained in PCRs, restricted genomic DNA was subjected to Southern hybridization using *porA*-specific probes in order to assess whether the *porA* gene was present. Fragments from restricted genomic DNA of both strains hybridized to the *porA*-specific probe indicating that at least part of the gene was present in these strains. Inability to amplify *porA* sequences from these two strains may be attributed to deletion or to mutations in the primer regions in these sequences. Following the report (8) that variable expression of the class 1 OMP is related to the spacing between the -10 and -35 regions of the promoter and the number of G residues between them , we amplified and sequenced the promoter regions of the 8 strains that did not express the class 1 OMP. Two control strains that produced the class 1 OMP had a stretch of 10 and 12 G residues, respectively, between the -10 and -35 regions. The strains that did not express the class 1 OMP had stretches of 9, 10, 11 or 12 residues but in 7 out of the 8 strains, one of the G residues in the stretch was changed to an A. Thus, variations of sequences between the -10 and -35 regions may determine gene expression in meningococci.

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Appearance of *N. meningitidis* serogroup B:ET15 in C[illegible]ada.

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Serogroup C meningococcal disease has increased in Canada during the past several years due to the emergence of a specific bacterial genotype, ET15 (1), which is a member of the ET37 complex (2,3). This increased incidence has been accompanied by a number of outbreaks (4-6) which have led to mass-vaccination programs to interrupt the spread of disease (5,6).

Prior to August, 1993, nearly 700 group C:ET15 disease isolates were obtained from patients while no group B:ET15 isolate was found. From August 1993, onwards, nine B:ET15 disease (blood/CSF) isolates and one urethral isolate were obtained from patients ranging in age from 4 months to 48 years. These B:ET15 strains were serotype 2a, subtype P1.2, P1.2,5 or P1.[-] and expressed opa protein epitopes which are also prevalent on C:ET15 isolates. Analysis of these isolates by pulsed-field gel electrophoresis using restriction enzymes *Bgl*II, *Spe*I and *Not*I (7) revealed that all B:ET15 strains had chromosomal genotypes that differed from each other, yet were similar to PFGE genotypes previously obtained for C:ET15 strains in Canada. This suggests that the B:ET15 strains examined so far are derived from multiple ancestral genotypes, all of which are very closely related to the more commonly recovered C:ET15 disease-causing strains.

Sharing of ETs between strains of different serogroups has been observed previously by Caugant et al. (2). Genes encoding enzymes required for synthesis of chemically different serogroup B, C, Y and W135 polysaccharides may be partly non-homologous (8). The B:ET15 phenomenon may, therefore, prove to be attributable to qualitative differences in the capsular-polysaccharide genes arising on an ET15 genotypic background, perhaps as a consequence of localized recombination. It is of special interest to note that, with the exception of one isolate, the occurrence of B:2a:ET15 among Canadian disease isolates did not begin until about 7 months after mass-vaccination campaigns were initiated. Therefore, the human immune response to group C polysaccharide may have contributed to the appearance of B:ET15 strains by conferring a selective advantage to rare genetic variants in the bacterial population. Regardless of the reasons for appearance of B:2a:ET15 strains in Canada, it is clear that continuous surveillance is needed to monitor the potential emergence of this genotype, particularly in view of the fact that there is still no satisfactory group B vaccine.

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Antimicrobial susceptibility test for evaluation of *Neisseria meningitidis* C isolated during an urban epidemic, Rio de Janeiro 1993-1995

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Geographical information about antimicrobial susceptibility of *Neisseria meningitidis* became a significant factor in the investigation. Emergence of *N. meningitidis* with relative resistance (penRR, MIC≥0.1 to 1μg/ml) and rarely resistant to penicillin (MIC>1 μg/ml) has been reported during the last decade (1). Most of the reports come from Europe (1). Nevertheless, a few reports from North America of penRR *N. meningitidis*, have appeared in medical literature recently (2,3). This phenomenon does not seem to be related to a particular serogroup (4). Another significant problem for Public Health, is the development of resistance to rifampicin (MIC≥0.25μg/ml), currently used for chemoprophylaxis of meningococcal disease (MD). Sulphonamide has not been used in clinical practice in most countries, because the presence of high prevalence of resistant strains. Although discrimination between resistant (MIC≥10μg/ml) and susceptible *N. meningitidis* to sulphonamide has been shown as an useful epidemiological tool (5).

Determination of the antimicrobial susceptibility pattern of *N. meningitidis* to penicillin (PN), rifampicin (RA) and sulphonamide (SD) is our aim. We have decided to work with these three drugs because of their clinical and epidemiological importance.

Minimum inhibitory concentrations (MIC) of 73 clinical isolates of *N. meningitidis* C to PN, RA and SD, were determined by the agar dilution procedure, according to NCCLS guidelines (6). *N. meningitidis* were obtained from the Instituto Estadual de Infectologia São Sebastião (IEISS), the Reference Centre for MD in Rio de Janeiro, Brazil. The strains were isolated between 1988-1995. 80% of the bacteria belongs to the epidemic wave registered from 1993 -1995, in the city of Rio de Janeiro (UVE/IEISS/SESRJ).

All *N. meningitidis* were fully susceptible to PN (MIC≤0.06μg/ml). Of the total, 45% were resistant to SD. Seven strains (9%) were resistant to RA. Three had a MIC value for RA of 0.25μg/ml and four of 0.5μg/ml.

Determination of regional MIC values of *N. meningitidis* is the first step for an efficient surveillance program. The high resistance level demonstrated for sulphonamide is a significant observation and may serve for epidemiological surveillance. Group C strains characterised in different epidemics registered in Brazil in the 90's were sulphonamide-susceptible (7). The epidemic registered in Rio de Janeiro is associated with a shift in the

age-distribution of the disease towards older children and teenagers (UVE/IEISS/SESRJ). Certain *N. meningitidis* are more likely to be associated with disease in older age-groups, like a particular sulphonamid sistant phenotype of the ET-5 complex described in England (5). Resistance to RA among clinical isolates poses a serious problem for the control of MD secondary cases. A significant proportion of MD secondary cases (6%) have been registered in Rio de Janeiro, where chemoprophylaxis with RA is current recommended (8). The presence of RA-resistance detected in this study may in part explain the failures of chemoprophylaxis in our region. Nevertheless, we must take into account that some secondary cases are the result of the failure to detect all close contacts of an index case (8).

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Epidemiology and molecular analysis of epidemic mer' ;ococcal disease related to group C *Neisseria meningitidis* in a Brazilian metropolis: Rio de Janeiro, 1993-1995

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Since the first report of epidemic meningococcal disease (MD) in Rio de Janeiro in 1921, three other epidemics have been reported: two in 1974 (groups C and A) and one in 1989 (group B) (1). Since 1992, disease incidence has steadily risen in Rio de Janeiro, a city in the south-east of Brazil, with a population close to 6 million. In 1995, the epidemic peaked, with a recorded incidence of 9.5/100,000. The increased incidence was related to the presence of *Neisseria meningitidis* C. Epidemics of MD serogroup C have affected some southern Brazilian cities in the 90's and more than 70% of strains have been characterised as C:2b:P1.3 sulphonamide-sensitive (ET-40; ET-11 complex) (2).

The general purpose of the study is to provide clinical and epidemiological information about MD during an epidemic wave and describe the causative strain. The investigation took place in the Instituto Estadual de Infectologia São Sebastião (IEISS), the local Reference Centre for MD in Rio de Janeiro. The IEISS has notified in the media, 72% of all known cases in the city of Rio de Janeiro. Cases study were carried out involving the investigation of all patients admitted to the hospital between 1989-1995. Data for 1995 is not yet definitive. Either clinical case definition (fever and a purpuric rash), or bacteriologic criteria (*N. meningitidis* isolated from blood or cerobrospinal fluid or a positive CSF latex test) eligible cases will be included in the study. The Bacteriology Laboratory of the IEISS characterises more than 90% of *N. meningitidis* isolates obtained in the metropolitan area and stores the bacteria at -70°C. Sero/subtype are currently determined in the Instituto Adolfo Lutz (IAL) using monoclonal antibodies (2a,2b,4,8,15,17,19,23; P1.2,P1.3,P1.4,P1.7,P1.9,P1.14,P1.15,P1.16) and whole-cell suspensions. The electrophoretic types (Ets) were determined by analysis of allelic variations in 13 enzymes (electromorph profile) as previously described (3). Minimum inhibitory concentrations (MIC) of sulphonamide (SD, sulphonamide-resistant MIC≥10μ g/ml) were determined following a standard guideline (4).

4161 MD patients have been identified between 1989-1995, 51% of which are residents in the city of Rio de Janeiro ('89-'92: 1115 cases; '93-'95: 1017 cases). Patients were classified as meningitis (32%), septicaemia and meningitis (60%) and septicaemia (8%). Lethality was 10% and clinical presentation was a significant determinant for a fatal outcome. Of the total, 60% presented a bacteriological criteria. Between '89-'92, 12% were group C and between '93-'95, 41% ('93: 20%, '94: 35%, '95: 59%). A shift in the age-distribution of the disease was observed during the epidemic period. The proportion

of cases in those 10-19 years was 19% (15-19 years: 6%) before 1993 and 24% (15-19 years: 12%) from 1993-1995. For group C disease, media age was 12 years and median was 10 years and for group B disease media was 11 years and median was 6 years.

Analysis of the epidemic period included 342 clinical isolates of *N. meningitidis*, 203 group B and 139 group C. 81% of group B strains were of serotype 4. Among group C strains, 73 were of serotype 2b ('93: 8; '94: 16; '95: 49) and 44 of 2a ('93: 10; '94: 14; '95: 20). Among group C: 2b strains in 1995, 90% were C:2b:nt. 84 group C strains were analysed by starch-gel electrophoresis. 78% were isolated during the epidemic wave ('93-'95). 60 (95%) group C strains of the serotypes 2a or 2b were related to a unique clone of the ET-40. The ET-40 was first described in the city of São Paulo (2). 14 new Ets were found among serotypes W135:23, C:4 and C:NT. 33% of the ET-40 strains were sulphonamide-resistant.

Multilocus enzyme electrophoresis was an important tool in defining the epidemic strain. The increase of serogroup C disease, associated to two different serotypes, has been investigated in Spain recently (5). The epidemic strain described in Rio de Janeiro shows striking differences when compared with reports of recent epidemics in our country, particularly the trend to affect older age groups (2,6). A new subtype may be associated with the C:2b:nt mentioned above. Although we have not included all monoclonal antibodies for the subtype screen that were previously described.

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Antigenic variation of the class 1 outer membrane protein in an emerging *Neisseria meningitidis* clone in the Netherlands

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The class 1 outer membrane protein P1 of *Neisseria meningitidis* is a vaccine candidate against meningococcal infection. Antigenic variation of the class 1 outer membrane protein P1, was studied. The prevalence of serogroup B P1.4 subtype among Dutch isolates increased 200 fold since 1980, and is associated with the five-fold increase in serogroup B meningococcal disease in the last 15 years. Screening of the strain collection of the Netherlands Reference Laboratory for Bacterial Meningitis for this subtype, revealed that P1.4 has been present in the Dutch population since 1965. Sequence analysis showed that *porA* genes of genotypically distinct P1.4 strains have different VR1 regions, but contain identical VR2 regions. This indicates that the VR2 region encoding the P1.4 epitope may have spread through the meningococcal population via horizontal gene transfer. Genotyping of P1.4 strains showed that one cluster of strains, the ET24/25 cluster, is responsible for the increase since 1980. ET24/25 strains not expressing the P1.4 subtype, seem to have lost the P1.4 epitope encoding region by replacement via horizontal gene transfer or by partial deletion via recombination between direct repeats.

Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA

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In the UK each year, an increased proportion of suspected meningococcal infections remain unconfirmed by culture, due in part to the increasing practice of pre-admission parenteral antibiotic treatment and reluctance to perform lumbar punctures(1). This is evident by the differential between the number of culture proven case isolates referred to the Public Health Laboratory Service (PHLS) Meningococcal Reference Unit (MRU) and the number of notified cases of meningococcal disease recorded by the Office of Population Censuses and Surveys (OPCS)(2). The rapid non-culture confirmation of meningococcal disease is of growing importance for case ascertainment and epidemiological surveillance.

Recently, there have been several publications describing the use of PCR for the detection of meningococcal DNA in clinical specimens(3,4,5,6), however, none of these PCRs provide epidemiologically useful information about the causative organism. The identification of the serogroup of the organism is essential for effective outbreak control and contact management. During recent years, the majority of meningococcal infections in the UK were due to either serogroup B (70%) or C (30%)(2). To combine the non-culture diagnosis of meningococcal infection from CSF, whole blood and serum with serogroup (B and C) identification, a polymerase chain reaction assay (PCR), based on a restriction fragment length polymorphism (RFLP) in the meningococcal serogroup B and C *siaD* gene, was developed.

The similarity between the biochemical nature of meningococcal polysialic acid B (α2-8 linked sialic acid) and C (α2-9 linked sialic acid) capsules(7) has so far hampered the identification of target sequences to differentiate meningococci expressing serogroup B and C capsules. Hybridization occurs between serogroup B, C, W135 and Y capsular genes involved in directing sialic acid synthesis, but not the serogroup-specific sialyltranferases involved in the polymerization of the sialic acid to the polysialic acid chain (8). Nucleotide sequencing of the sialyltransferase (*siaD*) genes of serogroup B and C meningococci (9) has enabled identification of sequence differences allowing the specific identification of serogroup B or C *siaD* genes.

The PCR assay was adapted to an ELISA format incorporating hybridization with serogroup-specific B and C oligonucleotide probes. The specificity for CSFs was 100% and sensitivities were respectively 81, 63 and 30% for CSFs, whole blood and sera.

The *siaD* PCR has been used in several outbreak investigations, whereupon non-culture identification of the serogroup from serum or whole blood DNA confirmed the serogroup to be the same as that of the outbreak strain in the absence of positive culture confirmation. In one instance this was substantiated by a subsequent throat swab isolate from the *siaD* PCR diagnosed case. Following an outbreak of meningococcal disease amongst a student community caused by a B:15:P1.7,16 sulphonamide resistant (R) strain, both serogroupable and non-groupable isolates were cultured from nasopharyngeal swabs from students investigated. The non-groupable (NG) and B:15:P1.7,16 R isolates examined were all identified as serogroup B by *siaD* PCR ELISA. During another outbreak investigation caused by a C:2a:P1.2 R strain amongst secondary school children, 3 NG and 3 C:2a:P1.2R were confirmed as serogroup C.

The serogroup-specific *siaD* PCR ELISA is a significant addition to currently available tests for non-culture diagnosis of meningococcal infection and outbreak investigation.

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Epidemiology of meningococcal meningitis in Niamey, Niger: 1989-1995

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Introduction: Although epidemics of meningococcal meningitis (MM) within the "meningitis belt" of SubSaharan Africa are well-described, few reports with population-based data describe MM between epidemics in the context of bacterial meningitis in general. Our objective was to describe the epidemiology of bacterial meningitis in Niamey, Niger (1995 population est.: 547,739) during 1989 to 1995, a period which included an epidemic (1994/5).

Methods. The bacteriology laboratory at CERMES evaluates all cerebrospinal fluid (CSF) specimens obtained at the National Hospital of Niamey, where all Niamey residents with suspected meningitis are hospitalized. We reviewed laboratory and hospital records associated with CSF specimens submitted between June 1989 and May 1995. A case of bacterial meningitis was defined as occurring when at least one of the following criteria were met: direct exam with $\geq$100 WBC/ml of CSF, detection of antigen from CSF by latex agglutination, or isolation of bacteria from CSF culture. To permit evaluation of information related to each meningitis season, we analyzed data for the 12 months from June to May, rather than for calendar years.

Results. Of a total of 3,706 bacterial meningitis cases, 2505 (67.6%) were due to *N. meningitidis*. Among those, 1808 occurred during the 1994-95 epidemic. The annual incidence (cases per 100,000) of meningitis due to each pathogen was:

Year	*N. meningitidis*	*S. pneumoniae*	*H. influenzae*
1989-90	38.7	16.3	15.6
1990-91	33.4	15.1	13.9
1991-92	52.2	18.8	9.1
1992-93	29.3	17.3	11.0
1993-94	3.8	11.9	12.3
1994-95	346.3	7.3	5.2

N. meningitidis was the principal pathogen associated with bacterial meningitis in each year except 1993-4, when the incidence of meningococcal meningitis fell substantially. During 1993-4, the incidence of meningitis due to *S. pneumoniae* and *H. influenzae* was similar to previous years. Seasonal variation was evident during both epidemic and nonepidemic years. The majority of cases occurred during the dry season, from December to May, with peak disease during March and April of each year. Incidence was consistently higher in males compared with females (male to female ratio: 1.5:1). The case fatality ratio for meningococcal meningitis was 7.8%, which was substantially

lower than case fatality of meningitis due to *S. pneumoniae* (45%) or *H. influenzae* (36%). The vast majority (82.5%) of meningococcal meningitis cases occurred among persons less than 20 years of age. In the nonepidemic years, the highest incidence of disease occurred among children 10-14 years of age (62/100,000). During the epidemic, high rates of disease occurred in all age groups <30 years old.

Age (years)	Annual incidence 1989-94	Annual incidence 1994-95
< 1 year	32	421
1-4	37	516
5-9	49	489
10-14	62	499
15-19	40	484
20-29	12	129
30-39	8	66
40-49	4	30

Among meningococcal cases, serogroup A predominated in all years except 1991-92. During 1991-92, serogroup C caused more than 50% of meningococcal meningitis. The incidence of serogroup C meningococcal disease peaked at 29 cases per 100,000 during 1991-2. The epidemic of 1994-95 was caused by serogroup A, subtype 4:1.9, clone III-1 (1).

Conclusions. These data suggest that *N. meningitidis* is the leading cause of meningitis in Niamey in endemic as well as epidemic years. Most disease is due to serogroups A and C, suggesting that serogroup A/C meningococcal conjugate vaccines currently under development offer the potential to prevent substantial endemic disease as well as preventing periodic epidemics. Meningococcal disease incidence fell markedly during 1993-4, the year preceding a major serogroup A epidemic. Since the incidence of meningitis due to *H. influenzae* and *S. pneumoniae* were stable during 1993-4, the meningococcal disease trough was unlikely to reflect changes in diagnostic practices or laboratory methods. The low rate of disease in 1993-4 may reflect the absence of circulating strains during that year. Subsequent introduction of the virulent III-1 clone into a highly susceptible population probably accounted for the 1994-5 epidemic. We are not aware of other meningococcal epidemics preceded by marked meningococcal disease troughs. Evaluation of longitudinal surveillance data in other meningitis belt countries would help determine whether meningococcal epidemics might be predicted by troughs in meningococcal disease in previous years, which might then provide an early alert to the risk of subsequent epidemics.

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Improved surveillance of meningococcal disease in Norway by continual connection of the epidemiological and bacteriological data

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At the end of 1994, the Department of Bacteriology, NIPH, Oslo, became National Reference Laboratory (NRL) for meningococcal disease. At the same time, the Norwegian Infectious Diseases Notification System was integrated into the Department. This new structure permitted a continual surveillance of the epidemiological situation and allowed early intervention on population groups at high risk of meningococcal disease.

In the 18-month period, from September 1, 1994 to February 29, 1996, a total of 220 cases were notified in Norway and the disease causing isolate was received at NRL for 203 (92%) of the cases. All isolates were analysed by serogrouping, serotyping, and multilocus enzyme electrophoresis (1). Of the 203 patient strains, 73% were serogroup B, 21% were serogroup C, and 4% were serogroup Y. The 9 cases caused by serogroup Y were significantly older: 7/9 patients were between 39 and 91 years old. While serogroup B disease was at 66% in the first 6-month period, it increased again to over 75% in the last year.

The proportion of ET-5 complex strains increased again from just over 40% in the first to 54% in the last six-month period, while the proportion of ET-37 complex strains remained low (under 10%). The main epidemiological change observed is that 50% of the serogroup C strains were serotype 15:P1.7,16, and belonged to the ET-5 complex. These strains were responsible for a large part of the new increase in disease due to ET-5 strains. Twelve cases (6%) were caused by clones of lineage III, that has been associated with the recent hyperendemic wave of disease in The Netherlands (2).

Fourteen cases (2 of them fatal) were caused by a clone-complex that only newly was associated with disease in Norway. While the first three cases occurred in the same region within a month, the additional cases were spread through the country. Most of these isolates were serologically characterized as B:4:NST. DNA sequence analysis of the *porA* gene of these strains revealed an identical sequence (P1.18,25) coding for a porin against which no monoclonal antibodies have been developed so far.

In the described 18-month period there were six pairs of associated cases and one group of three cases occurring within the same communities in the course of one day and up to ten weeks, and caused by identical isolates. In addition, a one-year old girl underwent two episodes of meningococcal disease 5 months apart, caused by the same strain (B:15:P1.7,16; ET-5).

Two outbreaks were identified. The first one occurred in the Sør-Trøndelag county from April to December 1995 and was caused by a clone variant of the ET-5 complex, with serological characteristics B:15:P1.7,16. It involved 10 cases (8 culture-verified cases and 2 cases likely due to the same clone), one of them being fatal. A carrier study around the cases showed a very low prevalence of the outbreak clone. Carriers of the virulent strain were treated prophylactically with ciprofloxacin. The second outbreak was in the Hordaland county from September 1995 to February 1996 and was caused by an unusual ET-5 strain in that it belonged to serogroup C. Following our recommendation, serogroup C vaccination of all children between the age of 2 to 5 and young people from 13 to 21 years old (3 950 individuals) was performed in February 1996 in the part of Hordaland county afflicted by the outbreak.

The new organisation of meningococcal surveillance in Norway has permitted the early recognition of two outbreaks, allowing intervention on the population at risk. It has also shown that the number of associated cases, outside the close contacts, may be higher than earlier estimated (3).

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Detection of *Neisseria gonorrhoeae* in clinical samples by the polymerase chain reaction

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The gold standard for diagnosis of gonorrhoea has always been the culture and the presence of gram-negative diplococci in smears of genital discharge (1). However, these methods have several disadvantages including false-negative results and several days may elapse before cultural diagnosis can be established (1). Non-cultural methods offer several advantages over conventional procedures because diagnosis is not dependent upon the presence of viable micro-organisms for microbial isolation and turnaround times can be significantly reduced (1). The development of the polymerase chain reaction (PCR) has been shown to offer a sensitive and specific alternative approach for the diagnosis of a variety of bacterial pathogens. In the present study, a PCR assay (2) was used for direct detection of *Neisseria gonorrhoeae* in clinical specimens of 200 consecutive adult males presenting with acute urethritis at a STD clinic in Durban, South Africa. Ho *et al.* (2) developed this assay by targeting the *cppB* gene found on the 2.6 MDa cryptic plasmid or integrated into the gonococcal chromosome. Swabs, containing urethral exudate, were collected and suspended in phosphate-buffered saline, pH 7.3. Samples were centrifuged and resulting pellets suspended in 100 ml 1 x PCR buffer with 0.45% non-ionic detergent Tween 20 and 200 mg/ml proteinase K. Cell suspensions were incubated at 50-60°C for 1 h, heated to 95°C for 10 min (2) and suspensions stored at -20°C for future use. Crude clinical lysates and 30 pure cultures of *N. gonorrhoeae* were amplified using two 20-mer oligonucleotide primers HO1 (5' GCTACGCATACCCGCGTTGC 3') and HO3 (5' CGAAGACCTTCGAGCAGACA 3') (2). 40 cycles of amplification were performed, each of which consisted of a 30s denaturation step at 94°C, a 1 min annealing step at 55°C and a 30s template elongation step at 74°C. Amplified product was analysed by electrophoresis in a 2% agarose gel, which was examined for the presence of a 390 bp fragment. The 390 bp amplimer was obtained for the pure cultures as expected, the specificity of which was confirmed by restricting the amplified product with *Msp*I, to give two fragments of 250 and 140 bp. A sensitivity of 100% by PCR was obtained for the pure culture. Initial PCR assays of clinical samples using pure culture parameters did not yield the expected 390 bp amplimer. Instead smears that stopped exactly at the 390 bp amplimer position or smears without a distinct stop were obtained. In order to optimise the PCR assay for clinical specimens, the amount of crude lysate used as the template DNA was decreased and the annealing temperature increased from 55°C to 60°C. The required 390 bp product was, however, not obtained for all samples. Decreasing the dNTP concentration, increasing the $MgCl_2$ concentration, chelating excess phosphate ions with sodium citrate, decreasing the number of reaction cycles and phenol-chloroform extraction of the crude

lysate, have been attempted in an effort to further optimise the assay. Compared to Ho *et al.* (2), who obtained a sensitivity of 100% and a specificity of 88.9%, preliminary evaluation of 50 clinical specimens showed that of 42 specimens that were culture positive, only 37 were PCR positive. Among the 8 culture negative specimens, four were found to be PCR positive. The large number of false-negative PCR results seen could be as a result of (i) the inability of primers to anneal with appropriate sites and prime amplification in all strains; (ii) inhibition of the *Taq* polymerase by a substance present in the extracts of clinical specimens; and (iii) low numbers of gonococci present in exudate as a function of length of infection. The use of the PCR assay as a diagnostic tool is promising but requires optimisation and further evaluation.

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Epidemiology, Typing and Diagnostics, Poster 156

Prevalence of *Neisseria meningitidis* serotype 22 in G· ·nany

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The serotypes/serosubtypes are important epidemiological markers of *Neisseria meningitidis* isolates. Antigenic variations of the outer membrane proteins (OMPs) of classes 2 and 3 define the serotype, those of class 1 the serosubtype of a meningococcal strain. Six hundred and forty-four (45,3%) out of 1421 *N. meningitidis* strains isolated between January 1989 and March 1996 from patients and carriers in Germany, were non-typable (NT) with the currently available monoclonal typing reagents. The National Reference Laboratory in Prague has produced a new serotype 22-specific monoclonal antibody (McAb) which reacts with the epitope of serotype-specific class 2 OMP (1). Its suitability for extending the panel of McAbs currently used for strain discrimination was tested. The isolates examined comprised all subculturable non-typable (NT) as well as non-subtypable (NST) strains of the years 1989-1993 and all NT strains from 1994 to March 1996. Forty-five (11,3%) of 397 meningococcal NT isolates of Germany tested reacted with this new McAb (serotype 22) in whole-cell ELISA (2). The highest prevalence of serotype 22 was seen in 1993 (25%, 7 out of 28 NT/NST strains). Thirty-three (73,3%) of meningococcal serotype 22 isolates derived from patients with invasive disease and 11 (24,4%) from carriers, the origin of one serotype 22 strain remained unknown. With the exception of one isolate, which was serogroup C, all serotype 22 strains belonged to serogroup B (44 strains) although 280 serogroup B and 49 serogroup C strains were examined. The prevalent antigenic formula of serotype 22 strains from 1994 to 1996 (27 isolates) was B:22:NST (10 isolates) and the second most common B:22:P1.2,5 (5 isolates). The geographic distribution of serotype 22 demonstrates that it is found almost exclusively in the region of the former FRG and shows a cluster in Berlin.

Conclusion: Employment of McAb 22 in meningococcal typing provides additional valuable epidemiological data.

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Random Amplified Polymorphic DNA (RAPD) genotyping of serogroup A meningococci yields results comparable to those of Multilocus Enzyme Electrophoresis (MEE).

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The RAPD genotyping method was applied to 1 representative each of the 84 Neisseria meningitidis serogroup A electrophoretic types (ETs) previously described (1). With few exceptions, the subgroup structure was similar by both methods but one strain (ET28 in subgroup I) was assigned to a different subgroup by RAPD and MEE. Further tests showed that six other isolates of ET28 were assigned to subgroup I by RAPD and MEE.

These results were extended by comparing additional strains from individual ETs of serogroup A. Again, the results of RAPD and MEE were fully consistent. Finally, an independent sample of 18 serogroup A strains isolated in The Netherlands was also analyzed by RAPD and MEE. These bacteria again yielded comparable results between the two methods and showed that isolates from the Netherlands included bacteria from subgroups which had not been formerly described. One such strain was assigned to the ET5 complex, which is more typical of serogroup B strains (2).

RAPD analysis was faster and involved less work than MEE. Strains can be linked to previously characterized genotypes in a computerized database, and dendrograms based on cluster analyses can be generated easily. These properties of RAPD make it suitable for quickly assigning new isolates of serogroup A bacteria to known subgroups, for defining new subgroups and possibly for similar purposes with epidemic outbreaks caused by serogroups B and C.

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Epidemic meningococcal disease and tobacco smoke: A risk factor study in the Pacific Northwest

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Background. *Neisseria meningitidis* causes an estimated 2,600 cases of bacterial meningitis and sepsis annually in the United States, with an incidence of one case per 100,000 population and a case-fatality rate of 10 to 15 percent. Since 1992, Oregon and southwest Washington have experienced a substantial increase in the incidence of meningococcal disease, with rates reaching four to eight times the national average. Most of these infections were caused by a group of closely related serogroup B meningococcal strains belonging to the enzyme type 5 (ET-5) complex. The only meningococcal vaccine currently licensed in the United States does not protect against serogroup B disease. Thus, identifying modifiable risk factors for meningococcal disease may be the only means of altering the course of this epidemic.

Antecedent viral infection, household crowding, tobacco smoke exposure, and bar patronage have all been implicated as risk factors for meningococcal disease. However, many of these studies were performed with small numbers of cases, and none assessed the independent contribution of each factor or evaluated the magnitude of risk associated with specific exposures. In this report, we describe the results of a large population-based study to define independent risk factors for meningococcal disease. Using these adjusted estimates of risk, we calculated the proportion of disease associated with specific exposures to develop public health recommendations.

Methods. We performed a case-control study of risk factors for invasive meningococcal disease, comparing 129 cases (67% serogroup B) identified through population-based surveillance in Oregon and southwest Washington (3.5 million aggregate population) with 274 age- and area-matched controls. A case-patient was defined as any resident of the study area from whom *N. meningitidis* was cultured from a normally sterile site between January 1 and December 31, 1994. Control households were identified through a process of random digit dialing, using the modified Mitofsky-Waksberg method. Study subjects or their guardians were interviewed via telephone by trained personnel using a standardized questionnaire. Matched odds ratios were calculated and assessed using maximum likelihood estimates. Dose-effect relationships were investigated for variables with more than two ordered levels. Conditional logistic regression analysis was performed to determine independent risk factors for disease. Adjusted population attributable risks were computed using methods appropriate for multivariate analysis with pair-matched data. Similar results were observed when the analyses were stratified by causative serogroup.

Results. Among children less than 18 years of age, having a mother who smokes was the strongest independent risk factor for invasive meningococcal infection (OR, 3.8; CI, 1.6 to 8.9). Low maternal education, lack of a primary physician and living in a household with other children, and attending school with 30 or more students per classroom were also independent risk factors for meningococcal disease in children. Using a humidifier in the month of interest was protective, and despite its strong correlation with both the smoking and education variables, church attendance was also associated with a decreased risk of disease.

Among adults, having a chronic underlying illness (OR, 10.8; CI, 2.7 to 43.3) and passive tobacco smoke exposure (OR, 2.6; CI, 1.0 to 6.9) were independently associated with invasive meningococcal infection. In addition, active smoking conferred an elevated risk of disease, although this association did not remain statistically significant after adjusting for the other factors (OR, 2.4; CI, 0.9 to 6.6). Risk of meningococcal disease increased with increasing passive smoke exposure in all age groups, and 37 percent (CI, 15 to 65) of meningococcal disease in children could be attributed to maternal smoking.

Conclusions. We found that having a mother who smokes was the strongest independent risk factor for meningococcal disease in children. Results were most dramatic for children less than 5 years old. Adults who were exposed to passive or active tobacco smoke were also more likely to develop disease. Previous studies have implicated tobacco smoke as a risk factor for invasive meningococcal infection (1,2). However, this is the first study to assess the magnitude of risk conferred by tobacco exposure when adjusting for all of the other factors identified, including socioeconomic status and household crowding.

Several studies have addressed the biologic plausibility of tobacco smoke facilitating invasive bacterial disease. Through either its mechanical effects on the respiratory mucosa or its functional effects on the immune response (3), tobacco smoke may directly promote the adherence and/or invasion of meningococci. The association between smoking and meningococcal disease may also be due in part to its indirect predisposition to viral respiratory illness (4). Finally, tobacco smoke, both active and passive, has repeatedly been identified as a risk factor for meningococcal carriage (5,6).

This study provides further evidence that tobacco smoke is a potentially modifiable risk factor for a significant proportion of meningococcal disease. Causality is supported by the strength of its independent association with disease, the demonstration of a dose-response relationship, and biologic plausibility. Reductions in tobacco smoking, especially among mothers with young children, may substantially decrease the incidence of meningococcal disease.

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Epidemiology of meningococcal disease in the Republic of Ireland

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Previous laboratory record review has documented *Neisseria meningitidis* as a cause of significant morbidity in Irish children (1). Establishing current meningococcal disease (MD) incidence rates and serogroup distribution is required to update epidemiological patterns of this disease. A laboratory based surveillance system for culture confirmed MD was established in November 1994 in the Republic of Ireland (ROI) (population 3.5 million). Monthly return forms provide details of cases (name, age, gender, site of isolate, clinical diagnosis, serogroup and survival status). Data are presented here for cases with *N. meningitidis* cultured from deep sites (blood, cerebro-spinal fluid (CSF), and joint fluid) for 1995.

The occurrence of 209 cases throughout 1995 constitutes an annual MD incidence of 5.9 per 100,000 population for the ROI with the rate varying between 8.5 per 100,000 population in the health board with the highest incidence and 0.5 per 100,000 population in that with the lowest incidence. Females comprised 100 (52.6%) cases and 104 (50%) cases occurred in the 4 month period December through March. Nineteen (9.1%) cases died. The highest age-specific annual incidence occurred in children under 1 year (88 cases per 100,000). Serogroup data were available for 195 (93.3%) cases, of which group B comprised 105 (53.8%) cases and group C 87 (44.8%) cases. While the national incidence of serogroup C disease was 2.5 per 100,000 population one health board region had a group C incidence rate of 4.3 per 100,000 population. The mean age of cases with serogroup B disease (7.5 years, range 0.08-68.90) was significantly lower than that for serogroup C disease (9.6 years, 0.17-88.00) ($p=0.02$). Of the two health board regions with the greatest overall MD incidence rates the Eastern Health Board had significantly more cases due to serogroup B than serogroup C (66 versus 34 cases) compared to the Southern Health Board (16 versus 23 cases) (Odds ratio=2.79, 95% confidence interval 1.21-6.46, $p<0.01$).

This surveillance system is the first to document the epidemiology of MD nationally in the ROI. The incidence rate of 5.9 per 100,000 population is one of the highest rates in western Europe and about 4.5 times that reported in the United States (US) (2). The fatality rate of 9.1% is a minimum rate as this data is provided by a laboratory-based system rather than by clinicians. The annual age-specific incidence for meningococcal disease in children under 5 years (43 cases per 100,000) is 72% higher than that for Haemophilus influenzae type b (Hib) disease prior to the introduction of Hib conjugate vaccine in the ROI (3). MD is now the most serious infectious disease which is currently non-vaccine preventable by primary immunisation in the ROI. The variation in MD

incidence rates between different regions in the ROI is wide with that in the highest incident health board being 17 times higher than the rate in lowest incident health board. The current lack of sufficiently effective vaccines for primary prevention of MD is a major drawback in controlling this disease. If the currently available polysaccharide meningococcal A & C vaccine was to be 100% effective and provided to all children at the earliest effective age of 2 years only 29% of all MD in the ROI could be prevented. However, when a conjugate meningococcal vaccine for group C disease becomes available the proportion prevented could be increased to 42% of all disease nationally (assuming vaccine efficacy from 2 months of age). Whatever impact a meningococcal group C conjugate vaccine can make in reducing the burden of this disease, an effective group B vaccine will be necessary to achieve the ultimate - meningococcal disease eradication.

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Production of monoclonal antibodies against *Neisseria meningitidis* that recognize specific and cross reactive antigens

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Meningococcal meningitis and septicemia caused by *Neisseria meningitidis* continue to be worldwide public health problems. The mortality rate is high, and the disease occurs in epidemics.

The monoclonal antibody directed against *N. meningitidis* was established by hybridoma technology (1). Clone 8C7Br1 was obtained from a fusion of mouse spleen cells with the murine myeloma line X63-Ag8.653. Mice were immunized with two doses of live meningococci given both intraperitoneally and intravenously three days before fusion (2). The meningococcal strain used in this study was B:4:P1.9 recovered from patients with meningococcal disease in Brazil. The selection of monoclonal antibodies was initially based on their binding with the homologous strain by ELISA. Immunoblotting of SDS-PAGE resolved, *Neisseria gonorrhoeae*, *Haemophilus influenzae b*, *Escherichia coli*, *Bordetella pertussis*, *Salmonella typhimurium*, *Shigella flexneri* and *Bacillus subtilis* were used to demonstrate antibody cross reactivity. The MAb 8C7BR1 recognized their target antigen, a 50 kDa protein, in different serotypes and subtypes of *N. meningitidis*, *Neisseria lactamica*, *N. gonorrhoeae*, *B. pertussis*, *S. typhimurium* and *S. flexneri*. In *E. coli*, *H. influenzae b* and *B. subtilis* the monoclonal recognized a peptide of 65, 60 and 70 kDa, respectively. The interesting finding that different electrophoretic mobilities were obtained when reacting MAb 8C7Br1 with Brazilian case and reference strains of *N. meningitidis* are under investigation. We have generated the hybridoma cell line which produces IgM monoclonal antibodies reactive against specific antigens of different serogroups, serotypes and subtypes of *N. meningitidis* . These monoclonal antibody provide new tools for antigenic analysis of *N. meningitidis*.

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Prevalence and variation in class 5 expression by serogroup A,C and B during epidemics in Brazil

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The incidence of meningococcal Disease (MD) in Brazil has been monitored since the serogroup A and C epidemics that occurred between 1971 and 1974[1]. In 1988, the incidence of B MD in the greater São Paulo, area state of São Paulo , was over 4.06 per 100,000 inhabitants ,suggesting a new epidemic wave in this region.

The major proteins of the outer membrane of *Neisseria meningitidis* are designated class 1 through class 5. Each strain of *N. meningitidis* also variably expresses up to four distinct class 5 proteins. These heat -modifiable proteins are 25-30 kDa strain variants that differ only in their expression pattern.

Until now, the frequency of most of the class 5 proteins expressed by the Brazilian meningococcal strains had not been determined. Here we present the results of such tests. The production of two novel monoclonal antibodies specific for epitopes present in the class 5 protein derived from a Brazilian *N .meningitidis* epidemic strains (5.8 and 5.9) will contribute greatly to the determination of class 5 proteins to be included as antigens in the future vaccine for meningitis.

We analyzed 63 strains of serogroup A and 60 strains of serogroup C (from the 1972-1974 epidemics), and 136 strains of serogroup B(from the 1988-1994 epidemics). The serotypes and subtypes of *N. meningitidis* B studied were, respectively B:4: P1.15, B:4:P1.9 ,B:4:P1.7, B:4:P1.3, B:4:P1.14, B:4:P1.16, B:4:NT and B:NT:NT. The strains were recovered from blood or cerebrospinal fluid of patients with systemic disease. The anti-class 5 MAbs selected for this study were : 3E6(5.1), 3B4-C7 (5.3) ,1B61C7(5.4), 3DH9F5G8(5.5), 5F1F4-T3 (5.3) and the two new monoclonal antibodies C14F10Br2 (5.8) ,7F11B5Br3 (5.9). To prepare *N. meningitidis* samples (whole cells) for Dot-blot we used the method described in (2).

Our results demonstrated that the expression of class 5 proteins in the *N. meningitidis* B Brazilian strains studied is highly heterogeneous. A vaccine for *N. meningitidis* B to be prepared in Brazil should contain not only the (5.5) antigen but also (5.4) and (5.c), because the expression of the latter one was found to be highly significant by us. The new monoclonal antibodies C14F10Br2 (5.8) reacted with (3.6%) of the serogroup B investigated, yet MAb 7F11B5Br3 (5.9) reacted with (8.8%) of serogroup A, (5%) of serogroup B and (5%) C strains of *N. meningitidis* .

We would like to emphasize the importance of establishing a broad set of antigenic characteristics of the prevalent meningococcal species found in each epidemic region, in order that truly effective vaccines and immunoprophylactic programs can be produced.

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A monoclonal antibody as a probable immunologica[1] differential marker to discriminate bacterial from non-bacterial meningitis

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In public health laboratory the diagnosis of *Neisseria meningitidis* infection is usually accomplished by bacteria culture, counter immunoeletrophoresis and latex agglutination (1).

A correct diagnosis of bacterial meningitis at an early stage of infection is of fundamental importance to the clinician to select the appropriate treatment promptly to avoid serious sequelae.

The partially treated bacterial meningitis cases, as well as those in which neither laboratory tests nor clinical symptoms show a clear distinction between bacterial and lymphocytic meningitis, and even cases of viral meningitis which present, at the on set of infection, a CSF (cerebrospinal fluid) pattern suggestive of a bacterial cause, continue to pose problems for correct diagnosis and, consequently, for early appropriate treatment.

Additional parameters indicating the presence or absence of a bacterial or viral agent would be helpful, especially in countries where meningitis is still frequent.

The new monoclonal 8C7Br1 directed against *N. meningitidis* was established by hybridoma technology (2). The monoclonal antibody was initially chosen based on their binding capacity with *N. meningitidis* strain by ELISA . The monoclonal antibody presented cross reactivity with *Neisseria gonorrhoeae, Haemophilus influenzae b, Escherichia coli, Bordetella pertussis, Salmonella typhimurium, Shigella flexneri and Bacillus subtilis*.

We used a new monoclonal antibody for testing by Dot-ELISA technique. A total of 168 CSF selected in diagnostic routine at Immunology Division of Adolfo Lutz Institute by counter immunoelectrophoresis and bacterial culture. The selected samples were obtained from patients with positive diagnosis of meningitis by *N.meningitidis B, N.meningitidis C, H.influenzae b, Streptococcus pneumoniae* and *E.coli*. Other CSF samples were from patients with some others neurological disorders. In order to see cross reactivity we used antigenic preparations of adenovirus, enterovirus and influenzae virus.

The new monoclonal antibody presented an agreement of reaction of 95% by Dot - ELISA reaction with the CSF of bacterial selected, meanwhile the MAb did not show

reactivity with the selected viral antigen preparations or with CSF samples from patients with other neurological disorders.

The potential use of the new monoclonal antibody in Dot-ELISA reaction as a possible differential immunological marker in discriminating bacterial from lymphocytic meningitis justifies future research.

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Neisseria gonorrhoeae is clonal - sometimes

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The incidence of Gonococcal infection in Grampian region was the highest in Scotland in 1992, 1993 and 1994. Despite a considerable decrease in 1994, it remained more than twice that of the whole country. Grampian also differed from other regions in that 1A isolates were commoner, accounting for around 78%, 73% and 75% of isolates; the 1A-2 serovar was particularly common. The proportion of 1B isolates in Grampian (22%, 27% and 25%) was low compared with the other centres where 1B accounted for around 70% of all isolates (Young & Moyes, 1996). Seventy-six 1992,1993 and 1994 clinical isolates of various serotypes were obtained from the Scottish *Neisseria gonorrhoeae* Reference Laboratory including 40 strains from Grampian and 36 strains from Lothian. Genomic DNA was subjected to pulsed-field gel electrophoresis (PFGE) after digestion with low-frequency cleavage (LFC) endonuclease (NheI). The restriction patterns generated were reproducible, stable and easy to read. PFGE was compared to restriction endonuclease analysis (REA) with high-frequency cleavage (HFC) endonuclease (Hind III). To analyse the results of the NheI and Hind III patterns Dice analysis as well as "Gel Compar" were carried out to facilitate further subdivision of the serogroups. Strains with values of >90% were considered to be clonally related. 37 patterns by PFGE and 36 patterns by REA were identified. A large number of the patterns, particularly those from 1A-2 serovars, were similar, many being identical. These findings clearly demonstrate the potential of PFGE as a highly discriminatory tool, for the epidemiological investigation of gonorrhoea. The temporal and geographical distribution of strains with these patterns indicated that a clone of *N. gonorrhoeae* was circulating in Scotland during the study period.

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Macrorestriction profiles of *Neisseria meningitidis* using pulsed-field gel electrophoresis: Novel epidemiological examples.

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The epidemiological characterization of *Neisseria meningitidis* is currently based upon variation in surface components using serological based methods. However, the epidemiology of meningococci is confounded by the underlying genetic diversity. One technique demonstrated to be useful for the genotyping meningococci is pulsed-field gel electrophoresis (PFGE) in which the genetic relatedness over the whole genome is considered (1,2,3) and is an important method for outbreak identification (4) and cluster analysis (2).

A practicable PFGE method for the characterization of meningococci received at the MRU was investigated, using the restriction enzymes *Sfi*I, *Spe*I and *Nhe*I. The standardized electrophoresis conditions used were; 200v, forward to reverse ratio = 1, switch times 20 to 25 seconds over a 22 hour time period, using the CHEF-DRII apparatus (Biorad). Four novel epidemiological examples are presented to illustrate the application of PFGE for genotyping *N. meningitidis*.

1. *In vivo* mouse model: The stability of the PFGE macrorestriction profiles were shown by the typing of isolates recovered from an infant mouse model of meningococcal infection (5). The original inoculum (B:15:P1.7,16R) and the isolates obtained from the nose, lungs and blood demonstrated identical PFGE DNA types using *Sfi*I and *Spe*I.

2. Stonehouse Meningococcal Survey (SMS): Epidemiologically related isolates obtained from volunteers in the SMS (6) were investigated using PFGE. Seven individuals with two or more isolates of phenotypes (B:15:P1.16R, B:15:NTR or NG:15:P1.16R) were PFGE typed. All the isolates had previously been considered genotypically similar based upon RFLPs using a random DNA-probe (7). PFGE typing demonstrated three main *Sfi*I and six main *Spe*I PFGE types, (where the type definition was based upon three or more fragment differences) (8). There was a single predominant PFGE type with either enzyme (with closely associated subtypes), but repeated isolates from some individuals demonstrated a diversity of profiles.

3. Military recruit study: The methodology was used to track the transmission and carriage of meningococci of a particular phenotype (X:4) amongst military recruits during a longitudinal study of *N. meningitidis* acquisition and carriage. The majority of the 59 X:4 isolates examined by PFGE were categorised into five *Sfi*I and four *Spe*I PFGE types. Two PFGE types predominated, corresponding to the X:4:P1.2 isolates and

a single X:4:P1.16 isolate. The identifying of PFGE types has allowed a more accurate study of the acquisition and carriage of meningococci in the troops. The X:4:P1.2 and X:4:NT isolates were shown to be acquired by several recruits of a particular troop halfway through training. The X:4:P1.16 PFGE type was more frequently isolated from a particular troop, in which carriage was limited.

4. School outbreak: Following a C:2a:P1.2R associated school outbreak investigation 44 of 48 isolates examined were indistinguishable by *Spe*I and *Sfi*I PFGE typing. All three NG:2a:P1.2R isolates were similar to the outbreak PFGE type whereas single C:2a:P1.2R and the C:2a:NT:R isolates demonstrated distinct genotypes.

Genetic stability during meningococcal disease was demonstrated *in vivo* using PFGE on isolates from an infection mouse model. This has not been previously described. Genetic stability following transmission was demonstrated for a large number of isolates during a school outbreak of serogroup C infection. PFGE genotyping of isolates obtained from military recruits facilitated studies of the acquisition, carriage and transmission of meningococci in a closed community. The SMS volunteers showed that some individuals acquired and carried phenotypically similar meningococci but of diverse PFGE genotype over the two year time period. This may suggest frequent genetic events or acquisition of new strains amongst individuals in an open epidemiological context.

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Molecular analysis of *Neisseria meningitidis* class 3 outer membrane protein in the strains recognized by the Monoclonal antibody CB-Nm.2

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The bactericidal monoclonal antibody (Mab) CB-Nm.2 (1,2), specific for class 3 outer membrane protein of *N. meningitidis*, was assayed in Enzyme linked immuno-sorbent assay (ELISA) with a panel of 86 *N. meningitidis* strains. Fifty six strains belonging to seven serogroups: A, B, 29E, L, X, Y, Z and five serotypes: 1, 4, 5, 12, and 13, reacted with CB-Nm.2. The *por*B gene, coding for four different class 3 proteins, was cloned and sequenced. The translated amino acid sequences were compared with five previously published sequences. Sequence alignment revealed the five amino acid region (S/T)VETG located into the major variable region (VR) VR1, which was present in all *N. meningitidis* strains recognized by CB-Nm.2 and was not present in the strains negative in ELISA. Synthetic peptides, containing the predicted antigenic determinant from strains B385 and H355, were designed. Mouse antiserum obtained against the synthetic peptides recognized *Neisseria* strains in whole cell Dot-blot, but synthetic peptides failed to react with the Mab.

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Meningococci causing disease in South Australia 1971 through 1995: A 25 year study

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For the 25 year period 1971 through 1995, isolates of meningococci from children, adolescents and adults with meningococcal disease in South Australia (SA) were examined by serogrouping, serotyping and serosubtyping. Serogrouping was by slide agglutination using Wellcome (now Murex Diagnostics) sera1. For the period 1971 through 1989 serotyping/subtyping was done by FA in Ottawa by ELISA with whole cell meningococci as coating antigen and monoclonal antibodies2. For the period 1990 through 1994 typing/subtyping was done by AL in Adelaide with ELISA using the RIVM meningococcal serosubtyping kit, which now (1996) includes a total of 19 monoclonal antibodies; the method used was as described in the kit product insert except that a commercial substrate was used (Behring). In 1995 a dot blot technique also with RIVM monoclonal antibodies was introduced; this technique uses less antibody than does ELISA and tests in parallel with ELISA showed equal specificity; the dot blot method was easier to read.

For the period of the study the incidence of meningococcal disease varied considerably from year to year. During the period there were no identified outbreaks of meningococcal disease. However, a small epidemic of meningococcal disease occurred in Central Australia in the late 1980's; this may have spread to involve aborigines in SA accounting for the increased number of group A meningococcal infections in SA in 1988 and 1989.

Serogroup B predominated in 22 of the 25 years studies and constituted 126 (55%) of isolates from 230 cases of meningococcal bacteremia and/or meningitis. Group C was next in prevalence, predominating in three years (1984, 1989, 1994) and constituted 55 (24%) of isolates. Of the other groups, Y (20 isolates or 8%) and A (17, 7%) were most common. There were 9 isolates of Group W135 and single isolates identified as groups X or Z; one isolate was non-groupable.

For the 19 year period 1971 through 1989, the main types were 4, 2a, 15 and 14 in that order, and the main subtypes were P1.2, P1.1 and P1.10, in that order3,4.

For the six year period 1990 through 1995 the commonest types were 4, 14 2b and 15; subtypes were diverse, the least uncommon were P1.2 and P1.4.

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Epidemiology, Typing and Diagnostics, Poster 167

PFGE-RFLP analysis of meningococci of the phenotype C:2b:P1.2 causing geographically diverse outbreaks of disease in Australia

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Background. Meningococci of the phenotype C:2b:P1.2 were associated with a number of outbreaks of disease in geographically diverse areas of Australia in 1990 - 1991. Historically, this phenotype was also the commonest sporadic isolate in some areas, but has declined in incidence in 1994 - 1995.

Aim. To compare PFGE-RFLP patterns of the C:2b:P1.2 outbreak isolates with the patterns obtained from randomly selected sporadic isolates 1990 - 1994.

Organisms tested. A total of 35 strains were examined. Twenty strains of serogroup C *Neisseria meningitidis* isolated from epidemiologically confirmed outbreaks, 12 sporadic invasive isolates from 1990-1991 and three strains from 1993 - 1994 were examined by PFGE-RFLP. These last three strains were selected to represent other serogroups and a non-invasive strain which shared the C:2b:P1.2 phenotype of outbreak strains. These organisms were: an invasive serogroup Y:NT:NT isolate, a serogroup B:2b:P1.2 strain and a non-invasive (throat) isolate of a C:2b:P1.2. Both outbreak and sporadic strains were from geographically diverse regions of Australia several thousands of kilometers apart..

Methods. Serotyping and serosubtyping. Serotyping and serosubtyping was performed using a modified immuno dot-blot method and monoclonal antibodies from RIVM, Netherlands.

PFGE-RFLP Whole genomic DNA from the 35 isolates were prepared and restricted with either SpeI or NheI. The restriction digestion fragments for each enzyme was examined by PFGE using the CHEF DRII system (BioRad), stained with ethidium bromide and photographed under ultra-violet transillumination using Polaroid 667 film. Banding patterns were visually compared with molecular weight markers.

Results. Serotyping and serosubtyping:- Nineteen outbreak strains were of the phenotype C:2b:P1.2 (and P1.5[1])and one was C:2b:NT. The 12 sporadic isolates belonged to a variety of phenotypes:

- six were C:2b:P1.2 (and P1.5[1]),
- one was C:2b:NT
- one was C:NT:P1.2
- two C:2a:P1.5,2.

- one B:NT:P1.12,16
- one B:NT:NT.

PFGE-RFLP - Visual examination of the fingerprints for both enzymes showed that 17 of the outbreak strains and seven sporadic strains all with the phenotype C:2b:P1.2 had identical fingerprints. A further two strains (one outbreak and one sporadic isolate) with the phenotype C:2b:NT also had fingerprints identical to the above 24 strains. The two remaining C:2b:P1.2 outbreak strains had two banding differences from the common fingerprint suggesting they were very closely related but based on the epidemiological links would be classified as the same strain.

The banding patterns obtained for the other seven strains, none of which had the phenotype C:2b:P1.2, differed from that obtained for the twenty outbreak and eight sporadic strains.

Discussion. The PFGE-RFLP results suggest that one clone with the phenotype C:2b:P1.2 was involved in invasive disease and associated with geographically diverse outbreaks in Australia during 1990 - 1991. Sporadic isolates of the phenotype C:2b:P1.2 from the same period appeared to belong to the same clone. Furthermore, the same fingerprint has been obtained for recent sporadic isolates of this phenotype. Ongoing PFGE of larger numbers of sporadic isolates belonging to this phenotype will indicate whether there is more than one clone of C:2b:P1.2 associated with invasive disease in Australia.

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Public Health Microbiology, Centre for Public Health Sciences, Cooper's Plain, Queensland.
Microbiology Diagnostic Unit, University of Melbourne, Parkville, Victoria
Department of Microbiology, Princess Margaret Hospital for Children, Subiaco, Western Australia.

[1]Not all strains had the P1.5 epitope detected.

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Examination of the usefulness of three genotype methods to characterize epidemiologically related meningococci

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Background. In October 1993, three children from one family presented to the Accident and Emergency Department of a local hospital suffering from meningococcal meningitis. Throat swabs collected from forty close household contacts yielded a further four strains of *Neisseria meningitidis*. One patient had a repeat isolate of *N. meningitidis* after rifampicin prophylaxis. One week after the three co-primary cases had occurred, an elderly person resident in the same local government area presented with meningococcal disease. No epidemiological link between these cases could be established. These ten isolates form the basis of this study.

Aim. To examine three co-primary cases and contact isolates and assesses the ability of three genotypic techniques to distinguish amongst these strains and the potential use of these techniques for future meningococcal strain differentiation.

Methods. All strains were serogrouped against serogroups A, B, C, W135, Y, Z (Murex Diagnostics). MICs were performed by a standardized agar plate dilution technique. All strains were serotyped and serosubtyped with monoclonal antibodies from RIVM, Netherlands, using a modified immuno dot-blot technique(1,2). Genotypic analysis was undertaking by the following methods: Random amplified polymorphic DNA fingerprinting (RAPD-PCR)(3,4), *por A* gene PCR amplicon restriction enzyme amplification (*por A* PCR-AREA)(5) and restriction fragment length polymorphism detection using pulsed field gel electrophoresis (PFGE-RFLP)(6,7,8).
The reproducibility/stability of banding patterns obtained with these techniques was evaluated.

Results. All invasive isolates and two of the five contact strains were found to belong to phenotype C:2b:P1.2. The strain isolated after rifampicin prophylaxis was also C:2b:P1.2. One contact was NG:2b:P1.2 and the other NG:NT:P1.15. All three genotypic methods found the three co-primary invasive cases and four of the five contacts strains to be identical. The other invasive case, the elderly patient, gave identical banding patterns to the three co-primary cases using RAPD-PCR and *por A* gene PCR-AREA. This same strain, when analysed using PFGE-RFLP, yielded results suggestive of being closely related to the three co-primary cases. The fifth contact strain, NG:NT:P1.15 had banding patterns distinctive from the other strains using all three methods.

RAPD-PCR banding patterns suffered from a lack of reproducibility when different batches of Taq polymerase was used.

Discussion. Phenotyping may not distinguish amongst isolates and in particular, nasopharyngeal isolates may not be typeable by the available phenotypic methods. However, all strains examined were typeable by the three genotypic techniques and all three techniques correctly identified organisms which were strongly epidemiologically related.

1) PFGE-RFLP is a widely used technique, can be used to establish clonal relationships(6,8) and has the added advantage that both the equipment and reagents can be applied to many other organisms. However, it is expensive and may take up to a week to obtain results.

2) The technique of *por A* gene PCR-AREA was found to be reliable and provide rapid results. However, this technique examines only a small segment of the genome. *por* A PCR-AREA may be used as an intermediary method for non phenotypeable strains and PFGE-RFLP performed as a confirmatory genotypic tool.

3) RAPD-PCR, whilst initially promising, suffered from reproducibility problems when different Taq polymerase batches and/or suppliers were used.

The need for further strain differentiation and the final analysis of the results thus obtained should always be made in conjunction with the epidemiological data to hand.

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Meningococcal disease isolate surveillance, New South Wales, Australia, 1994 - 1995

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Introduction. The Australian National Neisseria Network was established in 1994 as a means of laboratory based meningococcal disease surveillance and provides serogroup, MIC and outcome data on invasive disease. The NNN is affiliated with the European Monitoring Group on Meningococci. In New South Wales additional data on serotypes and serosubtypes of meningococci are available for 1994 - 1995. The results of laboratory and epidemiological data for 118 meningococcal isolates are presented. The data presented here relates to invasive isolates only and strains from either throat carriage or other sites were not included in this analysis.

Results. Age distribution and seasonality. In 1995, the seasonal variation in incidence of invasive disease differed from the pattern observed in 1994. More than 25% of cases of invasive disease occurred between January and March 1995 (summer) as opposed to 12% of cases in the corresponding period in 1994. However, the peak incidence for both 1994 and 1995 still occurred during the winter period, July - September. The age distribution for both years was bi - modal with the majority of cases being reported in children under 4 years of age with a second peak in 15-19 years age group.

Serogroup and serotype/subtype data. In 1995, serogroup B accounted for 69% and serogroup C for 27% of invasive strains. This pattern was markedly different from 1994 where serogroup B accounted for only 40% of strains and serogroup C accounted for 57% of invasive strains. The commonest phenotypes of the 1995 serogroup B strains were B:2b:P1.10 (20%) and B:NT:NT (17%). Sixteen phenotypes were found amongst the remaining 25 serogroup B strains. In 1994, the commonest phenotypes of serogroup B were 2b:P1.10 (38%) and NT:NT (17%). Forty-one per cent of the 1995 serogroup C strains were C:2b:P1.5,2 and 31% were C:2a:P1.5,2. In 1994, the commonest phenotypes of serogroup C were 2b:P1.5,2 (44%) and 2a:P1.5,2 (38%). There were two serogroup Y isolates and two non-groupable isolates included over this period. No serogroup A strains were received/reported.

Mortality Outcome data for 1995 was provided for 45 of 58 cases with two deaths being reported. Both fatal cases had serogroup B meningococci isolated from cerebrospinal fluid and /or blood cultures. One fatal case was a 19 year old male and the other was a male of unknown age. In 1994 there were 6 deaths noted in 44 of 60 cases where outcome was notified. One death had serogroup B meningococcus isolated from CSF. The other five deaths were due to septicemia. One patient had serogroup B isolated and four had serogroup C meningococci isolated.

Antimicrobial susceptibilities 1994 - 1995. The penicillin MICs of 117 strains were determined using standardized agar dilution methods. Forty six (39%) strains were "sensitive" (≤ 0.03 mg/L) and 71 (61%) strains were "less sensitive" (0.06 - 0.5 mg/L). The MICs ranged from between 0.016 and 0.25mg/L. All 117 strains tested (one strain was non-viable) were sensitive to ceftriaxone, rifampicin and ciprofloxacin. One isolate had a raised MIC to chloramphenicol.

Discussion. The cases reported during 1995 were all sporadic with no reports of clusters or outbreaks of cases. However, in 1994 there were several interesting cases including two children from the same school who were both infected with a C:2a:P1.5 strain. Staff and children were offered vaccination. .
The increase in serogroup B invasive disease in 1995 was not associated with a corresponding increase in one particular phenotype. No significant change in serogroup C phenotypes was found between 1994 and 1995 although the frequency of serogroup C strains markedly decreased. The four common phenotypes were the same in both years. The penicillin MIC data for 1994 - 95 as summarized, indicates that penicillin based treatment regimens remains suitable for use in the State of New South Wales, Australia.

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Stability of the meningococcal *porA* gene in serial isolates from military recruits

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The meningococcal *porA* gene encodes the class 1 outer membrane protein, which determines sero-subtype specificity and is an important component of candidate vaccines against meningococcal disease. Sequence variations in two specific regions of the *porA* gene (VR1 and VR2) encode differences in the predicted amino acid sequence of two surface-exposed loops of the proposed porin structure (1). Only a limited number of subtypes are recognized by currently available sero-subtyping monoclonal antibodies and sequence variations can occur within a subtype which are not detected by these monoclonal antibodies.

Sequence variation of VR1 and VR2 has been used to examine serologically identical isolates from cases of meningococcal disease and household contacts. Whilst cases and contacts showed a high degree of sequence homology, significant differences were detected in some family groups (2). Such differences suggest that it is possible to use sequence information to differentiate between potential sources of infection, which appear identical using serological methods. An alternative explanation might be the rapid emergence of variants during meningococcal carriage, due to direct mutation within the *porA* gene, or recombination through horizontal exchange between strains. Sequence data obtained from isolates taken at a single time point, or from different individuals cannot differentiate these possibilities. Therefore, we have used DNA sequence analysis to assess the stability of the *porA* gene, studying serial isolates of meningococci cultured from the same individuals over a 30 week period.

Isolates were obtained as part of a longitudinal study of meningococcal throat carriage in cohorts of military recruits undergoing a 30 week training program (3). Many isolates were not readily identified by serotyping, most being either non-groupable, non-typable or non-subtypable. Serologically indistinguishable isolates were selected from individuals who exhibited serial colonization by meningococci and sequence analysis of VR1 and VR2 performed to determine the stability of the *porA* gene over the period of study. Similarly indistinguishable isolates from additional subjects within the cohort and from subjects from other cohorts within the study were also studied. Twenty-five isolates from eight subjects were sequenced, with up to five serial isolates per subject. Single isolates from a further five subjects were also sequenced. Sequence data for VR1 and VR2 from these isolates show the dynamics of transmission and the stability of *porA*.

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Epidemiology, Typing and Diagnostics, Poster 171

Optimizing ascertainment of meningococcal infection in England and Wales - impact of novel diagnostic techniques and reconciliation of available datasets between the national reference laboratory and other surveillance schemes

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Meningococcal disease remains one of the most feared bacterial infections with a predilection for children and young adults and a mortality of 10-12% in England and Wales. Attempts to lower mortality and serious morbidity have been made by United Kingdom health departments recommending that primary care physicians give parenteral penicillin when arranging hospital admission of suspected cases.

The stepwise fall in laboratory ascertained meningococcal infection in England and Wales from a high of 1,500 cases in 1990 to 1,129 in 1994 (1) may be part of the normal cyclical pattern seen in this country, however, the decrease in culture proven cases has not been mirrored by clinical case notifications and the discrepancy between these totals has increased over time. A possible explanation is that preadmission penicillin has adversely affected laboratory diagnosis while media publicity and heightened doctor awareness has increased notifications. This impression is supported by analysis of the mortality and notification data which show a falling mortality rate while notifications of predominantly septicemic meningococcal infection have risen.

Conjugate group C meningococcal vaccines are undergoing immunogenicity trials and early results show promise. The proportion of meningococcal disease burden represented by group C infections in England and Wales fell from 39% in 1988 to 26% in 1994. This, along with the fact that group C infections in England and Wales are more uniformly distributed across age bands than group B disease with about 25% cases occurring in patients aged over 25 years, means that any assessment of vaccine efficacy will need optimized case ascertainment and characterization of infecting organisms from a wide population base. Non-culture diagnostic methods have been developed to this end.

Serodiagnosis based on finding IgM and IgG antibody to outer membrane proteins (OMPs) and group B and C polysaccharide has been available for some years. Cases identified by this means rose from 84 in 1992 to 174 in 1994, the increase in numbers being due to greater use of the investigation by clinicians.

In autumn 1995, an evaluation of PCR for diagnosis of meningococcal infection was commenced. PCRs for the *IS1106* (2), class 2/3 OMPs (*porB*) and sialyltransferase (*siaD*) genes were developed, the last two providing epidemiological as well as

diagnostic information. Guidelines for optimal laboratory investigation of suspected cases which included specimens to obtain for non-culture diagnosis were published and widely distributed (3).

The start of the PCR evaluation period (October - December 1995) coincided with a sharp upsurge in meningococcal disease activity in parts of England. In addition to an increase in culture proven cases to the highest recorded total for this quarter, specimens from large numbers of cases from whom no isolates could be obtained were submitted for investigation by non culture methods - both PCR and serology. Initial assessment of the PCR tests was carried out on 476 evaluable specimens sent from 311 patients. These gave positive reactions on material from 138 of the patients and an isolate was subsequently obtained from 47 of them. These in all but one case, confirmed the PCR group and/or typing when this was available. The 91 cases identified by PCR alone represented an 20% increase in laboratory ascertained infection and serodiagnosis further added to this total. Additional details along with data from the rest of the evaluation phase will be presented.

An attempt was also been made to identify overlap between cases investigated by laboratories and those statutorily notified (where no laboratory confirmation is necessary) and assess the extent to which clinical judgment alone was used to designate cases as meningococcal infection and also to see what proportion of laboratory proven cases were never formally notified.

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Clonal distribution of disease-causing *Neisseria meningitidis* in the county of Telemark, Norway 1987-95; studied by PCR Amplicon restriction endonuclease analysis (PCAREA)

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Sequence analysis of the dhps gene of *Neisseria meningitidis* shows the presence of both conserved and variable regions (1). This allows construction of PCR primers that are homologous with conserved regions, but whose amplicons contain variable regions. We have shown that such primers can be used both for detection (2) and typing (3) of meningococci.. Detection is based on visualization of an amplicon of expected size, while typing is done by HhaI amplicon restriction endonuclease analysis (PCRAREA).

In the present study, we used PCR AREA to determine the clonal distribution of all meningococci from culture-confirmed meningococcal disease in the county of Telemark, Norway (165.000 inhabitants) during the period from November 1987 to March 1995. There were 42 isolates from 42 patients. For typing, we used the NM7/NM6 set of primers which amplify a sequence of 634 bp. All isolates were additionally serogrouped, serotyped, their sulfonamide susceptibility determined. Chromosomal DNA fingerprinting cleaving DNA with HindIII was performed on all isolates. We also collected information on the patients age, sex, clinical presentation and outcome, and place of residence.

We were able to identify 11 different meningococcal PCRAREA classes among the 42 isolates. The predominant classes were class I, II and III containing 15, 8 and 6 isolates, respectively. The apparent intraclass identity and interclass variability of these three classes were confirmed by chromosomal DNA fingerprinting. Class I isolates were all sulfonamide resistant; were of serogroup B, and 12 of 14 which were typed, contained the serotype 15 protein and ten isolates were of subtype P1.7,16. All class II isolates were sulfonamide resistant, were of serogroup C and of serotype 2a:P1.2,5. The class III isolates were all sulfonamide sensitive, of serogroup B and contained the serotype 16 protein. The remaining classes (IV-XI) were more heterogeneous.

Epidemiologically, class I isolates were predominant in 1987 and 1992, but were absent in 1989, 1990 and 1994. Class II isolates were present each year until 1992, but have not been seen since. Class III appeared in 1991 when it was the predominant class, was present in 1992, but has since disappeared.

The Grenland area, which is the most densely populated area of Telemark (120.000 inhabitants), had an incidence over the study period of 20 cases/100.000 inhabitants. The corresponding figure for the 45.000 inhabitants of remaining Telemark, was 38. Ten out of 15 class I isolates were from outside Grenland; an incidence during the study period of 22/100,000 inhabitants. In Grenland the incidence of class I was 4/100,000 inhabitants. Class II and class III isolates, however, were almost exclusively seen in the Grenland area.

24 of the patients were below 6 years of age, 12 were between 11 and 20 years, and 6 were between 39 and 77 years of age. There was no patients between 20 and 39 years of age. The mean age of the patients was 14.3 years, but we observed that the mean age varied from year to year; in 1987 (5 patients) the mean age was 6.5 years, in 1988 (8 patients) 19.5 years, in 1989 (2 patients) 38.5 years, 1991 (7 patients) 15.2 years, 1992 (8 patients) 6.1 years and in 1993 (2 patients) 0.8 years. The most predominant classes among children from 0-5 years of age were classes other than class I,II, and III (39%), class I occurred in 35%, class II in 13% and class III in 17% of these patients. Twenty-nine (69%) patients were male and 13 (31%) female. The different classes of meningococci were equally distributed in the two sexes.

A total of 17 patients were diagnosed as having septicemia alone, 12 had septicemia and meningitis, eleven had meningitis and 2 had benign meningococcemia. We observed no significant association between any class of meningococci and clinical presentation. Four patients (9.5%) died from their disease. Those who died had disease caused by class I or II meningococci.

There is a need for a rapid and reliable method to differentiate between clones of meningococci. The PCRAREA method based on primers from the dhps gene, has the advantage that it can be used both for diagnosis of meningococcal disease and for typing of the bacterium directly in CSF. PCRAREA can also be used for detecting and typing meningococci in pure culture, in mixed culture (primary isolation) and may also be used on throat specimens and blood. PCRAREA may therefore, be applied for the rapid identification of the disease-causing strain in healthy contacts of patients with meningococcal disease, for screening selected populations for pathogenic meningococci (military recruits), and for the surveillance of variation in clone distribution in a population giving valuable information for vaccine recommendations. The PCRAREA band patterns can be digitized and stored in digital databases. There exist PC programs which compare band patterns, automatically recognize and identify patterns, and allow the construction of dendrograms. Our work progresses along these lines.

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Molecular epidemiology of meningococcal disease in Iceland 1977-1995.

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Iceland is an island in the North-Atlantic ocean with approximately 270,000 inhabitants. There are >100,000 annual arrivals and departures of travellers from the island.

There is one central microbiology laboratory serving the whole country where most meningococcal isolates have been identified and data on patients has been collected by one physician. These facts make Iceland a unique place to study the molecular epidemiolgy of meningococcus.

An epidemic of meningococcal disease started in Iceland in 1975 reaching a peak incidence of $37.7/10^5$ cases in 1976. The incidence declined during 1977-78, but remained hyperendemic until 1988. In 1989 the incidence rose again reaching $11.3/10^5$ in 1994, but declined to 5.4 in 1995. Since 1977 isolates of meningococci from patients have been stored frozen and by the end of 1995 the collection consisted of 231 viable isolates. In addition, 150 isolates were obtained during an investigation of a cluster of cases in 1991, and carrier rate of college students in 1993.

A recent study compared different molecular methods to differentiate among meningococcal isolates and found that pulsed field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MEE) and ribotyping were comparable, both with respect to typeability and discrimination of strains (1).

The available patient strains were analysed by serotyping (ST), sero-subtyping (SST) and multilocus enzyme electrophoresis (MEE) (141 isolates). Pulsed field gel electrophoresis (PFGE) on *SfiI* digested whole chromosomal DNA (381 isolates) (2) but modified according to Maslow et al. (3). Susceptibility to penicillin (P), sulfadiazine (S) and rifampin (R) (231 isolates) was determined using the E-test™.

From 1976 through 1984 serogroup B (predominantly B 2b:P1,2) was most prevalent with serogroup A seen sporadically in 1976 and in 1980 through 1982. None of the group A strains were analysed by ST or SST. However, by PFGE seven of eight isolates were identical while one was unrelated. Serogroup C was initially isolated in 1978. It remained sporadic until 1982 but has subsequently been hyperendemic. Analysis of the serogroup C isolates by ST and STT showed three contiguous "clones" since 1982. These data correlate well with the genotyping data as determined by both PFGE and MEE. The incidence of serogroup B was low between

1984 and 1990 when a surge in the number of serogroup B cases occurred. Initially ST and STT analysis of the serogroup B isolates showed mostly group 21:P1.16 (seen in serogroup C isolates also) with occasional 15:P1.7,16. From 1992 the latter has, however, predominated and was in fact responsible for an outbreak between 1992 and 1995 (ET-5 complex).

By PFGE, the isolates (n = 231) were allocated to 35 genotypes (clones) and further into 11 subgroups according to previously published criteria (4). One hundred forty one isolates were analysed by MEE and they were assigned to 42 electrophoretic types. The results were well correlated between both methods (PFGE and MEE). By PFGE fourteen genotypes included ≥ 3 isolates and five genotypes > 10 isolates. With the exception of two years (1988-89) three of the five genotypes caused disease yearly. The three most common genotypes have been in our community since 1980, 1981 and 1989. Two of these (ETs 19-22 and ET-37 complex) are of serogroup C; one of them (ETs 19-22) so far has only been identified in Iceland and one of group B (ET-5 complex). Twenty one of 35 PFGE genotypes (60%) occurred as sporadic cases representing 10% of patient isolates.

All patient isolates were susceptible to R and P. One genotype showed an intermediate susceptibility (MIC = 0,125μg/ml) to P. Resistance to S (MIC > 125 μg/ml)was noted in several genotypes (ET-5 complex).

The study demonstrates that more than one strain was responsible for meningococcal disease at any given time. Usually there was a dominant epidemic strain occurring with one or more sporadic strains. A particular clone may cause sporadic disease for several years (up to 10 years) before causing an epidemic. Further, among sporadic cases, at least 5 years may lapse between occurrences of cases infected with the same strain.

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Antigenic variations within the genetic clone ET-15/37 of *Neisseria meningitidis* occurring in the Czech Republic

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A new genetic clone of *Neisseria meningitidis* appeared in the Czech Republic in 1993 and caused an unusual epidemiological and clinical situation in invasive meningococcal disease characterized by increased morbidity and fatality rates, a changed age distribution, local outbreaks, and changed clinical courses. The clone was recognized using multilocus electrophoresis as ET-15 belonging to ET-37 complex and was practically identical to the clone identified in Canada at the beginning of the 1990's (1) This clone, which was quite new in the Czech Republic, occurred in 1993 in two districts of the country. It showed the following phenotypic characteristics: C:2a:P1.2(P1.5). The variability of the P1.5 serosubtype seems to be attributable to the conditions of *Neisseria meningitidis* culture. Class 5 proteins were uniform (P5.II, P5.III) and pili as well (II b). The agent was responsible for a high fatality rate (20%) and the highest morbidity in the age group 15-19 years (2). In the district with the highest incidence of invasive meningococcal disease, a targeted vaccination with A+C polysaccharide meningococcal vaccine in the most affected age group (with the age specific morbidity 57 per 100,000) prevented the spread of the disease caused by *Neisseria meningitidis* C (3,4)

The new clone spread all over the country later (1994-1996) and changes were recognized in the age distribution of invasive meningococcal disease: a shift to the age groups 1-4 years, 0-11 months and adults (5). Two antigenic variants in which either serotype or serosubtype had not been determined by WCE were found in 1994 and in the first part of 1995: *Neisseria meningitidis* C:**NT**:P1.2(P1.5) and *Neisseria meningitidis* C:2a:**NST**, respectively. In the second part of 1995 a new very important antigenic change was revealed: serogroup B appeared in combination with serotype 2a and serosubtype P1.2(P1.5). These strains **B**:2a:P1.2(P1.5) belong to the genetic clone ET-15/37 like the previous antigenic serogroup C variant. Alarming is the high fatality rate due to serogroup B variants (20%), while that due to serogroup C variants has a decreasing trend: 20% in 1993, 13.8% in 1994 and 8.4% in 1995.

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The new serotype of *Neisseria meningitidis*

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A high percentage (50-80%) of the *Neisseria meningitidis* strains isolated in the Czech Republic since 1973 remained non-typable (NT) and/or non-subtypable (NST) in spite of a large collection of monoclonal antibodies (MAbs) used for the whole-cell ELISA (WCE) (1). A hypothesis arose that the isolated *Neisseria meningitidis* strains might be different from those used in the world for MAbs production and therefore new meningococcal serotypes and/or subtypes might be present among meningococci isolated in the Czech Republic. A project focused on the problem of NT/NST *Neisseria meningitidis* was started and a new serotype candidate has been identified (2, 3).

Construction of producing hybridomas was made by the classical method of fusing a murine myeloma line SP2/0-Ag14 cells and activated lymphocytes obtained from BALB/c mice immunized by the whole-cell antigen. Cell fusion was initiated using PEG 1550 in the conditioned RPMI 1640 medium. The highly positive clones were selected for production of murine ascites. The Ig class and sub-class of MAb was determined by means of Isotype AB-STAT^{TM}-I test (SangStat Medical Corporation). The hybridomas, clones and ascites were tested by WCE and immunoblotting and showed good sensitivity and specificity. The new MAb does not react with any of the serotype/subtype reference strains but does react with the strain used for its production at a 1:1,000 dilution. The immunoblotting has allowed to recognize that the new MAb reacts with the epitope of the serotype-specific Class 2 OMP. Therefore this new MAb was designated serotype 22-specific, as the last number of serotype-specific MAb available is 21 of Dr. Zollinger.

A collection of 97 *Neisseria meningitidis* B:NT strains isolated from cerebrospinal fluid or blood of patients with invasive meningococcal disease in the Czech Republic between 1973 and 1994 was serotyped with the new MAb and 37% of these strains gave positive WCE result. The period of 22 years was divided into two sub-periods: 1973-1992 and 1993-1994, to allow to see whether there was a difference in the strains incidence after the appearance of the new clone *Neisseria meningitidis* C:2a:P1.2(P1.5), ET-15/37 in the Czech Republic in 1993. A lower percentage of the new serotype 22 has been found in the later period but the difference is not statistically significant (40.4% versus 35.6%).

In 1995, the new serotype 22-specific MAb has been included into our MAbs collection used for routine WCE serotyping. Fifty-nine *Neisseria meningitidis* B:NT strains isolated from individuals in different clinical situations (i.e. invasive meningococcal disease, respiratory disease, contact, carrier) in the Czech Republic from January 1995 to May 1996 were serotyped and 26 of them (44.1%) appeared positive with the new serotype

22-specific MAb. Seven of the *Neisseria meningitidis* B:22 strains were isolated from patients with invasive meningococcal disease mostly aged 0-4 years. The significance of the new serotype 22 candidate was underlined recently: it was recognized in the *Neisseria meningitidis* B strains isolated from three died patients. The geographical distribution of the new serotype meningococci is general all over the Czech Republic.

These results indicate the epidemiological and clinical significance of the new serotype candidate 22. The new MAb was offered to seven European laboratories (in Athens, Bilthoven, Glasgow, Graz, Heidelberg, Manchester and Oslo) and their first results are shown in other presentations. An international discussion of the importance of this new meningococcal serotype is desirable, including a suggestion of its final numbering, because the number 22 has been already used for the serotype-specific monoclonal.

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The molecular characterization of a new meningococcal serotype: serotype 22

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It is now recognized that *Neisseria meningitidis* particularly serogroup B organisms are genetically diverse due in part to frequent recombination between strains. This results in constant emergence of new serotypes often heralded by increasing numbers of nontypable strains. Large numbers of Group B meningococci isolated in the Czech Republic were non-serotypable (NT) with the available panel of monoclonal antibodies (MAbs) (1). Recently, a significant new meningococcal serotype designated serotype 22 was identified amongst nontypable isolates from the Czech Republic which is found in 37% of the Czech B:NT strains isolated from invasive meningococcal disease within a 22 year period (1973-1994) (2). This demand necessitated the production of a serotype 22 specific monoclonal antibody for inclusion in the serotype reagent panel, illustrating the need for constant expansion of the reagent panel to include new serotypes. Following the introduction of the serotype 22 MAb, 44% of the Czech B:NT isolates from 1995 were identified as serotype 22. Since then serotype 22 strains have been identified outside the Czech Republic including England and Wales where both serogroup B and C serotype 22 isolates have been identified.

DNA-based typing methods for *Neisseria meningitidis* may overcome many of the problems inherent to the serological typing system. The characterization of the serotype 22 *porB* gene of Czech and UK isolates by molecular fingerprinting techniques, namely T-tracking and direct nucleotide sequence analysis identified two class 2 *porB* sequence types amongst the Czech isolates. These were designated type 22a and type 22b. Amongst the UK isolates analyzed only the serotype 22b type was identified. The two serotype 22 types have common sequences in variable loops I and VI of the gene. Sequence data was used to design a serotype 22 specific oligonucleotide probe for incorporation in a *porB* serotype specific DNA probe panel for PCR-based *porB* DNA typing. Furthermore, the molecular characterization of the serotype 22 including the design of the serotype specific probe can be performed in a reasonably short time allowing the rapid expansion of the serotype probe panel in response the emergence of new serotypes.

This presentation illustrates the potential of DNA-based methods for the rapid characterization of *porB* variation and the identification of new epidemiologically important serotypes. Furthermore, the increased discrimination achieved using DNA-based typing as shown here by the identification of serotypes 22a and 22b provides

enhanced surveillance for the better understanding of the epidemiology of meningococcal disease.

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Antigenic phenotype of meningococcal strains isolated from patients and carriers in Greece using the new monoclonal antibody designated 22

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The National Meningococcal Reference Laboratory in Greece started serotyping and subtyping of *Neisseria meningitidis* strains in 1989 using the whole-cell ELISA (WCE) (1-3). In spite of a large collection of monoclonal antibodies (MAbs) used, the majority of the Greek *Neisseria meningitidis* isolates do not react with the serotype- and subtype-specific MAbs available at present. The percentage of the non-typable (NT) and/or non-subtypable (NST) isolates ranges from 40 to 60% indicating, that the antigenic phenotypes associated with the outbreaks in Britain and Scandinavia are not common in Greece (4).

A new serotype preliminarily designated 22 resulted from the research of the National Reference Laboratory for Meningococcal Infections in the Czech Republic and a new MAb has been produced recently. We are presenting the attempt to serotype the Greek meningococcal non-typable isolates with the new serotype 22-specific MAb.

A collection of 430 non-typable meningococcal strains from both patients (52) and carriers (378) was tested against the type 22-specific MAb. Approximately 10% and 9.5% of the meningococcal strains isolated from patients and carriers, respectively, were serotypable with the new serotype-specific MAb. The most frequent phenotype combination found was B:22:P1.13 among the patients' isolates and B:22:P1.7 and A:22:P1.5 among the carriers' isolates.

It was shown that the significant portion of Greek meningococcal strains remained still non-serotypable with the enlarged serotype and subtype reagents panel. On the contrary, a high percentage (29.7%) of the carrier strains isolated from immigrant schoolchildren originated from Russia, which were previously non-typable, reacted with the new MAb.

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Factors relating to carriage of *Neisseria meningitidis* and the Lewis antigen phenotype

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There is a long history of investigations on carriage of *Neisseria meningitidis* in the Czech Republic. Carriage was about 10 % up to the late 1970s when it reached 80 % among the population in the 15-19 year age range; however, this was not associated with an increase in the incidence of invasive meningococcal disease. The *N. meningitidis* strains isolated from carriers were very heterogeneous for capsular and non-capsular antigens (1,2).

The new meningococcal clone ET-15/37 appeared in 1993 in the Czech Republic and caused a dramatic increase in the morbidity and fatality rates of this disease. A study of carriers of meningococci was initiated to analyze the factors influencing carriage and possible development of invasive meningococcal disease. It has been suggested that various meningococcal clones differ in their transmissibility and virulence among different populations.

The investigation began in February 1996 in the Czech district of Olomouc which had the highest incidence of invasive meningococcal disease. The new clone first appeared here in 1993 and a successful targeted vaccination program was carried out among the 15-19 year age group to control the outbreak (3). Two groups were investigated in this voluntary study: 116 children in the 1-4 year age range, 415 in the 15-19 year age range. The first sampling included a nasopharyngeal swab, blood and saliva specimens. The parents filled in a questionnaire on socioeconomic and health factors. Participants from whom *Neisseria* species were isolated were sampled again two weeks later for carriage and blood and saliva specimens were collected. A second questionnaire concerning the period between the two samples was administered.

The first sample found no meningococci in the younger age group and a carriage rate of 7.7 % for *Neisseria lactamica*. All these children were negative for both species when retested. *N. lactamica* was not isolated from any of the children in the older age group but the carriage rate was 6.2 % for *N. meningitidis*. All but 2 of the 33 carriers were positive for meningococci when retested (93.9%). At both samplings isolates from 38.7% of the carriers had the same capsular phenotype and 96.8% of the isolates had the same non-capsular antigens (serotype and subtype).

Analysis of ABO and Lewis blood groups has been completed for the older group. There was no difference in the distribution of ABO blood groups among the 34 carriers compared with the distribution of these groups among the population tested: A = 48%; B = 0%; O = 28%; AB = 3%. Assessment of Lewis blood group antigens found that there was an increase in the proportion of carriers among those individuals whose red cells were not agglutinated by either monoclonal anti-Lewisa or anti-Lewisb (Lewis-negative). There were 384 subjects whose red cells were agglutinated by the antibodies and 27 (7%) were carriers. There were 57 Lewis-negative subjects and 8 (14%) were carriers, but the differences were not statistically significant.

The sera are being investigated for bactericidal and opsonizing activities in relation to antibodies detected to bacterial components by ELISA. The data from the questionnaires are being analyzed and the preliminary results will be presented.

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Automated direct nucleotide sequence analysis in the study of meningococcal antigenic variation.

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The surface proteins of *N. meningitidis* are antigenically highly variable. Our previous studies of two meningococcal such proteins, PorA and PorB, which are important as epidemiological markers and potential vaccine components, indicated that, for a variety of reasons, serological analyses underestimate this variability (2,3,6,8,9). In addition, the monoclonal antibodies that identified meningococcal serotypes and subtypes provide information that is of limited use in understanding the mechanisms whereby antigenic variation arises. If multivalent vaccines which use cocktails of surface proteins are to be introduced, it is important that we have a comprehensive understanding of the diversity of these antigens in meningococcal populations, the mechanisms by which they change, and rate at which they change.

To overcome the problem of strains that could not be characterised by serological means, a number of groups developed DNA-based approaches for rapid characterization of isolates (e.g. Refs. 1 and 5). However, even these approaches do not necessarily guarantee comprehensive strain characterization (2). In the last few years automated nucleotide sequence technology has become increasingly available, rapid, and cost-effective. Determining the nucleotide sequences of potential antigens and typing targets has several advantages. It provides accurate, unambiguous and detailed data which, in addition to characterizing strains, can be used for detailed epidemiological and population genetic analyses. Further, the same methodology can be used for any gene target, requiring only oligonucleotide primers for that gene. Once complete a complete genome sequence is available for the meningococcus, any gene can be studied in multiple isolates by this approach.

We have instituted a program to assess the feasibility of using nucleotide sequence analysis for the routine characterization of meningococcal isolates and are sequencing the PorA protein from all strains isolated in the UK in the current year (November 1995 onwards; between 1000-2000 isolates). These data will be used in the following analyses:

(i) Comprehensive identification of PorA in the UK population of meningococci, providing a baseline of data for retrospective studies of antigenic change of these antigens over the last 20 years;
(ii) Comparisons these data with data obtained by serosubtyping;
(iii) Establish the feasibility of using nucleotide sequence analyses routinely;

(iv) Production of data suitable for testing of mathematical models of the evolution, persistence of antigenic variation, and strain structure (4,7).

In conclusion, direct automated nucleotide sequence analyses provide the prospect of comprehensive and accurate typing data that can be also applied to population studies and easily extended to the study of any genetic locus of the meningococcus.

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Confirmation of meningococcal sepsis using diagnosti- PCR of urine samples - a case report

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The life threatening nature of sepsis necessitates aggressive treatment upon patient presentation. *Neisseria meningitidis*, one causative agent of sepsis, is frequently difficult to culture from patient fluids as a result of empirical antibiotic treatment (1). Oftentimes, diagnosis of meningococcemia is presumptive, rather than confirmed. In an effort to confirm the etiology of such a case, a previously described nested polymerase chain reaction (nPCR) methodology (2) was modified for use with serum and urine. This technique confirmed the presence of meningococcal DNA, subtype P1.16, in the urine of a patient.

The patient was a young active duty male who was stationed in the DMZ in South Korea. He presented at an outlying clinic with systemic toxicity with fever, petechial rash, monoarticular arthropathy and conjunctivitis. He was treated empirically with a meningitic dose of IV ceftriaxone, fluids and hydrocortisone and was evacuated to the 121st General Hospital in Seoul where cultures were subsequently obtained.

He reported no recent insect bites, no recent sexual history nor any recollection of meningococcal vaccination. Laboratory evaluation and ancillary studies found coagulopathy/mild thrombocytopenia. Cerebrospinal fluid (CSF) examination was within normal limits with no antigens detected for *N. meningitidis* groups A and B, *S. pneumoniae* or *H. influenzae*. Skin lesions were biopsied, and rare Gram negative diplococci were observed. Urinalysis was normal. Cultures of blood, CSF, urine and skin biopsy tissue were negative. The patient was treated with intravenous penicillin G, showed immediate clinical response and was discharged after 10 days.

Concern remained that definitive diagnosis of meningococcal disease had not been made due to the remote possibility of gonococcal contacts. Frozen CSF, sera and urine samples were sent to WRAIR to attempt to confirm the diagnosis of meningococcemia by nPCR. Samples of CSF were treated as previously described (2). Controls included previously identified CSF samples as well as frozen urine samples from which gonococci had been cultured in a range from 0 CFU/ml to 8,000 CFU/ml (provided by Dr. K. Schmidt). Five hundred ml samples of serum and urine were centrifuged, and the pellet was resuspended in water. After heating, the urine and serum samples were processed like CSF samples. Nested PCR was performed as previously described, except that the concentration of meningococcal *porA* specific primers was increased to 250 pM/reaction. An ethidium bromide gel confirmed the amplification of DNA of the appropriate size from the urine as well as from the positive CSF controls, but not from the urine samples

containing gonococci or from the serum samples. The DNA product from duplicate urine samples was gel purified and partially sequenced through the subtype specific variable region 2. Sequence analysis of this region dete: ıed that the DNA was meningococcal subtype P1.16.

Detection of meningococcal meningitis by nPCR has been demonstrated in retrospective analyses of CSF samples from vaccine trials (3,4). Suspected but unconfirmed cases of meningococcemia from these trials could not be assayed using CSF samples. Whole blood samples, rather than serum, might yield detectable amounts of DNA in association with white blood cells of the patient. However, whole blood is not usually collected and preserved. Urine samples are commonly collected and tested for meningococcal antigen by latex agglutination methods. Preservation of urine or urine sediment for nPCR analysis even with a normal urinalysis would be a simple addition to sample collection procedures. Further development of this nPCR with urine samples collected from meningococcemia patients will help to expand the effectiveness of PCR in confirming all cases of suspected meningococcal disease.

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Epidemic of serogroup B meningococcal disease in New Zealand has parallels with that observed in the Netherlands, 1980-1990 .

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New Zealand has been experiencing a meningococcal disease epidemic since mid-1991(1). Total numbers of cases have increased from an average of 51 per annum for the 1989-90 pre-epidemic period to 394 cases in 1995. This is an increase in the rate of disease over the total population from 1.5 per 100,000 for the years 1989-90 to 11.7 per 100,000 in 1995. Based on current numbers it is predicted that about 600 cases will occur in 1996 giving a rate of 17.5 per 100,000. These rates contrast with the rate of 2.2 per 100,000 recorded in Australia in 1994 (2). Disease rates are highest among our Maori and Pacific Islands children under the age of five with the highest rate of 278.8 cases per 100,000 occurring in Pacific Islands children under one year of age. In contrast a smaller peak in rates of disease, occurring in the 15-19 year age-group, has been experienced by Causasians. A total of 21 deaths occurred during 1995 giving a case-fatality rate of 5.3%. The case-fatality rate has been constant since 1992.

Most disease has been caused by serogroup B meningococci. In 1995 serogroup B accounted for 182 cases (76.8%) where a viable meningococcus was recovered. Serogroup C accounted for 49 (20.8%) of cases. Serogroup B with phenotype B:4:P1.4, emerged as dominant in mid-1991 coincident with the start of the epidemic (1). Since then isolates with this profile have increased as a proportion of all serogroup B meningococci tested. In 1995 B:4:P1.4 isolates represented 72% of all serogroup B meningococci. Although not included in this percentage, there have been some isolates of subtype P1.4 which are non-serotypable or are serotype 14. Numbers of such isolates appear to have increased in 1996. Macrorestriction fragment length polymorphism typing using *SfiI* and pulsed field gel electrophoresis has shown that the majority of B:4:P1.4 isolates belong to a single pattern type although some have demonstrated distinct restriction fragment patterns.

A representative set of serogroup B and serogroup C isolates of varying serotypes and serosubtypes were sent to Dr. Dominique Caugant, Norway, for multilocus enzyme electrophoresis typing. Results show that all 13 isolates with phenotype B:4:P1.4 recovered since 1991, belong to clones of the same lineage III. Of note, two isolates recovered in 1989, one with phenotype B:4:P1.4 and the other with phenotype B:4:non-subtypable, did not belong to this lineage. The results are consistent with the concept that meningococci with phenotype B:4:P1.4 appear to have been introduced into New Zealand around 1991.

A similar increase in meningococci with phenotype B:4:P1.4 and belonging to lineage III was observed in the Netherlands in the 1980's (3). The insidious increase in incidence of meningococcal disease that has accompanied the recognition of meningococci of this phenotype in New Zealand since 1991 also has parallels with the pattern of meningococcal disease observed in the Netherlands. The current development of outer membrane vesicle vaccines containing the Por A $P1.7^{h},4$ (4) offers some hope for the protraction of our epidemic.

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PCR-SSCP of clinical specimens for non-culture-based sub-typing of the meningococcus in clinical specimens

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Introduction. In the absence of a microbial culture, confirmation of the diagnosis can be made using the polymerase chain reaction (PCR) which can detect presence of meningococcal DNA in both CSF (1-4) and blood specimens, even after antibiotic treatment. However, present PCR tests do not give information about the sub-specific typing of meningococcal strain present in the disease. Sub-specific typing of strains from cases and carriers of the meningococcus is important for the following reasons: (i) a vaccine is currently available for serogroup A and C strains and it is therefore important to identify outbreaks associated with these serogroups; (ii) typing may be used to demonstrate epidemiological links between cases, and between cases and carriers in an outbreak situation; (iii) typing is important to monitor the changing epidemiology of disease; and (iv) multivalent serogroup B vaccines based on outer membrane proteins are presently being evaluated.

We have therefore developed a PCR-SSCP technique to obtain sub-specific typing information on meningococci present in blood and CSF. We amplified a segment of the variable region VR1 of the *por*A gene since VR1 is the target for many of the sero-subtyping antibodies and a great deal of information is available concerning sequence variation at this locus (5-8). PCR amplification was followed by single stranded conformational polymorphism (SSCP) analysis of the PCR product to detect differences in base sequence at the target locus. PCR-SSCP was used to demonstrate both identity and non-identity of meningococcal strains from clinical specimens and between clinical specimens and cultured strains.

Methodology. Clinical samples included blood and CSF cultures, boiled CSF, or DNA extracted from buffy coat or serum, as described (Newcombe et al) were obtained from Gloucester, Plymouth, Hereford and Cheltenham PHL. All meningococcal cultures had previously been typed by Meningococcal Reference Laboratory, Manchester PHL. DNA was extracted from meningococcal cultures using standard DNA extraction protocols. The DNA was amplified using PCR WITH fluorescein labelled 12-dUTP incorporated into the PCR reaction mix. The products were analysed either by electrophoresis on the Applied Biosystems ABI 373A Automated DNA Sequencer.

Results. We investigated strains belonging to five different sero-subtypes. Each strain of the same sero-subtype tested was found to generate a distinct SSCP banding pattern. We next investigated eight strains of the same sero-subtype but differing in serogroup

and/or serotype designations. Each of the eight strains gave identical SSCP banding patterns. We then demonstrated that clinical specimen produce the same banding patterns as DNA extracted from microbial cultures. We next examined four clinical specimens (2 serum 2 CSF) obtained from four different patients where subsequent *N. meningitidis* cultures were found to be of the same p1.16,7 sero-subtype. Again, banding patterns that could be superimposed were obtained. Lastly, we examined DNA extracted from four clinical specimens (all CSF) obtained from four patients from whom *N. meningitidis* strains of differing sero-subtypes were isolated. Distinct banding patterns were obtained for each specimen.

Discussion. PCR-SSCP analysis may be used to demonstrate either identity or non-identity of strains present in clinical specimens taken from different patients. This technique may be used to establish the existence of an outbreak and to investigate where active transmission may be occurring within the community. Direct PCR-SSCP of clinical specimens may also be important for disease surveillance in situations where microbiological culture of patient specimens is negative.

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Meningoencephalitis. Notified cases between 1969 and 1995 in Argentina.

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An analysis of meningoencephalitis notified cases from 1969 to 1995 was made from the existing National Ministry of Health database. This study showed two outbreaks. The first occurred between 1974 and 1978 with the peak in 1976 and a rate of 15.2 cases per 100,000 inhabitants due to *Neisseria meningitidis* group C.

The second one began in 1992 with a peak in 1994 and a rate of 11.4 cases per 100,000 inhabitants. This last outbreak was due to a succession of outbreaks in restricted geographical areas of *Neisseria meningitidis* group B and group C with incidence rate between 0.2 and 18 cases per 100,000 inhabitants. *Neisseria meningitidis* group B was mostly observed in children under one year old. Meanwhile *N. meningitidis* group C was mostly detected in older children. During this second outbreak better microbiological detection agents were used and a better epidemiological study was performed. The mortality has not been variable during the last outbreak.

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Polymerase chain reaction of peripheral blood for the diagnosis of meningococcal disease.

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Confirmation of the diagnosis of meningococcal disease is becoming increasingly difficult. Confirmation of the diagnosis can be made from CSF in almost 90% of patients (1), but there is a a trend away from lumbar puncture especially in young children. Pre-admission antibiotic treatment reduces the chance of a positive blood culture to 5% or less (2). We demonstrated previously the value of a PCR designed to amplify a segment of the meningococcal insertion sequence IS*1106* (3), for specific and sensitive detection of *N. meningitidis* DNA in clinical CSF specimens (4). We here evaluated a DIG-PCR ELISA for the detection of meningococcal DNA in blood.

Patients and specimens. Peripheral venous blood samples (for standard haematological tests and anticoagulated with EDTA) were those taken on hospital admission from patients with suspected meningococcal disease and from controls. Samples were collected from patients in Gloucester, Hereford, Plymouth and Cheltenham. Almost always after overnight storage at 4°C, samples were centrifuged and divided into buffy coat, serum and red cell fractions. The samples were coded, stored at -20°C, and transported to the University of Surrey for DNA extraction and PCR analysis.

PCR sample preparation. DNA was purified from 50 ml blood buffy-coat or serum sample based on the method of Boom *et al* (5).

PCR amplification. Samples were handled in batches of 10 with 2 extraction blanks. 3 ml of diatom eluent was used in a 25 ml PCR reactions.

Detection of PCR products using DIG-PCR ELISA. This was available as a kit and performed essentially as described by the manufacturer (Boehringer manheim). An ELISA plate reader was used to record the result. Primers for the PCR and capture probe sequence were based on the meningococcal-specific insertion sequence IS*1106*(3).The PCR capture probe sequence was internal to the PCR product.

Coded clinical specimens from 80 patients with confirmed and suspected meningococcal disease and controls were examined by DIG-PCR ELISA. The sensitivity of the test in patients with confirmed meningococcal disease -either by culture or by the presence of gram-negative intracellular diplococci in CSF n these groups was 89% for serum specimens and 100% for blood buffy-coat. Using 95% confidence limits, the minimum value for the sensitivity of the test on blood buffy-coat was 86%. The specificity of the

test was determined using specimens from control patient specimens and was found to be 100% for both serum and blood buffy-coat. Ten buffy-coat specimens were from patients with strong suspicion of meningococcal disease (fever with haemorrhagic rash) but from whom no organisms were isolated (Table 3 patient category 3). Using the PCR test on blood buffy-coat, confirmation of meningococcal infection was obtained in all of these patients. Four of the 25 patients with confirmed invasive meningococcal disease and 7 of the 10 patients with suspected meningococcal disease had been treated with parenteral benzylpenicillin before the specimen was collected. Blood cultures were uniformly negative on these patients but they all gave a positive PCR result. Buffy-coat specimens from a further eighteen patients with evidence of bacterial meningitis but without a haemorrhagic rash (Table 3 patient category 4) were examined and 5 of these were found to be positive for meningococcal DNA. None of these patients had been treated with antibiotics.

Conclusions. The PCR-DIG ELISA test is as rapid (result in less than 24 hours), as conventional culture (12-36 hours) but does not seem to be affected by prior antibiotic therapy. The PCR blood test is practical and convenient and could be readily applied in clinical laboratories. The test described here may therefore be of particular benefit for diagnosis of patients in whom lumbar puncture is contraindicated or in whom antibiotic treatment had rendered blood or CSF sterile, before specimens were taken.

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Comparison between DNA sequencing and fingerprinting by pulsed-field gel electrophoresis for strain typing : Preliminary results using 12 meningococcal strains and isolates of 4 further *Neisseria* species

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Neisseria meningitidis shows a high level of interspecific genetic diversity. Various typing methods have been employed for strain characterization including the examination of allozymes (MLEE) and the study of restriction fragment length polymorphisms RFLPs (either by cutting small PCR generated DNA fragments or by digesting the whole genome, which is known as pulsed-field gel electrophoresis PFGE). The finest resolution of strains is achieved by sequencing DNA but this method is expensive and not routinely used so far. Information on the genetic variability among strains may then be used to reconstruct the genetic relationships of the strains studied. Various studies concerning the genetic diversity of *Neisseriaceae* led to the conclusion that at least particular serogroups of meningococci are of more or less panmictic population structure (e.g. 1). This is a result of horizontal inter- and intraspecific genetic transfer (recombination). Interspecific horizontal transfer of DNA fragments could be detected for some gene loci (e.g. 2). By sequencing a fragment of the *rpoB* gene, coding for the second largest subunit of DNA directed RNA polymerase, no hints for recombination were found in 18 DNA fragments belonging to meningococci and four other *Neisseria* species. However, the meningococcal strains used showed a more or less clonal population structure, although belonging to various serogroups (3).

While the study of allozymes is on the protein level, both the analysis of RFLPs or DNA sequencing is on the genomic level. When evaluating genetic relatedness, point mutations which are recognized by both techniques to a different degree are used for the construction of clusters in a dendrogram. The aim of the present study was to clarify, whether there are differences in the genetic relationships between the strains, when dendrograms had been generated using the RFLP pattern or by using the sequence data.

A subgenic PCR fragment within the *rpoB* gene from 12 strains of meningococci and isolates of 4 other species of the genus *Neisseria* was sequenced. The meningococcal strains covered various serogroups which were studied previously (3, 4) together with *N. lactamica*, *N. gonorrhoeae*, *N. flava*, and *N. subflava*. Sequence analysis was performed on a 471 bp *rpoB* fragment. The sequences were aligned using the program CLUSTALW (5). Phylogenetic trees were constructed using various methods like UPGMA or neighbor joining (6, 7, 3).

The same strains were subjected to fingerprinting by PFGE. The neisserial genome was digested either with the restriction enzymes *Nhe*I or *Sfi*I. Restriction fragment patterns

were analyzed using the program GelCompare. The program utilizes the UPGMA (6) or neighbor joining method (7) for reconstruction of the relationships among the strains by examining the patterns generated by the restriction enzyme. Observed RFLP patterns were analyzed separately for each enzyme and afterwards for both enzymes in combination. The resulting genetic relationships between the strains, obtained by both methods, were compared.

Our preliminary results indicate that by applying different statistical methods for analyzing the results of each technique (PFGE or sequencing) the clustering of strains is comparable. When comparing the clustering between both techniques, however, certain differences are observed. These results are in accordance with the assumption of a more or less panmictic population structure.

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The high incidence of meningococcal disease in indigenous Australians

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Objectives. To describe the incidence and control of meningococcal disease in Aboriginal Australians.

Introduction. Aboriginal Australians constitute 1.6% of the country's 17.5 million people, and about 6% reside in the arid region of central Australia. Like many other indigenous population groups in developed countries, Aboriginal Australians suffer poor health. They have a very high incidence of infectious diseases and of the diseases of transition, such as diabetes. The annual incidence of invasive *Haemophilus influenzae* type b (Hib) disease in Aboriginal children under 5 years of age in central Australia in the pre-Hib vaccine era was 871/100,000, among the highest ever reported in the literature (1). Similarly, the mean annual incidence of invasive pneumococcal disease between 1985 and 1990 in this age group was 935/100,000 (2).

Methods. Data on notifications of meningococcal disease were obtained from the Communicable Diseases Network of Australia and New Zealand. Since 1991, the notifiable diseases dataset has included a field for identifying Aboriginality, but this is not obligatory. Three states with relatively high Aboriginal populations (Western Australia, Northern Territory and New South Wales), usually provided this information with some consistency; together they have a total population of 7.7 million, of which just over 151,000 are Aboriginal. Reports on outbreaks were obtained from peer reviewed journals and communicable disease bulletins. The definition of an outbreak was based on a publication reporting an outbreak in which at least 2 or more cases occurred in a defined Aboriginal community within 3 months.

Results. ***Incidence***: Aboriginality of the subjects notified with meningococcal disease was recorded as "unknown" in 35% of cases in the three states. To obtain minimal estimates of the incidence in the Aboriginal population, all cases with an "unknown" status were assumed to be non-Aboriginal. The mean annual incidence per 100,000, based on notifications in the three states between 1991 and 1994, was: 13 for the Aboriginal population (61 for children under 5 years), and 2.2 for the non-Aboriginal population (14 for children under 5 years). A study from northern Queensland reported a mean annual incidence per 100,000 of 20 for the Aboriginal population (61 for children under 5 years) and 1.6 for the non-Aboriginal population (15 for children under 5 years).

Outbreaks in Aboriginal communities: An outbreak between 1987 and 1991 in the central arid region of the country resulted in a mean annual incidence of 21/13228 in the Aboriginal population (3); it was caused by serogroup A, clonal subgroup I-1 (4), and

none of the non-Aboriginal population of 25,000 people was affected. Between 1990 and 1994, outbreaks were reported in three communities in the northern part of the country, with attack rates of 12/1250 (5), 3/1000 and 7/6000. The f er two were caused by *Neisseria meningitidis* C2b:P1.2 and the latter by C2a:P1.2. All the outbreaks were controlled with community-wide vaccination programs.

The Aboriginal population in the arid centre was the only group in the country affected by the outbreak of serogroup A disease. Clonal subgroup I-1 also caused an epidemic in Auckland, New Zealand between 1985 and 1986, with high attack rates in the Polynesian population (6), and in the United States North West Pacific between 1975 and 1977, with high attack rates in native Americans (7). The same region in central Australia was affected by a larger outbreak of serogroup A disease between 1971 and 1974 (8) (clonal subgroup unknown), and outbreaks were not reported elsewhere in the country during this period.

The outbreak in the Aboriginal population in 1987 foreshadowed a steady rise in disease caused by serogroup B and C in the general population around the country. This incidence (based on notifications) increased from below 1, to a peak of 2.3 per 100,000 in 1994.

Conclusion

Aboriginal Australians have a high incidence of meningococcal disease. The causes of the high incidence of infectious diseases, and of overall poor health, are complex and probably related to adverse historical, cultural, social and economic factors. These, in turn, are reflected in measures of housing, education, employment and income.

The new meningococcal vaccines will have a role in controlling the disease in Aboriginal people. However, this strategy will have to be considered in the context of competing needs for resources to address the social and environmental determinants of ill health.

Several questions on the outbreak of disease caused by serogroup A remain unanswered: Why was the outbreak geographically restricted? Why were the non-Aboriginal residents in the epidemic area not affected? What is the significance of the temporal relationship between the outbreak and the subsequent rise in the incidence of disease caused by serogroup B and C around the country?

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Cochrane Reviews: The way ahead in the control of meningococcal disease

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Objective
To seek a commitment from individuals to work collaboratively in preparing and maintaining systematic, up-to-date reviews on the effect of interventions for controlling and preventing meningococcal disease.

The Problem
The effectiveness of interventions to control and prevent meningococcal disease has been reported by many original research studies.

As an example, studies on the serogroup A and C vaccines provide a spectrum of results and/or recommendations on: the age at which the vaccine is clinically protective; the duration of protection; the age at which a booster dose should be given; the need for vaccinating close contacts when a sporadic case occurs; the impact of vaccine on carriage of *Neisseria meningitidis*; and the population or setting the results are generalizable to.

Conclusions from a review of these studies will be determined by how the studies will be selected and scrutinized. They will vary in quality and generalizability; these, in turn, will depend on the study design and methods, including methods for selecting the study population, for measuring the outcome variables, and for conducting the analysis.

It is unreasonable and inefficient to expect all practitioners who want reliable information on the effectiveness of the interventions to unearth all the relevant evidence from original research. Most people rely on reviews of the primary research as a way of coping with the information overload confronting them. However, the quality of reviews often leaves much to be desired (1,2); this is because reviewers may not have approached their task with a respect of scientific principles, and in particular, a consideration of the control of biases and random errors. Furthermore, they may not have conducted exhaustive searches for all studies, including those published in the non-English language literature.

We need a systematic review of studies on the effectiveness of the interventions for controlling and preventing meningococcal disease, including specifically studies on the vaccines and the chemoprophylactic agents. The aims of the review would be:

- to identify all studies through an exhaustive search of the English and non-English language literature, including studies completed but not published;
- to select studies based on minimum quality criteria;
- to synthesize the information and provide estimates of effectiveness of the intervention measure, taking into account the expected heterogeneity of the results;
- to link the results of research to improved health outcomes; and
- to inform future research. For example, we could provide reliable estimates of the effectiveness of current vaccines against which the new vaccines can be compared, and identify important questions that researchers may have not have explored, or left unanswered in the earlier studies.

The Proposal

This conference provides a unique opportunity to identify individuals from many different countries, and with fluency in different languages, who will share in the vision of the Cochrane Collaboration (3).

The aims of the Collaboration are to prepare and maintain systematic reviews of randomized controlled trials (RCT) of the effects of health care, and to make this information readily available to decision-makers at all levels of the health care system. In the absence of RCT, the best available evidence is used. The Collaboration is guided by six principles: the shared will of contributors to collaborate with each other; building on people's commitment, enthusiasm and specific interests; minimizing unwarranted duplication of effort; avoiding bias by responding to criticism and disagreements through collegial resolution; keeping up-to-date with new evidence; and ensuring that the "*Cochrane Database of Systematic Reviews*" is available as widely as possible.

Working within this framework "...requires an ego that is satisfied by unselfish collaboration and group, rather than individual, recognition.....and the subservience of personal convictions to scientific evidence" (David Sackett 3).

The Cochrane Handbook provides a 'tool kit' of scientific strategies and tactics for preparing, updating, and disseminating the reviews, and for encouraging criticisms through a process of open peer review.

Conclusion

The Collaboration has identified the Acute Respiratory Infections Collaborative Review Group (ARI-CRG) as the initial site for locating individuals with an interest in meningococcal disease. The co-authors and the administrative centre of the Group, are based at the National Centre for Epidemiology and Population Health, at the Australian National University in Canberra, Australia.

Individuals interested in this initiative should contact Mahomed Patel at the conference. He invites colleagues to collaborate with him in developing further, a draft protocol for a systematic review of studies on the serogroup A vaccine. He is also keen, on behalf of the ARI-CRG, to talk with individuals willing to review other specific interventions, and plans to convene an exploratory meeting of interested colleagues during the conference.

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Typing of *N. meningitidis* in Moscow: Prevalence of non-European strains.

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Seventy-five strains of *Neisseria meningitidis* were isolated from patients with systemic meningococcal disease in Moscow in 1993-1995. In contrast to most European countries 21% of the isolates were group A strains, 56% of strains group B, and 15% group C. In 1993-1995 the percentage of group A strains has a tendency to increase from 11% to 33% and group B to decrease correspondingly. In contrast, the European countries reported in 1994 less than 25 group A isolates from total number of 3594 isolates (<1%) (1).

Using the current panel of antibodies to class 2/3 outer membrane proteins, 81% of group B, 73% of group C and 31% of group A strains were non-serotypable. The same proportion of non typable strains was found among group B isolates obtained in Moscow in 1983-92 (2). Thus, either additional specific monoclonals for Russian strains or, preferably, genetic typing methods have to be elicited for class 2/3 proteins-based classification of such meningococci. According to their class 1 outer membrane protein, 24% of group B, 18% of group C, and 6% of group A strains were non-subtypable.

Twenty four different serotype/subtype combinations were found among 53 strains of group B and C. This heterogeneity was also in contrast to the situation in Europe; for example, in the Netherlands, having approximately the same territory and population as the Moscow region, 23 most frequent sero/subtype combinations were responsible for 75% of 475 Dutch group B and C cases in 1994 (3). Even for group A meningococci, five different subtype/serotype combinations were found among 16 Moscow strains, although usually only one group A clone predominates in certain region (4). No predominant serosubtype was found in Moscow; relatively most strains had the formulas B:NT:P1.2,5 and B:NT:P1.14 (24% and 14% of group B strains, correspondingly), C:4:P1.10 and C:NT:P1.2,5 (18% and 18%), A:4:P1.5,10 and A:NT:P1.10 (31% and 25%). Such strains are very rare in Western Europe whilst the common European strains, such as B:15:P1.7,16, B:NT:P1.4, B:4:P1.4, B:4:P1.15, B:2b:P1.10, C:2a:P1.2, C:2b:P1.10, C:2a:P1.5, C:2b:P1.2,5, C:2b:P1.2 (1, 3), were not found in Moscow. All strains were sensitive to penicillin (MIC < 0.16 mg/l), chloramphenicol (MIC < 2 mg/l) and rifampicin (MIC < 0.25 mg/l), whereas about 7% of European strains were reported to be penicillin-resistant in 1994 in total (1), although some countries reported no resistant strains.

In conclusion, meningococcal strains from Moscow showed considerable diversity of sero- and subtypes, probably evolved in post-epidemic situation in Russia. Differences in circulating strains and presumable immunity of population in Western Europe and

Russia increases the probability of mutual exchange of strains and stresses the need of group B vaccine protecting both from West and East European variants of meningococci.

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Heteroduplex mobility analysis for identification of pathogens causing bacterial meningitis.

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Introduction. The classical bacteriological diagnosis of purulent meningitis by culturing takes at least 24 h and is strongly affected by antibiotic treatment before sampling (1). PCR techniques using a set of genus- or species-specific primers to diagnose meningitis are of limited value because many PCR runs should be performed to identify the possible pathogen. The use of universal PCR primers for amplification of the 16S rRNA gene of most pathogenic bacteria followed by hybridization with specific probes (2) is an alternative but cumbersome approach.

Objective. To evaluate heteroduplex mobility assay (HMA) for the identification of bacteria causing meningitides to the genus and/or species level.

Materials and methods. The bacterial strains used in this study were either clinical isolates from collection of the RIVM and the Reference Laboratory for Bacterial Meningitis, University of Amsterdam, the Netherlands or isolates stored at the Russian Collection of pathogenic microorganisms of the State Research Institute For Standardization and Control of Medical and Biological Preparations, Moscow, Russia. All strains were cultured on plates by standard methods. DNA samples for PCR were prepared according to Boom et al. (3) PCR and HMA were made as described elsewhere (4). Briefly, after 35 cycles of PCR with universal primers to bacterial 16S rRNA gene (1), amplicons produced from two different strains were mixed, denatured 2 min by heating at 95C, and cooled to form homo- and heteroduplexes. Then homo- and heteroduplexes were separated by 5% PAAG-electrophoresis at standard condition. Relative mobility of heteroduplexes was estimated using digitalized images of ethidium bromide stained gels.

Results. Thirty 16S rRNA gene sequences from representatives of 10 genera causing meningitis were picked up from Genbank and their 1 kb parts were aligned manually. Two primers in conserved regions were chosen. The length of primer-flanked fragment was 889 bp. DNA distances were calculated by counting mismatches after removal of small unpaired gaps using MEGA software. A theoretical possibility to differentiate all analyzed genera and even species within some genera by HMA was demonstrated. The estimated genetic distances between the analyzed 16S rRNA sequences of closest but different genera were always more than 12%. The distances between different species within a genus never exceeded 5%. The chosen primers were tested in PCR with DNAs from a panel of 40 bacterial species, isolated from CSF or blood of patients with bacterial meningitis.

Specific PCR products were observed for all the DNA tested. The collected PCR products were used as a panel of reference probes for HMA identification of any analyzed genus. A strong correlation between heteroduplex mobility and genetic distance was found for every pair of bacterial strains. Unspecific PCR amplification of the 16S rRNA gene in combination with HMA distinguished ten following genera: *Neisseria*, *Streptococcus*, *Haemophilus*, *Staphylococcus*, *Escherichia*, *Listeria*, *Moraxella*, *Klebsiella*, *Pseudomonas*, *Campylobacter*. Moreover, as it was predicted by theoretical calculation of genetic distances, the discrimination of species within a genus by PCR-HMA was also possible for some genera. For example, *Haemophilus parainfluenzae* vs. *H. influenzae*, *Staphylococcus aureus* vs. *S. epidermidis* were simply discriminated. Almost all tested species within genus *Streptococcus* could be distinguished with exception of *S. pneumoniae* vs. *S. mitis* or *S. sanguis*. The total time for PCR-HMA test was only 5 h (2 h for PCR and 3 h for HMA).

Conclusions. PCR amplification of the 16S rRNA gene combined with HMA is a rapid, simple and relatively inexpensive method for identification of bacterial genera. For some genera the differentiation of species is also possible. This approach would be valuable for identification of bacteria found in the CSF, blood or other normally sterile body fluids. Such method is also promising as rapid screening method for phylogenetic classification of newly cultured unclassified microorganisms.

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Comparison of different methods to diagnose bacterial meningitis in Russia

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From 200 patients with clinical diagnosis of systemic meningococcal disease or bacterial meningitis of unknown etiology admitted to Moscow Hospital for Infectious Diseases in 1993-95, cerebrospinal fluid (CSF) and blood samples were obtained. AN additional 25 control samples were taken from patients with diseases other than bacterial meningitis. The laboratory diagnostic methods included bacterial culture, antigen detection (counter immune electrophoresis and latex agglutination) and DNA identification using polymerase chain reaction (PCR). PCR assays for the detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* were modifications of approaches elaborated by Ni et al. (1) (assay for detection of meningococcal insertion sequence IS1106) and Radstrom et al. (2) (assay for detection of bacterial 16S rRNA gene). The primers for IS element were changed that eliminated the occurrence of multi-band pattern, occasionally observed in (1). More specific primers for rRNA gene provided us the possibility to substitute seminested strategy (2) by one-run assay. Model experiments with 30 reference strains of different bacterial species and their extracted DNAs demonstrated 100% sensitivity and specificity of our PCR assays having detection limit of about 30 genome equivalents per sample. Control experiments included 30 CSF samples from Dutch patients having culture-confirmed bacterial meningitis (12, 10 and 8 cases caused by *N .meningitidis*, *S. pneumoniae*, and *H. influenzae* correspondingly). Sensitivity and specificity were equal 100% also.

Positive cultures were obtained from 66 of 200 Moscow patients (33%). The proportion of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* type b strains was 52%, 33%, and 10%, respectively. Low number of positive culture was caused by pre-clinical use of antibiotics (3). Comparing to microbiological methods, the antigen detection had the high specificity (100%), but a low sensitivity (about 67%).Using latex agglutination additionally 51 cases were diagnosed (meningococcal infection was found in 43 cases (84%) and pneumococcal meningitis in 8 cases).

PCR-based tests of our clinical specimens had the high specificity (97-98%) and sensitivity (more than 90%) in comparison to culturing as golden standard. Only two false-positive results was obtained. Both meningococcal PCR-tests (IS element and 16S RNA gene) were positive in the CSF samples of patient with cerebral insult and of patients with culture-positive pneumococcal meningitis. Both results were caused probably by contamination at the moment of sampling. Two false-negative PCR results were obtained in case of culture-positive pneumococcal meningitis. Six cases of meningococcemia without meningitis, confirmed by blood culture, were studied. From

that, three CSF samples were positive in our PCR-assay, and 3 samples were negative. The latter results were considered also as false-negative, decreasing the sensitivity. The former results indicated that the traces of meningococcal DNA may be found even in the culture-negative CSF samples from patients without clinical meningitis.

As far as a few false-positive PCR results were observed, our PCR-based diagnosis might be considered as reliable, even if other tests were negative. Using PCR- tests, 95% of cases diagnosed microbiologically and/or immunologically were confirmed, and 56 cases was diagnosed additionally as meningococcal infection. Thus, in total the causative agent was identified in 173 cases (87%) of clinically suspected meningococcal infection or bacterial meningitis. The PCR-based tests were most informative. The application of PCR-methods for blood samples and a test to differentiate the sero(sub)types and serogroups of meningococci, would be the next stage of improvement of diagnosis.

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Outbreak of group A meningococcal disease in Moscow

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In January 1996 18 patients with systemic meningococcal disease were admitted to Moscow Hospital for Infectious Diseases, that was approximately the same as in corresponding period of previous years. 33% of cases were caused by group A, 50% by group B, and 17% by group C meningococci, that was also as in 1995 (1). The number of patients increased dramatically in February (51 patients), March (78) and April (41). The outbreak of meningococcal disease occurred in Vietnamese community in Moscow. Most patients worked in the same trading company and/or lived in the same hostels. First episode was registered at fifth week of 1996. The outbreak peaked at 10th week and practically stopped at 16th week. As a result, 26, 24, and 5 cases were registered among Vietnamese in February, March, and April correspondingly. Meningococci were isolated from 26 patients, all of strains were group A. The vaccination of the members of community and their contacts in public institutions was started at eighth week, using group A polysaccharide vaccine produced by Gabrychevsky Research Institute of Epidemiology and Microbiology. In total, about 3000 persons were vaccinated, that promoted the cessation of outbreak.

Five of 55 Vietnamese patients died (9%), male : female ratio was 62% to 38%. All non-survivors were males, four of them were younger than 2 years. Median age of patients was 11 years; 24 patients were younger than 6 years, nine belonged to 6-25 years group, 21 were from 26 to 50 years. The age distribution was affected probably by shifted age distribution among all Vietnamese migrants.

With a slight delay the incidence of meningococcal disease increased among other Muscovites. Some patients might be considered as the contacts of Vietnamese patients; patients25, 54, and 36 cases were registered in February, March, and April correspondingly (Vietnamese cases excluded). The increase started at eighth week of 1996 and reached a maximum at 12th week (17 cases per week). At end of April the situation was partly normalized (5 cases per week), that might reflect both the effect of vaccination and the seasonal effect. (Usually the meningococcal season in Moscow is February-March.) This increase was caused solely by group A meningococci, because the absolute number of group B and C episodes was stable from January to April. As a result, the percentage of group A cases among Muscovites increased up to 75% in March.

Among Muscovites with group A disease the case-fatality ratio was 12% and male : female ratio was 56% to 44%. The median age of patients was 19 years; 20% of patients

were younger than 6 years, 39% belonged to 6-25 years group, 41% were from 26 to 70 years.

To date the genetic relationship between the strains, caused this group A outbreak, and the group A strains, circulating in Moscow in previous years (1), are not yet studied. Thus we could not conclude either the new strain was introduced by Vietnamese migrants or the outbreak reflected their low resistance to common Moscow strains. Historically the highest epidemic of meningococcal disease in Russia during last fifty years started in late sixties, and the incidence of 27 cases/ 100000 individuals/ year was registered in 1970 in Moscow (2). Epidemiological (3) and genetic (4) data suggested that this epidemic might be caused by group A strain carried by Vietnamese migrants traveling in 1968 to Russia through China.

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Molecular typing of *Neisseria gonorrhoeae* by repetitive element sequence-based PCR in comparison with arbitrarily primed PCR analysis

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In this study, characterization of *Neisseria gonorrhoeae* isolates by serotyping using a panel of six monoclonal antibodies (1) raised against Protein IB encountered problems of non-reproducibility of co-agglutination reactions. Upon repeat testing, six of the IB-2 isolates were characterized as IB-3 and three have changed their serovar designations from IB2 to IB-6. Two PCR methods [arbitrarily primed PCR (AP-PCR) (2) and repetitive element sequence-based PCR (rep-PCR) (3)] were evaluated as rapid subtyping tools for *Neisseria gonorrhoeae* isolates. Rep-PCR identified eight distinct types while AP-PCR only identified three amongst the 19 isolates. Rep-PCR could clearly discriminate three strains present within cohort 1 from four epidemiologically unrelated strains isolated from a second cohort. With its higher discriminatory power and good day-to-day reproducibility, the rep-PCR is a rapid and sensitive subtyping tool which can complement the serotyping scheme. In laboratories where there is a lack of reagents or facilities to carry out the auxotype-serovar (A/S) classification scheme, rep-PCR typing may act as a substitute for immediate epidemiological tracking of *N. gonorrhoeae* infections in a community.

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Analysis of serogroup C *Neisseria meningitidis* causing sporadic meningococcal disease

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Serogroup C currently causes 64% of invasive *N. meningitidis* disease in metropolitan Atlanta and a similar proportion in much of the United States (1). An increase since 1991 in the number of outbreaks due to serogroup C meningococci in the United States has been noted (2). These outbreaks have been caused by strains with identical, or closely related enzyme types (ET), but little is known about the spread of endemic or epidemic strains within the United States. Understanding the epidemiology of endemic (sporadic) meningococcal disease may be critical to prevention of meningococcal epidemic outbreaks and case clusters. We used prospective population-based surveillance and molecular epidemiologic techniques to study sporadic serogroup C meningococcal disease in a metropolitan population of 2.34 million persons. During the five-year surveillance, in which no case clusters or outbreaks were noted, seventy-one cases of sporadic serogroup C meningococcal disease occurred (annual incidence 0.51/100,000). Eighty-four percent (52/62) of the serogroup C strains available for further study were members of the ET-37 complex by multilocus enzyme electrophoresis (MEE), including two enzyme types (ET's 17 and 24) responsible for 8 of 12 serogroup C outbreaks in the United States since 1991. Pulsed-field gel electrophoresis (PFGE) and serotyping confirmed the relatedness of these isolates. Sporadic cases caused by strains identical by all typing methods occurred over periods up to three years. Our study indicates that group C meningococcal strains which cause sporadic disease are the same as those causing epidemic outbreaks and suggests slow spread of these strains in a human population.

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A study of the sero/subtypes and antimicrobial resistance of *Neisseria meningitidis* isolated in Argentina during 1991-1996.

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The annual incidence of meningoencephalitis in Argentina remained within endemic levels until 1973. In 1974, a peak coinciding with the epidemic due to *N. meningitidis* in Brazil, with serogroup C predominance, was registered. Since 1978, a return to endemic levels and the emergence of serogroup B have occurred (1). The incidence of serogroups B and C remained within the expected levels until the '90s, when there was an increase in the number of cases due to serogroup B (in coincidence with other Latin American countries) (2). Epidemiological surveillance of the prevalent serotypes and subtypes is vital in order to orient vaccine production and to foresee their immunogenicity, as in the case of those for serogroup B meningococcus, which have been developed since 1985. From March, 1991 through May, 1996, 816 *N. meningitidis* isolates of patients from different regions of the country with a diagnosis of meningitis and/or meningococcemia, were studied. They were identified by biochemical technics and serogrouped by plate agglutination. The frequency of the different groups changed gradually. Up to 1994 inclusive, serogroup B predominated (79.1 %, n = 477) over the other ones circulating throughout the country (C, W135, X and Y). The growth in serogroup C was marked during the same year and it equalled serogroup B (50.2 %, n = 103 and 49.7 %, n = 102 respectively) in 1995-1996. Serotype and subtype determinations by ELISA were performed in 58.5 % of the isolates of serogroup B (n = 340) and in 41.3 % of those of serogroup C (n = 89) obtained during the 1991-96 period. The predominant combinations were: B:2b:P1.non-typable (NT), (25.0 %) and B:2b:P1.10 (24.1 %). followed by: B:15:P1.7,16, (7.3 %) and B:NT:P1.NT (6.4 %). The remaining 37.2 % belonged to other serotype and subtype combinations. As regards serogroup C, the prevalent combinations were: C:2b:P1.NT (51.7 % and C:2a:P1.2 (15.7 %). The remaining 32.6 % belonged to other combinations. Between 1991 and 1995, the susceptibility of 198 isolates to penicillin (PEN), ampicillin (AMP), ceftriaxone (CRO), rifampicin (RFA), trimethoprim-sulphamethoxazole (SXT), tetracycline (TET) and ciprofloxacin (CIP), was studied by the method of macrodilution in agar. 19 % revealed moderate susceptibility to PEN and AMP (MICs = 0.12-1 µg/ml) and no beta-lactamases were detected. None of the clinical isolates studied were resistant to CRO, RFA, TET or CIP. 51 % of the isolates had MICs for SXT > 0.5 µg/ml, while the MICs of the remaining population ranged between 0.004 and 0.064 µg/ml.

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Evolution of the second pandemic due to strains of *Neisseria meningitidis* A:4:P1.9/clone III-1. Survey in four African countries Niger, Burkina Faso, Cameroon and Chad October 1995-May 1996

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Since 1991(1), we have organized an epidemiological surveillance of the progression of a second pandemic due to strains A:4:P1.9/clone III-1 of *Neisseria meningitidis* based in four African countries of Niger, Burkina Faso, Cameroon and Chad. By the end of October 1995, we had analyzed 62 strains recovered from 76 registrations. The techniques used were serogrouping, sero and subtyping associated with multilocus enzyme electrophoresis (MLEE).

From Niger, 29 strains belonged to type A:4:P1.9/clone III-1, except three isolated in the same area which belonged to serogroup X. In general this year in Niger, the outbreak was weaker than in 1995 and was located in the south. The number of cases was less than 15 per 100,000 inhabitants.

In Burkina Faso, 32 strains were subcultured from 36 registrations. In 1995, only one strain belonged to the type A:4:P1.9/clone III-1. This year they are all of this type. In 1995, the endemic strains were Y:2a:P1.2,5/ET-37 complex.

From Cameroon, we received three strains this year, one W135:2a:P1.2,5 isolated in Garoua (North). From Yaoundé (South) one was B:4:P1.7,16 and one was A:4:P1.9/clone III-1.

Only one strain was studied in Chad and, it was identified as W135:NT:P1.2,5/ET-37 complex. From 1988(2) to 1996, the second pandemic constitued by serogroup A spread in Africa was responsible for severe outbreaks. The immunization with A+C vaccine is active to prevent the extension of the outbreaks, nevertheless we need to remain attentive to the detection and extension of the serogroups B,X,Y and W135.

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Evaluation of the cross reactivity of antisera raised to ...mbinant Transferrin binding protein 2 variants from *Neisseria meningitidis* against a genetically diverse collection of serogroup B strains.

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Transferrin binding protein 2 (Tbp2) is variable among strains of *Neisseria meningitidis* (1, 2) and is able to induce cross-reactive and bactericidal antibodies in the B16B6-like strains (3). To assess whether the same was true among M982-like strains in which Tbp2 is variable in size, specific antisera were produced against two recombinant Tbp2 variants from strain M982: one corresponding to the full length Tbp2 and one corresponding to the N-terminal half of the molecule, described as the human transferrin binding domain (5). A genetically diverse collection of serogroup B strains representing different genotypes, serotypes and subtypes and having different geographic origins was analyzed and the *tbp2* gene from each strain was amplified so as to determine the size of the gene. Strains with a 2.1 kb *tbp2* gene were selected and the reactivity of the antisera was tested on intact meningococcal cells in a dot blot assay. Ninety eight percent of the 58 strains reacted with the antiserum specific for the N-terminal half of Tbp2 while 74% of the strains reacted with the antiserum raised to full length Tbp2. In parallel, the bactericidal activity of the antisera was evaluated against M982-like strains. The results indicated that the N-terminal half of Tbp2 was sufficient to induce cross-reactive antibodies reacting with the protein on meningococcal cells but the presence of the C-terminal half of the protein seemed beneficial for the induction of cross-bactericidal antibodies.

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Serogroup Y meningococcal disease in the United States

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Background. Endemic disease is caused by heterogeneous meningococci belonging to a variety of serogroups while epidemics are usually caused by clonal *Neisseria meningiditis* strains belonging to a single serogroup. Among *N. meningitidis* strains causing endemic disease, the proportion belonging to a particular serogroup can vary dramatically over time. The reasons for this are not well understood but may be related to circulation of new clonal strains within particular serogroups or changes in population immunity.

From 1989 to 1991, serogroup Y meningococcal disease (SYMD) accounted for only 2% of endemic disease in U.S. surveillance (1), but by 1995, the proportion of SYMD increased to 31%. In the 1970s, SYMD was also recognized as a common cause of endemic disease in some U.S. populations (2,3) and was associated with several outbreaks in military personnel (4-6). In some military studies (4,5), but not others (6), serogroup Y was more likely than other serogroups to be associated with non-meningitic disease, especially pneumonia.

We used the recent increase in the proportion of endemic meningococcal disease caused by serogroup Y in the United States as an opportunity to: 1) better understand the variation in serogroup distribution of endemic meningococci and 2) more fully characterize the epidemiology and clinical illness associated with SYMD.

Methods. In 1992-1995, surveillance was conducted in three counties in the San Francisco metropolitan area, eight counties in the Atlanta metropolitan area, four counties in Tennessee, and the entire state of Maryland for an aggregate population of 12 million. A case was defined as a resident of the surveillance areas who had isolation of *N. meningitidis* from a normally sterile site.

Multilocus enzyme electrophoresis (MEE) (7) was used to characterize serogroup Y isolates from: 1992-1995 U.S. surveillance (n = 40), 1972 U.S. surveillance when SYMD accounted for 18% of the 324 isolates submitted to CDC (n = 7) (3), and 1970-1974 surveillance of U.S. military personnel (n = 12) (kindly provided by Dr. W. Zollinger, Walter Reed Army Institute of Research).

Results. In the active surveillance areas, the rate of SYMD increased from 0.12 per 100,000 persons in 1992 to 0.28 per 100,000 in 1995. The proportion of meningococcal disease caused by serogroup Y increased from 18.5 % in 1992 to 31.1% in 1995. The median age of the SYMD patients was 21.8 years, compared with 14.0 years for all other serogroups ($p = 0.005$). Fifty-one percent of SYMD patients were classified as black and

42% as white; only 25% of patients with other serotypes were classified as black and 67% white (odds ratio (OR) 2.5; 95% confidence interval (CI) 1.8-3.4). SYMD cases were more likely to be associated with pneumonia than were the other serotypes (OR 4.2; CI 1.8-9.7). Serogroup Y did not differ from the other serogroups in case-fatality rate (10.3% vs. 9.6%).

Two closely related enzyme types (ET-508 and ET-501) represented 45% of the isolates tested from 1992 to 1995; however, these enzyme types were not present among the 1972 U.S. surveillance strains or the strains from the U.S. military personnel..

Conclusions. Serogroup Y meningococcal disease now represents a significant proportion of meningococcal disease in the United States. When patients with SYMD were compared to patients with meningococcal disease caused by other serogroups, they were older, more often black, and more likely to have pneumonia. At least part of the increase in SYMD may be due to the emergence of a distinct clone as characterized by MEE. Development of a serogroup Y conjugate component may be necessary to supplement meningococcal vaccines designed for control of endemic disease.

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Outbreaks of meningococcal disease in England and Wales in the winter of 1995/6

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Thirteen outbreaks of meningococcal disease in school settings were reported to CDSC between 1/10/95 and 31/12/95. Forty four cases were identified in these outbreaks, of whom five had died. Thirty were diagnosed as due to septicaemia, 11 as meningitis and three as both. Twenty six were confirmed by laboratory methods. Of the 13 clusters, eight had at least one serogroup C case, two had at least one serogroup B case and three consisted only of clinical cases.

The 13 outbreaks during a three month period represented a considerable increase when compared with the eight school outbreaks a year identified by CCDCs during the previous two years. A greater proportion of serogroup C cases were observed than would have been expected from the proportion of serogroup C cases identified at the Meningococcal Reference Unit during this period.

In two separate community outbreaks age specific attack rates of serogroup C disease reached levels of 40/100,000 and 160/100,000 within two month periods, compared with an attack rate of 4,3/100,000 in 1-19 year olds in England and Wales for the whole of 1995. A&C meningococcal vaccine and antibiotics were offered to 19,000 2-18 year olds in these outbreaks.

Considerable difficulties in managing these outbreaks, especially in relation to large scale immunization in outbreaks of serogroup C disease were encountered.

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Characterization of two porin genes present in *Neisseria flavescens*

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The genus *Neisseria* includes the pathogens, *N. meningitidis* and *N. gonorrhoeae* together with a variety of less disease-causing species which are considered to be 'commensal'. It has been postulated that carriage of commensal *Neisseria* species may stimulate the production, in humans, of cross-protective antibodies (1,2) but there is little evidence to confirm or refute this idea. Comparisons of predominant antigens in pathogenic and commensal *Neisseria* may therefore help to authenticate this theory and may assist in the development of new vaccines against pathogenic strains.

Extensive studies in *N. meningitidis* have identified porin proteins as being major surface antigens and this has led to their inclusion in novel vaccine approaches (3,4). The porins of different *Neisseria* species are related at the primary structural level and are thought to be similar in their tertiary structures, all having a β-barrel conformation with variable loop regions (5). Two porin alleles are found in *N. gonorrhoeae*, but individual gonococcal strains express only one of the alternative porin classes, PIA or PIB. However, *N. meningitidis* strains commonly express two major porin classes simultaneously, PorA and PorB, of which only PorB is essential for growth. It is thought that the commensal *Neisseria* usually contain only one porin gene which is characteristic for each species.

Nucleotide sequence analysis of porin genes in *N. meningitidis* has demonstrated that extensive variation exists in the loop regions of porins from different meningococcal strains. The same regions are responsible for serological differences recognized by monoclonal antibodies used in typing of clinical isolates. The greatest variation is seen in the loops of PorA and this has fueled speculation that PorA has an immunological role in helping the meningococcus to evade the human immune system. Horizontal genetic exchange has been described as one mechanism by which variation between *por*A genes from different meningococcal strains is perpetuated (6,7). As all the *Neisseria* are naturally competent for DNA uptake, horizontal genetic exchange also occurs between, as well as within, *Neisseria* species. An example of inter-species recombination is the development of penicillin resistance in *N. meningitidis*. An altered *pen*A gene in resistant meningococci was found to contain segments of DNA that had been acquired from *N. flavescens* (8). The common gene pool of *Neisseria* is hence a potential source of genetic variation.

Previous work showed that *N. flavescens* contained two porin genes (9). These genes were of almost identical size and so it was impossible to determine their nucleotide sequences by direct methods or to distinguish, by SDS-PAGE, whether there were one or two porins expressed. In the current work, the nucleotide sequences of the two genes were elucidated by cloning and subsequent PCR amplification of each gene separately. The presence of two porin genes in each of two further strains of *N. flavescens* was established by Southern blotting experiments. The results confirmed that the existence of two porin genes in *N. flavescens* was not a strain-specific phenomenon. The two *N. flavescens* porin genes were compared with each other and with porin genes from other *Neisseria* species. One of the porin genes from *N. flavescens* shared more similarity (greater than 70% homology) with the porin gene from *N. sicca* than with the other porin gene from *N. flavescens*. The porin genes from both *N. sicca* and *N. flavescens* did not closely resemble any of the other porin genes examined. It is possible that inter-species transfer of a whole porin gene had occurred by horizontal genetic exchange as has been described previously for *N. meningitidis* and *N. gonorrhoeae* (10). Alternatively, *N. sicca* and *N. flavescens* may have previously existed as a single species and the second porin gene in *N. flavescens* was acquired upon species divergence. Further isolates need to be examined before these questions can be resolved.

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Molecular typing of *Neisseria meningitidis* strains using polymorphism of *pilA* gene.

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The identification and the characterization of meningococcal strains is quite important for the management of the infection and for the control of epidemics in the population.

A new molecular typing method for identification and characterization of *Neisseria meningitidis* is reported using the polymerase chain reaction (PCR)(1). We designed primers in the *pilA/pilB* locus and amplified the corresponding fragment which is subjected to restriction endonuclease analysis using three different enzymes. The restriction endonuclease patterns (REP) obtained were compared.

The choice of *pilA* locus for PCR-REP analysis is advantageous as *pilA* is universally present as a conserved gene in all meningococcal strains. Strains tested were from serogroups A, B, C, Y, W135, X and Z. Clonal isolates clustered together in distinct restriction endonuclease patterns (each corresponding to a particular *pilA* allele). PCR-REP-based classification coincided with electrotypes as determined by multi-locus enzyme electrophoresis (MLEE). Strains of serogroup A are less variable than those of serogroup B and C. Indeed, strains from serogroup A clustered in few PCR-REP types while strains from other serogroups (particularly B and C) showed extensive polymorphism in *pilA* with numerous PCR-REP types. Correlation serotype and serosubtype is incomplete particularly in strains belonging to serogroup B indicating the insufficience of serological classification alone in outbreaks and epidemics surveillance.

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The molecular epidemiology of *tet*M genes in *Neisseria gonorrhoeae.*

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Neisseria gonorrhoeae isolates with high-level (MIC>8mg/l) resistance to tetracycline (TRNG) were first detected in the USA in 1984 and first isolated in the UK in 1987. Tetracycline resistance in gonococci is mediated by a *tet*M determinant carried on a 25.2MDa conjugative plasmid (1). The restriction endonuclease map of the conjugative plasmid from a TRNG strain imported from the USA has been found to differ from a map derived from a strain isolated in Holland (2). These two types of *tet*M carrying conjugative plasmids were designated American and Dutch respectively. More recently the nucleotide sequences of the *tet*M genes from the +American and Dutch conjugative plasmids have been determined and found to differ significantly from each other (3).

We have designed three oligonucleotide primers that will hybridise with the *tet*M gene in a polymerase chain reaction (PCR). A universal forward primer that hybridizes with both variants is combined with reverse primers specific to each variant. This PCR assay amplifies the genes to produce products of 777 and 443 base pairs with the American and Dutch types of *tet*M respectively.

The specificity of the PCR assay was challenged using DNA preparations from 18 tetracycline sensitive isolates carrying the 24.5MDa conjugative plasmid and DNA preparations from three strains of *N. gonorrhoeae* with tetracycline MICs of 8mg/l. No product of any size was detected in the PCR assay. Twenty strains of *N. gonorrhoeae* carrying *tet*M conjugative plasmids of known restriction endonuclease pattern type (2) were also tested. Nineteen strains of these yielded the corresponding PCR product and one produced an American size PCR fragment from a Dutch type plasmid. This was reproducible and was confirmed as the correct result by another laboratory (4).

Four hundred and fourteen strains of TRNG isolated in the UK, but originating from infections caught in 48 countries worldwide as well as the UK, were investigated for *tet*M type. These strains had previously been typed using the auxotyping method of Copley and Egglestone (5), serotyped following the scheme of Knapp *et al* (6) and had the plasmid profile determined using a modification of the method of Birnboim and Doly (7).

Two hundred and ninety eight TRNG strains produced 777bp PCR products (American type *tet*M) and 116 produced 443bp PCR products (Dutch *tet*M). All TRNG isolated from strains originating in the Far East yielded the Dutch type and all strains originating in Africa, except one from S. Africa yielded the American type. Strains from other areas of the World yielded a mixture of types but with a predominance of the American type.

b-lactamase-production was detected in 71% of strains carrying the American *tet*M and 66% of strains containing the Dutch *tet*M. The American type was found in 79% of strains carrying the 3.2MDa b-lactamase plasmid and the ~ tch tetM type was found in 61% of strains carrying the 4.4MDa plasmid. Of the 15 strains originating from Indonesia 12 (80%) contained this latter combination of plasmids but these belonged to 9 different auxo/serotype (A/S) combinations. Of strains originating in the African continent, infections caught in Nigeria and Kenya contributed most strains, 18/25 (72%) contained the 3.2MDa b-lactamase plasmid. These comprised 6 A/S types, however 10/18 were a proline requiring IA6 serovar. Over the period of collection of TRNG strains (1988-1995) there was an increase in incidence of strains containing American *tet*M with either the 3.2MDa plasmid or no b-lactamase plasmid. Since 1991 there has been little overall change in other combinations of plasmids and *tet*M type.

These results are consistent with data previously collected on the global distribution of variants of *tet*M (4) and extend our knowledge of the molecular epidemiology of tetracycline resistance in the gonococcus.

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Assessment of serological response to meningococcal outer membrane proteins and capsular polysaccharide in the diagnosis of meningococcal infection

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Since 1991 the use of pre-admission antibiotic therapy of suspected cases of meningococcal infection in England and Wales, has resulted in a significant fall of culture confirmed cases when compared with those notified clinically. Attempts to try to confirm the diagnosis and additionally identify the serogroup of the infecting strain, where these are B or C by serodiagnosis have been made by the reference laboratories in England and Wales, and in Scotland.

Collections of acute and convalescent phase sera from patients with culture proven or clinically suspected meningococcal disease, with varying clinical presentations, were examined. Paired sera from patients with other proven infections served as controls.

Sera were initially screened using an enzyme-linked immunosorbent assay (ELISA) previously developed for the serodiagnosis of meningococcal infection. This ELISA uses outer membrane vesicles purified from three phenotypically diverse meningococci as antigen (1). Sera were then tested for antibodies to the serogroup A, B and C capsular polysaccharide by ELISA, using a modification of the CDC protocol (2, 3, 4).

Although most individuals responded to both the protein and the relevant polysaccharide antigen, some patients responded to one antigen only. However, the data indicate that a combination of antigens provides an extremely useful tool for the diagnosis of all forms of culture negative non-fatal meningococcal disease.

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P1.19 specificity of a previous P1.15 reference monoclonal antibody demonstrated by blotting methods, *porA* sequencing and peptide mapping

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A difference in specificity of the two P1.15 reference monoclonal antibodies (Mabs) produced by WD Zollinger (2-1-P1.15) and JT Poolman (MN3C5C) has been reported (1). MN3C5C recognizes a sequence of three amino acids in the variable region (VR) 2 of the Por A molecule (2), while the specificity of 2-1-P1.15 has not been reported.

Among meningococcal strains infecting patients in Norway in the period 1987-1995, we identified 37 strains reacting on dot-blots with 2-1-P1.15 of which 25 also bound MN3C5C. No strains that were only positive with MN3C5C, but not with 2-1-P1.15, were found. Furthermore, some strains which only bound 2-1-P1.15, also reacted with Mabs specific for other VR2 epitopes, thus identifying P1.1,15, P1.2,15 and P1.14,15 subtypes.

To understand the difference in specificity between the two P1.15 Mabs, sequence analyses of parts of the *PorA* encoding the two VR of PorA were undertaken. The *porA* of 8 strains, reacting with 2-1-P1.15 Mab, showed a sequence coding for a VR1 related to the P1.19 subtype (3), whether or not they reacted with MN3C5C (PPSKSQPQVKVTKA, P1.19, 6 strains; PRSKSQPQVKVTKA and PLSKSQPQVKVTKA, one strain each, designated P1.19b and P1.19c, respectively). These results suggested that 2-1-P1.15 could be a P1.19 subtype-specific Mab. Strains, that in addition reacted with P1.1, P1.2 or P1.14 specific Mabs, showed *porA* sequences compatible with these reactions, whereas strains binding MN3C5C had sequences encoding NNT or NNA in VR2. One isolate, reacting only with 2-1-P1.15, had the NNT sequence in VR2, but this was preceded by a proline instead of a glutamine, which seemed sufficient to annul the reaction with MN3C5C.

Partial peptide mapping of the epitope for 2-1-P1.15 was done by examining its binding in ELISA to 25 to 29-mer synthetic peptides which corresponded to loops 1, 4 and 5 of strain H355 porA. The results verified that 2-1-P1.15 did indeed react with a peptide corresponding to loop 1, thus confirming the P1.19 specificity of that Mab. On immunoblots, 2-1-P1.15 bound weakly to the P1.19b variant, whereas no binding to P1.19c was observed, indicating that the epitope for 2-1-P1.15 must be located to the N-terminal end of VR1.

The Cuban B:4:P1.15 vaccine contains the P1.19,15 subtype protein (4). The demonstration of strains with subtype P1.19,15 and P1.19 strains without the P1.15

subtype made it possible to analyse theVR response in vaccinees receiving the Cuban vaccine. For this purpose, sera with class 1 protein antibody activity from five volunteers given the Cuban vaccine in the Icelandic trial (5), were immunoblotted against P1.19 strains with different VR2 subtypes and against unrelated strains. The postvaccination sera responded mainly against the P1.19 region, and they showed the same specificity against the P1.19 variants as 2-1-P1.15, that is low or no binding.

In conclusion, the demonstration of a P1.19 specificity of the prior P1.15 reference Mab 2-1-P1.15 explains the difference in reaction between this and the other P1.15 reference Mab MN3C5C, and it enlarges the repertoire of VR1 specific Mabs. The use of different P1.19 strains showed that the class 1 protein response in vaccinees given the Cuban vaccine was directed against VR1 in contrast to the VR2 reaction found against protein P1.7,16 in volunteers receiving the Norwegian 15:P1.7,16 vaccine (6).

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School-based clusters of meningococcal disease in the United States: descriptive epidemiology and a case-control analysis

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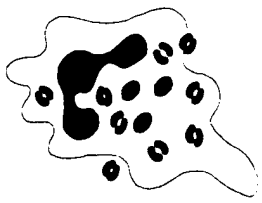
Background. The occurrence of multiple cases of meningococcal disease (MD) in a school causes substantial concern in the local community. Routine postexposure chemoprophylaxis or vaccination is not recommended for school contacts. The approach to disease control in the setting of a school-based cluster of MD is problematic and limited by a lack of information on the epidemiology of and risk factors for such clusters.

Methods. We surveyed state health departments for school clusters of MD (defined as ≥2 infected students in grades K-12 with MD within a 30 day period) from January 1989 through June 1994. Each school with a MD cluster was geographically-matched to a control school with only 1 case of MD. We collected data on school characteristics, case-patients' school activities, and genetic relatedness of meningococcal isolates using multilocus enzyme electrophoresis (MEE) with 24 constitutive enzymes. Absenteeism data were reviewed to assess the role of antecedent illness.

Results. *Descriptive epidemiology*: We identified 22 clusters of MD in 15 states from all geographic areas of the United States, with a seasonal distribution similar to that seen with sporadic disease. The estimated incidence of secondary MD among schoolchildren 5-18 years of age was 2.4/100,000 population, a relative risk of 2.2. The median number of students/cluster was two (range 2-4). The number of school clusters with ≥2 students/cluster was significantly higher from that expected in a Poisson distribution of MD among children attending school (summary χ^2=284,282). Among cluster schools, 10 (36%) of 28 secondary cases occurred ≤2 days and 22 (79%) occurred ≤14 days after the index case. Among the 8 school with >2 cases, 50% of the additional cases occurred ≥2 days after the second case. *Case-control study*: Secondary schools (grades 7-12) accounted for 14 (74%) of 19 cluster schools compared with 9 (42%) of 19 control schools (p<0.05). In 16 (73%) of 22 clusters, interaction between case-patients was noted. The index patient in cluster schools was more likely than matched controls to have participated in a school-based activity ≤14 days before illness (OR=7.0, 95% confidence interval=0.9,57). Cluster and control schools did not differ with respect to school attendance prior to the onset of MD. *Laboratory*: Thirteen (59%) of 22 clusters were due to serogroup C, and 7 (32%) due to serogroup B, similar to the distribution among control schools. Isolates from among 10 (91%) of 11 individual school clusters were clonal by MEE.

Conclusions. Three fourths of school clusters occurred in a secondary school. Subsequent cases usually occurred within 2 weeks of the index case. After 2 cases of MD

in a school, rapid initiation of a chemoprophylaxis program may prevent a substantial proportion of cases in this setting. Continued surveillance may help determine if participation in group activities or circulating illnesses increase the risk of transmission in schools.



Environmentally Regulated Proteins

Neisserial iron regulated stress proteins: What do they do? What can we do with them?

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A decade of work by many able investigators has identified many proteins in the pathogenic Neisseria that are produced in increased amounts during various types of stress, including iron deprivation and oxygen deprivation. This minireview will summarize briefly what is known about iron-regulated proteins, and their roles in pathogenesis and vaccine development. Space limitations prevent review of work on the anaerobically expressed proteins (Ani) (1, 2).

Iron regulated proteins. After one to two mass doublings in vitro under conditions of reduced or absent iron availability, *Neisseria gonorrhoeae* (Ng) and *Neisseria meningitidis* (Nm) produce a variety of iron repressible proteins (Frps or Irps) (3). Many of these are on the cell surface, either in the outer membrane or in the periplasm, and they have been studied intensively. Others are probably in the cytoplasm. By means of two dimensional gel electrophoresis, a surprisingly large set of Ng Frps recently was identified, the genes for which apparently are under transcriptional control of the iron-dependent regulatory protein Fur (4). Two dimensional gels also identified a relatively large set of Ng proteins that are expressed only during exposure to high concentrations of iron (iron inducible proteins, or Fips) (4). Some of the Fips also are located in the outer membrane (S. Carson, C. Elkins personal communication), and may possibly be involved either in protection against iron-related oxygen derived toxic radicals or in cell adherence; the latter inference is based on studies that demonstrate increased Ng adherence in the presence of high iron concentrations (5). Virtually nothing is known about the structure, function and regulation of Fips.

Iron transport: two component receptors. Many of the Frps are involved in uptake of essential iron from the environment. Since iron can be toxic, it is not surprising that systems evolved that regulate iron uptake, depending on the intracellular iron concentration. We now understand that both Ng and Nm produce specific receptors for binding different iron ligands, and that these receptors apparently are built on a similar model. Each appears to be a two component system, with a pair of genes under control of a single Fur-regulated promoter. This concept is well established for the transferrin (Tf) receptor, and is suggested by still incomplete data for each of the lactoferrin (Lf) and hemoglobin (Hgb) receptors. The first gene in the operon apparently encodes a lipoprotein, and the second an integral outer membrane protein that belongs to the family of TonB-dependent receptors. This has been extensively studied in the case of the Ng and Nm Tf receptors, which are very closely related in every detail (6). Each receptor is

made up of two Tf binding proteins (Tbp). The lipoprotein Tbp2 is the product of the Fur-regulated gene *tbpB*, (7, 8) which is located immediately upstream of the gene *tbpA* for the integral outer membrane protein Tbp1 (8, 9). The Nm and Ng Lf receptors also appear to be made up of a lipoprotein (Lbp2) and a TonB dependent integral outer membrane protein (Lbp1) (10 and G. Biswas, unpublished data). The Nm and Ng hemoglobin (Hgb) receptor designated Hpu also may be similarly designed, with both a lipoprotein (L. Lewis and D. Dyer, personal communication) and a TonB dependent integral outer membrane protein working in concert to functionally bind Hgb and/or HgB plus haptoglobin.

The Tf receptor. Tbp1 is essential to entry of Fe from Tf, whereas Tbp2 facilitates Tf binding but is not essential for Fe entry (7). Tbp2 binds iron-loaded Tf preferentially, whereas Tbp1 does not discriminate between iron-loaded or iron-free Tf (11). Both Tbp2 and Tbp1 are surface-exposed, and Tbp2 may be only loosely tethered to the outer membrane, perhaps by its lipid moiety, since it is easily released from whole cells or membranes by certain detergents (11). Formal proof has not been obtained for either physical proximity of Tbp1 and 2 or for protein-protein interactions between these proteins in the membrane, but a variety of evidence suggests that they occur together in patches on the membrane, with more copies of Tbp2 than Tbp1 (11). Recent evidence shows that the Tf receptor exists in more than a single conformational state (12), depending on whether it is effectively energized by the newly-discovered Ng TonB ExbBExbD system (13). A model has been constructed which suggests that Tbp2 helps to promote binding of FeTf to the receptor whereas Tbp1 may serve as a gated TonB dependent channel for entry of Fe released from Tf (6, 11), analogous to the now well-documented TonB-dependent gated porin activities of the *E. coli* iron-siderophore receptors FepA (14) and FhuA (15).

Function of the Tf receptor in vivo. The role of the Ng Tf receptor in pathogenesis of human mucosal infection has been studied very recently in male volunteers in Chapel Hill (16). The question addressed was whether expression of the Ng Tf receptor was necessary for establishing urethral infection in the volunteers. To answer this question, a *tbpB tbpA* deletion mutation was constructed in FA1090, which is a natural Lf^- (lactoferrin receptor deficient) strain. Thus the challenge strain for the experiments was unable to express either a functional Tf or Lf receptor, although it was wild-type in every other parameter. Results showed that 0 of 5 volunteers inoculated with over 10^6 cfu of the Tf receptor mutant strain (Tf^-Lf^-) developed urethral infection, as compared to 3 of 3 volunteers inoculated with a similar dose of the isogenic Tf^+Lf^- strain, and over 90% of historical controls inoculated with the same Tf^+Lf^- FA 1090 strain in previous experiments (12). Thus, under these experimental conditions the Tf receptor appeared to be essential for successful colonization of the normal male urethra, which is surprising in some respects because mucosal surfaces are rich in Lf but normally have very little Tf or other serum proteins. These experiments do not disprove the notion that expression of the Lf receptor would assist in establishment of mucosal infection; it is entirely possible that challenge with a Lf^+ derivative of FA1090 would result in an ID80 of less than the $1x10^6$ cfu dose calculated for the Tf^+Lf^- FA1090 used for all human challenge in Chapel Hill to date (16). It also is conceivable that experimental urethral inoculation of male

volunteers with a small catheter results in sufficient transient trauma to allow serum proteins such as Tf to exude onto the urethral mucosa, which could result in an apparent dependence on the Tf receptor for optimal infection of the volunteer. Despite these qualifications, these results are exciting because they strongly suggest that the Ng Tf receptor is very important to mucosal infection, in addition to its presumed essential role in systemic (blood, joint, central nervous system) sites. Presumably, the same conclusion is pertinent to meningococci, and to other mucosal pathogens such as *Hemophilus influenzae* that make similar Tf receptors.

The Lf receptor. All Nm and about 50% of clinical isolates of Ng produce a functional Lf receptor (17). The Lf receptor in both Nm and Ng contains a TonB-dependent integral outer membrane protein designated Lbp1, which is quite similar in predicted amino acid sequence to Tbp1 (10, 18). In addition, limited DNA sequencing upstream of *lbpA* (the structural gene for Lbp1) shows a gene in both Nm and Ng that is very similar to *tbpB*, tentatively identified as *lbpB* (10, and unpublished data of G. Biswas). Transposon insertions into the upstream gene in Ng abolish Lf receptor binding activity (G. Biswas, unpublished data). This suggests that Nm and Ng also produce a Tbp2-like protein, although it has not yet been identified by biochemical techniques, and the entire DNA sequence of *lbpB* has yet to be determined in either Nm or Ng.

The functional importance of the Lf receptor is unclear. Without doubt, mucosal colonization and infection does not absolutely depend on a Lf receptor because many nonpathogenic Neisseria successfully colonize the nasopharynx despite being unable to use Lf as a sole iron source in vitro (17). Moreover, all tested *H. influenzae* (19) and 50% of Ng are unable to utilize Lf as a sole iron source, (17), and experimental male urethral infection with the Lf$^-$ Ng strain FA1090 is successful. Little else is known about the Nm or Ng Lf receptor except it is functionally dependent on the TonBExBExD system in Ng (13) and the molecular basis for failure of many Ng strains to produce a functional Lf receptor is mutation either in *lbpA* or in a region immediately upstream of *lbpA* (18). Presumably a functional Lf receptor assists but is not essential to mucosal infection by helping to scavenge iron from Lf, although alternative functions such as binding of otherwise bactericidal peptides derived from Lf have not been experimentally examined yet.

The Hgb receptors. Both Nm and Ng produce iron-repressed receptors that bind Hgb, or Hgb plus its binding protein haptoglobin (20, 21), and which are essential for iron uptake and growth from Hgb under conditions where HgB is the sole available iron source. Nm produce two similarly sized (ca 76kDa) but genetically and structurally unrelated receptors with apparently similar function, now designated either HpuB (20, and L. Lewis and D. Dyer, personal communication) or HmbR (21) respectively. Ng apparently possesses genes for both of these Hgb receptors, but only HpuB are expressed and functional in vitro (22). The Ng *hmbR* gene is mutated in strain MS11, apparently explaining its lack of expression in gonococci (J. Stojiljkovic, personal communication to C.E. Elkins). Recently, Lewis and Dyer showed that there are two adjacent genes in an *hpu* operon, designated *hpuA* and *hpuB*. The *hpuA* DNA sequence predicts that HpuA is a lipoprotein, whereas *hpuB* encodes a TonB-dependent family member of integral

outer membrane protein (20, and L. Lewis and D. Dyer, personal communication). Mutations in the Ng *tonB*, *exbB*, or *exbD* genes abolishes function of the Ng HpuB receptor (13), analogous to the effects of a specific *hpuB* mutation (22). Interestingly, the Ng HpuB receptor undergoes a high-frequency in vitro apparent phase variation from "off" to "on" when grown on media with Hgb as a sole iron source (22), possibly due to slipped-strand mispairing in a polypyrimidine tract observed in the upstream *hpuA* gene (L. Lewis and D. Dyer, personal communication). There are no obvious explanations for the advantage of having two Hgb receptors (HpuB and HmbR) in Nm. Phase variation of a single expressed gonococcal HpuB receptor could be a selective advantage in women during menses.

Other iron uptake systems. Both Nm and Ng can utilize heme, citrate, aerobactin and possibly enterochelin as iron sources in vitro (23). Less is known about the mechanism or physiological significance of iron uptake from these sources. Mutations in Ng *tonB*, *exbB*, or *exbD* do not reduce iron uptake from heme or citrate (13), suggesting that if there are specific receptors for these systems, they are not in the TonB-dependent family of receptors.

Much work has focused on another major iron repressed outer membrane protein found in all strains of both Nm and Ng designated FrpB, whose predicted protein sequence shows it is a member of the TonB-dependent family of outer membrane proteins (23, 24). Nevertheless, careful experiments failed to prove that FrpB plays a specific role in iron uptake from any presently studied iron source (23). DNA sequencing in Ng downstream from *frpB* (25) identified three open reading frames that encode proteins that are highly related to the periplasmic and cytoplasmic proteins involved in uptake of phenolate-siderophores in *Vibrio anguillarum* and *Campylobacter coli*, implying a possible role for such a system in Ng.

Other iron-repressed proteins. In addition to the outer membrane receptors for various specific iron sources, both Nm and Ng produce a periplasmic iron-transport protein designated Fbp1 which binds Fe^{3+} from any source after it gains passage through the outer membrane (26, 27). Transport through the cytoplasmic membrane apparently is mediated by the products of two linked genes, *fbpB* and *fbpC* (28).

Vaccine implications. Considerable attention is being given to potential new vaccines for Nm based on the Tf receptor. Bactericidal and mouse-protective anti-meningococcal Tbp2 antibodies have been demonstrated, and they exhibit considerable cross-reactivity against various Nm strains despite extensive variability in amino acid sequence of Nm Tbp2 proteins (29-32). Antibodies are produced to these proteins during natural infection (33). There is somewhat less variability of Ng Tbp2 proteins (34), suggesting that Ng Tbp2 is also an attractive potential vaccine target. There is convincing evidence for surface-exposure of Ng Tbp1 including protease accessibility, Tf binding to whole cells, and ability of certain polyclonal anti Tbp1 sera to bind whole gonococci (34). Since Tbp1 is much less variable than Tbp2 in both Nm and Ng (6, 34), Tbp1 deserves further study as a vaccine target. Another iron-repressible protein with vaccine potential is FrpB, since it is the target for Nm strain-specific bactericidal monoclonal antibodies

(35-36) and is relatively antigenically conserved in both Nm and Ng (J.T. Poolman personal communication, unpublished data of M Beucher, J Fu and PF Sparling). Antibodies are also produced against FrpB in natural infection (37).

Concluding remarks. Much has been learned about stress proteins in the pathogenic neisseria, but at least as many questions remain unanswered. Some of these proteins are certainly important in pathogenesis (even essential), and some may be vaccine targets. There is much to be learned about all of the actual or putative iron uptake systems, including why so many are needed, and in what circumstances they are functional.

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Function and virulence studies of the gonococcal transferrin receptor

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The gonococcal transferrin receptor consists of two iron-regulated proteins, Tbp1 and Tbp2. Tbp1 shares homology with TonB-dependent, integral, outer membrane receptors, is necessary for iron-uptake from transferrin (Tf) (1) but is not capable of discriminating between apo and ferrated forms of Tf (3). Tbp2 is lipid-modified, is not essential for iron-uptake from Tf (2), and binds ferrated Tf preferentially (3). We have proposed a model of Tf-iron utilization in which Tbp1 serves as the integral outer membrane pore through which iron traverses the outer membrane after it is removed from transferrin by an unknown mechanism (4). Tbp2 may serve to increase the specificity of the receptor for ferrated Tf and be important in release of apo Tf from the high affinity receptor after iron has been removed (4).

We have evaluated physical association and function of Tbp1 and Tbp2 using Tf-binding and protease-accessibility assays. The gonococcal Tf receptor has a high affinity for Tf with a Kd in the range of 1-16 nM. The Tf-binding parameters of Tbp1 and Tbp2 in isogenic mutants are distinct from those of the wild-type parent, which expresses at least two conformational states of the transferrin receptor (3). Similarly, protease-accessibility experiments indicate that Tbp2 is in a trypsin-sensitive state in a Tbp1$^-$ mutant, while Tbp2 is partially protected from trypsin in the wild-type strain. We have developed a model of the transferrin receptor in which the two Tf-binding proteins in the wild-type strain functionally interact to create a receptor that is capable of assuming at least two conformations. One conformation has a high affinity for transferrin (Kd = 1nM) and Tbp2 is in an "open", protease-sensitive state. The other conformation has a lower affinity for transferrin (Kd = 16 nM) and Tbp2 exists in a "closed", protease-resistant state. We created a Tbp1 mutant analogous to "TonB-box" mutants described in *E. coli* (5). This mutant, in which Ile16 of Tbp1 is changed to Pro16, is unable to grow on or internalize iron from Tf while it remains Tf-binding competent. Analysis of this and other deenergized mutants (6; and Biswas, Anderson, and Sparling, in preparation) suggests that interconversion between the two Tf receptor conformations is dependent upon the energy status of the cell.

To test the virulence contribution of the Tf receptor, we created a *tbpA/B* deletion mutant of gonococcal strain FA1090 that cannot utilize Tf-bound iron and determined its infectivity in a human challenge model of gonococcal urethritis (7). Strain FA1090 is natively unable to utilize lactoferrin (LF)-bound iron and does not express the phase-variable hemoglobin receptor. None of five volunteers inoculated with 1-2 x 10^6 CFU of the Tf$^-$ mutant exhibited signs of urethritis during the course of the four to six day

challenge. In contrast, 1 x 10^6 CFU of wild-type FA1090 causes urethritis in 90-100% of subjects. Some volunteers inoculated with the mutant excreted viable gonococci in their urine; however, compared to wild-type controls, th umber of gonococci recovered was decreased 2-3 logs. Of the few gonococcal survivors that were recovered from the urine, all retained the phenotypes of the original inoculated mutant. We conclude that in the absence of functional lactoferrin and hemoglobin receptors, the Tf-receptor deficient gonococcal mutant is greatly attenuated for initiation of urethritis and for survival in the male urethra.

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Periplasm-to-cytosol free iron transport by pathogenic *Neisseria*

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High affinity iron acquisition by pathogenic *Neisseria* has become a paradigm for other bacterial pathogens that specifically sequester host transferrin or lactoferrin to their cell surfaces by way of receptors (1). Subsequent to this binding event is the removal of ferric iron, Fe(III), from the host protein and its transport across the outer membrane in an energy-dependent manner (2). The energy required for the removal and transport of Fe(III) is presumably mediated by a TonB-dependent process, however the precise mechanism for this process has not been elucidated. What is clear is that iron removed from transferrin or lactoferrin is reversibly deposited on the periplasmic Fe(III)-binding protein referred to as FbpA (2). This initiates the process of periplasm-to-cytosol Fe(III) transport by way of a classic active transport process.

Biochemical, crystallographic, and site-directed mutagenesis studies from our laboratory have demonstrated that FbpA is a member of the classic periplasmic binding protein family (3). FbpA is a novel member of this group in that it is the only known Fe(III)-binding protein. The property of Fe(III) binding and its periplasmic binding characteristics led us to hypothesize that FbpA is a functional homologue of a half-transferrin. The range of metal ions bound by Fbp and transferrin have been previously reported and include gallium, Ga(III), and terbium, Tb(III). We have prepared site-directed mutations in suspected residues involved in Fe(III) binding by FbpA. This analysis has demonstrated three classes of mutations: (i) those that do not affect metal binding, (ii) those that significantly decrease metal binding, and (iii) those that abrogate metal binding. The remarkable similarity of the contingent of amino acids utilized for metal coordination by FbpA and transferrin demonstrates the functional homology of these proteins.

The genetic organization of FbpA as the protein product of the first gene, *fbp*A, in a three gene operon has been recently reported (4). In addition two linked genes, *fbp*B and fbpC, are proposed to make up a classic ABC transporter that is critical to the periplasm-to-cytosol transport of Fe(III) across the cytoplasmic membrane. Analysis of the predicted FbpB amino acid sequence demonstrates hydrophobic and putative membrane-spanning regions characteristic of a cytoplasmic permease. A similar analysis of FbpC reveals classic Walker motifs that are associated with nucleotide-binding proteins. However, biochemical analysis of FbpC demonstrated additional characteristics of membrane association and substrate specificity that were not predicted based on homology to described nucleotide-binding proteins that operate in active transport

processes. Both the cytoplasmic permease and nucleotide-binding properties are required for classic ABC transporter activity (4, 5).

The extrapolation of the preceding genetic predictions have been investigated by establishing a functional model for studying iron transport by FbpABC in an *E. coli* background (4, 5). These results have demonstrated the absolute requirement for all three components of the FbpABC operon as well as the toxicity associated with the FbpB component. Furthermore, single amino acid mutations that decrease the affinity of FbpA for metal ions by only one order of magnitude can result in rescue of the organism from the Fbp-mediated toxicity to Ga(III)

These studies describe the biochemical basis for the periplasm-to-cytosol transport of Fe(III) characteristic of pathogenic *Neisseria*. Furthermore, they underscore the importance of the FbpABC operon as an important contributor to success of these pathogens. In a larger context, these studies provide further evidence for the importance of pathogenic *Neisseria* multiplication within the host environment as a critical component associated with disease outcome.

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The *N. meningitidis* hemoglobin receptor genes and interaction between the hemoglobin receptor and the hemoglobin molecule

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We have recently cloned and characterized the hemoglobin receptor gene, *hmbR*, from *N. meningitidis* serogroup C isolate (1). The *N. meningitidis hmbR* mutant was unable to use hemoglobin as an iron source but was still proficient in heme utilization (1).The hemoglobin utilization-deficient mutant of *N. meningitidis* was attenuated in the infant rat model of meningococcemia, underling the importance of hemoglobin as an iron source for meningococci (1). Both *N. meningitidis* and *E. coli* cells expressing HmbR protein were able to bind biotinylated hemoglobin and the binding was specifically inhibited by unlabeled hemoglobin but not heme (2, 3). The HmbR-mediated Hb binding activity of *N. meningitidis* cells was shown to be iron-regulated. Presence of hemoglobin but not heme in the growth media stimulated HmbR-mediated hemoglobin binding activity. The efficiency of utilization of different hemoglobins by HmbR-expressing *N. meningitidis* cells was shown to be species specific; human hemoglobin was the best source of iron followed by horse, rat, turkey, dog, mouse and sheep hemoglobins. N,N'-methylated hemoglobin was the worst source of iron for *N. meningitidis* cells (2). These results indicate that the HmbR receptor is able to recognize globin part of hemoglobin molecule. We have cloned and characterized *N. meningitidis* serogroups A, B, and *N. gonorrhoeae MS11 hmbR* homologues (1, 2). The deduced amino acid sequences of these Neisserial receptors were highly related with an overall 84.7% identical amino acid residues (2). The phenotypic characterization of HmbR mutants in some clinical strains of *N. meningitidis* suggested the existence of two unrelated hemoglobin receptors. The HmbR-unrelated hemoglobin receptor was shown to be identical to Hpu, the hemoglobin-haptoglobin receptor of *N. meningitidis* (2, 4). The Hpu-dependent hemoglobin utilization system was not able to distinguish between different sources of hemoglobin; all animal hemoglobins were utilized equally well (2). *N. gonorrhoeae* is efficient in hemoglobin and heme utilization and all tested strains possess *hmbR*-related sequences (5, 2). However, the nucleotide sequence of MS11 *hmbR* gene contains a stop codon suggesting that another receptor, most probably Hpu, is responsible for Hb utilization in some *N. gonorrhoeae* isolates (2).

Peptide scanning approach was utilized in order to identify domains of the HmbR receptor and the hemoglobin molecule that interact with each other. A set of peptides derived from amino acid sequences of hemoglobin a and b chains and the HmbR protein were synthesized and their ability to inhibit hemoglobin utilization was determined. Two out of twenty two HmbR-derived peptides and two out of seventeen peptides originating from similar regions of Hb a and b chains, significantly inhibited hemoglobin utilization in the growth promotion plate assay. This results are another proof that the HmbR receptor recognizes globin part of hemoglobin molecule. Deletion analysis of the

HmbR-surface exposed loops is currently carried out in order to corroborate the results of peptide scanning.

E. coli cells expressing the HmbR protein were not fully capable to use hemoglobin as an iron source indicating that additional *N. meningitidis* proteins are involved in hemoglobin utilization (1). In order to identify additional proteins involved in Hb utilization we have reconstituted the hemoglobin utilization system in *E. coli*. A *N. meningitidis* cosmid library was introduced into a heme-requiring mutant of *E. coli* expressing the HmbR protein. Transformants were plated onto Hb-supplemented media. Cosmids from five colonies which able to use hemoglobin as both porphyrin and iron source were further studied. When retransformed into *E. coli hemA*, cosmids allowed growth on Hb only in the presence of a *hmbR*-expressing plasmid. Nucleotide sequence analysis of DNA fragments subcloned from one of the Hb-positive cosmids identified three ORFs homologous to *Ps. putida*, *E. coli* and *H. influenzae exbB*, *exbD* and *tonB* genes. In order to understand the role these genes play in hemoglobin utilization, a *tonB* mutant of *N. meningitidis* was constructed. Preliminary data indicate that utilization of transferrin, lactoferrin, hemoglobin and haptoglobin-hemoglobin complexes as sole sources of iron is a TonB-dependent in *N. meningitidis*. Conversely, the *N. meningitidis tonB* mutant was still able to use heme indicating the existence of TonB-independent heme-uptake system in *Neisseriae*.

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Cloning, sequencing and genetic characterization of *tonB-exbB-exbD* genes of *Neisseria gonorrhoeae*

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N. gonorrhoeae is able to utilize efficiently iron (Fe) bound to transferrin (TF) and lactoferrin (LF). To understand the mechanisms used by gonococci to scavenge Fe from TF and LF, we isolated chemical mutants of strain FA19 that were unable to grow with Fe bound to either TF(TF^-) or LF(LF^-) or to both TF and LF($[TFLF]^-$)(3). The TF^--specific or LF^--specific mutants lacked receptor activity for TF or LF respectively(3), and they have been used successfully in cloning receptors for TF and LF(1,2). The $(TFLF)^-$ mutant designated *tlu* bound both TF and LF well, but the biochemical defect in the mutation was not known(3). In this report, we characterized the *tlu* loci through cloning, sequencing, transposon mutagenesis, and phenotypic studies.

An approach described previously was used to clone a wild type gene that is able to repair the defect in *tlu* mutations(1,2). A $(TFLF)^-$ mutant strain was transformed with pools of wild type FA19 DNA cloned in pBluescript, selecting for TF^+ transformants. A transforming clone was identified containing 404 bp gonococcal insert. By chromosome walking, 3488 bp of contiguous FA19 DNA were cloned. Nucleotide sequence identified three contiguous open reading frames (ORF) designated as ORF1, ORF2 and ORF3 that are arranged in tandem. The first two ORFs are separated by 66 bp, and the third ORF is situated 18 bp downstream of the second ORF. The deduced amino acid sequence of the 852 bp long ORF1 encoded a protein that exhibited 26% identity with the TonB protein of *E .coli* (5), whereas the 648 bp long ORF2 and 435 bp long ORF3 was 27% and 36% identical to that of of the ExbB and ExbD protein respectively of the same organism (4). The three ORFs are situated on a 4.5 kb gonococcal *Hinc* II fragment, and were able to repair six independently derived *tlu* mutations by DNA contained within these ORFs.

Each of the three ORFs in Bluescript vector were mutagenized with the interposon omega, and were then transformed into FA19. Each of the resulting mutants lost the ability to utilize either TF, LF, or human hemoglobin (Hgb), but could utilize hemin or Fe-citrate for growth. ^{55}Fe uptake assays with $^{55}FeTF$, $^{55}FeLF$, ^{55}Fe-hemin or ^{55}Fe-citrate showed that the same strains were unable to take up ^{55}Fe from TF or LF but internalized ^{55}Fe from hemin or citrate. However, the mutants bound TF, LF and Hgb normally as determined by dot blot assay.

Thus, the similarity in amino acid sequence homology and the pleiotropic nature of Fe related phenotype between the gonococcal ORF1-ORF2-ORF3 complex and *E. coli* TonB-ExbB-ExbD proteins suggest that ORF1, ORF2, and ORF3 represent *ton*B, *exbB*,

and *exbD* genes in gonococcus. Moreover. the results show unambiguously that the TF, LF and Hgb receptors, but not the heme and citrate-iron uptake pathways, are dependent on the TonB system.

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Genetic and serological analysis of lactoferrin receptors in the Neisseriaceae: evidence for the antigenically conserved nature of LbpB

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The utilization of iron from transferrin (Tf) and lactoferrin (Lf) by pathogens in the *Neisseriaceae* is believed to occur via a multicomponent pathway which includes host specific Tf (TbpA+B) and Lf (LbpA+B) receptors on the organism's cell surface, a periplasmic iron binding protein (FbpA) which shuttles the ferric iron to the cytoplasmic membrane, and cytoplasmic membrane components which provide energy for the active transport of the Fe from the extracellular milieu to the periplasm (TonB with associated proteins Exb and ExbD), and across the cytoplasmic membrane (FbpB and FbpC) into the cytoplasm. The outer membrane receptor complex is believed to consist of a lipidated-hydrophilic component (TbpB and presumably LbpB), and a membrane spanning component (TbpA and LbpA) which may act like a gated pore, to allow entry of the Fe into the periplasm. The TbpA and LbpA proteins of a number of different organisms are highly homologous, and are 43% identical for *Neisseria meningitidis*.

The recently described LbpB molecule has been shown to reside in the human pathogens *N. meningitidis* (strains M982 and P3006), *Moraxella catarrhalis* (strain 4223) and the bovine pathogen *Moraxella bovis* (strain n112). In each case, the growth and binding characteristics of these organisms Lf receptors were specific for the host Lf [1]. Previously, we have demonstrated that the *lbpA* gene was the present in all clinical isolates of *N. meningitidis* and *N. gonorrhoeae*, as well as being present in virtually all commensal *Neisseria spp.* [2]. However, there is neither genetic or biochemical evidence which examines the ubiquity of the *lbpB* gene in pathogenic *Neisseria spp.* Therefore, we used a specific *lbpB* gene probe to examine, by Southern blot analysis, several pathogenic *N. meningitidis* and *N. gonorrhoeae* strains for the presence of this gene. The presence of the *lbpB* gene product was confirmed in these strains using polyclonal antisera against the *N. meningitidis* P3006 LbpB. We were also able to include a limited number of clinical gonococcal isolates in this analysis that were unable to utilize Lf as a sole iron source.

Several reports, to date, have shown that TbpB proteins (formerly designated Tbp2) from different meningococcal strains are quite heterogeneous in both molecular weight and serological cross-reactivity. However, there is currently no information about LbpB antigenic heterogeneity (or lack thereof). Our analysis consisted of using the western blot technique to test the ability of rabbit polyclonal antisera derived against either the *N. meningitidis* (strain P3006) LbpB, the *M. catarrhalis* (strain 4223) LbpB, or the *M. bovis* (strain n112) LbpB protein to react with the LbpB molecules from different *N. meningitidis, M. catarrhalis* or *M. bovis* isolates. Our results have indicated that, unlike the TbpB molecule, the molecular weight of the LbpB is approximately identical in all strains tested. In addition, the sera derived against the *N. meningitidis* (strain P3006)

LbpB molecule reacted with all meningococcal strains tested, and also reacted weakly , with the *M. catarrhalis* (strain 4223) and *M. bovis* (strain n112) LbpB molecules. Sera against the either the *M. catarrhalis* (strain 4223) or *M. bovis* (strain n112) LbpB molecules demonstrated varying degrees of reactivity with a number of meningococcal isolates. In addition, we demonstrate the ability of antisera against the LbpB molecule to interact strongly with whole cells grown in iron restricted conditions, and only weakly with cells grown in an iron sufficient environment. These observations suggest that LbpB is surface exposed, is expressed when free iron is limiting, and is a potentially useful vaccine target due to its conserved antigenic nature and ubiquity among pathogenic strains.

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Characterization of the interaction between *Neisseria meningitidis* transferrin binding proteins and transferrin by gel filtration and surface plasmon resonance

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Neisseria meningitidis produces two iron repressible transferrin binding proteins; Tbp1 (100kDa) and Tbp2 (65-85kDa depending on strain) (1, 2). Both proteins are surface-exposed and are associated with the sequestration of iron from human serum transferrin (HST 80kDa) (3, 4). Iron uptake from this source requires the expression of Tbp1 and Tbp2 suggesting that these proteins interact and together form the functional HST receptor (5). In this study gel filtration and surface plasmon resonance (SPR) (6) have been used to characterise the association between Tbp1 and Tbp2, and the binding of HST by meningococcal Tbps.

Tbp1+2 was isolated from iron limited cultures of *N. meningitidis* using HST-Sepharose affinity chromatography, as described previously (7). Separate Tbp1 and Tbp2 were obtained by ion exchange chromatography using a Mono S column (Pharmacia) on FPLC. Analysis of affinity purified Tbp 1+2 and recombined separate Tbp1 and Tbp2 by gel filtration on a Superose 12 column (Pharmacia) indicated the formation of a ~300kDa complex. This complex bound HST resulting in a mass increase estimated at ~100kDa.

Separate Tbp1 had an apparent molecular weight of ~200kDa on gel filtration, suggesting the formation of a dimer. This complex bound HST and the resultant species had a molecular weight of ~300kDa. Indicating that Tbp1 bound a single molecule of HST. Thus it appears that the intact Tbp1+2 receptor and putative Tbp1 dimer each bind HST in a 1:1 ratio.

Similar analysis of purified Tbp2 by gel filtration indicated the formation of~400-600kDa multimers. These structures were absent from native mixtures inferring that the interaction between Tbp1 and Tbp2 may prevent their formation. The high and variable molecular weight of such aggregations indicates that they may be of non specific-structure, possibly resulting from the inherent adhesive nature of lipidated Tbp2.

The interaction between Tbps and HST has also been analysed by SPR using the Biacore X biosensor (Pharmacia). HST immobilised on the dextran matrix by amine coupling bound Tbp1 forming a highly stable complex. Tbp2 also bound to HST although the resultant complex underwent rapid dissociation. Immobilised HST was treated with Tbp1 until no further binding was observed. At this point resonance data indicated that each molecule of HST bound more than 1 molecule of Tbp1. The HST-Tbp2 interaction was

examined in the same way and results suggested a 1:1 ratio of binding. Prior saturation with Tbp1 did not inhibit subsequent Tbp2 binding and appeared to stabilise the resultant complex. These results indicate that Tbp1 and Tbp2 bind separate distinct regions of HST and may interact following this association. This is consistent with gel filtration results indicating that the functional HST receptor is formed by two molecules of Tbp1 and one molecule of Tbp2.

It has been shown that gonococci expressing only Tbp2 preferentially bind iron loaded (HOLO) HST over iron free (APO) HST (8). This has been demonstrated using the isolated high molecular weight form of meningococcal Tbp2 in an ELISA based study. Further gel filtration and SPR studies are in progress to investigate the kinetics and stoichiometry of APO/HOLO HST binding by meningococcal Tbps.

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Analysis of respiration linked nitrite reduction in *Neisseria gonorrhoeae*: AniA, the major anaerobically induced outer membrane protein, is probably not the terminal nitrite reductase.

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Neisseria gonorrhoeae (GC) is one of the primary causes of pelvic inflammatory disease (PID). Gonococcal PID is most likely an anaerobic infection as half of these cases are mixed infections with GC isolated along with obligate anaerobes (1). In order to grow under anaerobic conditions, GC uses nitrite as a terminal electron acceptor for respiration (2).

Previously, the outer membrane lipoprotein AniA (Pan1) was identified (3,4) and the *aniA* gene cloned and sequenced (5). Recent database searches indicate that AniA has 30-35% homology with copper-containing nitrite reductases (NiR) from other species. Outer membranes containing AniA reduced nitrite when methyl viologen was added as a gratuitous electron donor. In contrast, NiR from other species are typically periplasmic or bound to the cellular membrane (6), nor have any terminal reductases been found in the outer membrane in other bacterial species. Even though AniA appears to be capable of reducing nitrite, it is hard to envision that AniA could function as a respiration linked NiR with an outer membrane location.

In order to differentiate respiration linked NiR activity from total NiR activity, a physiological electron donor system was contrasted with an artifical gratuitous system. When glucose was added to measure respiration linked to the oxidation of NADH, there was a ten-fold increase in NiR activity in anaerobically vs. aerobically grown GC. If methyl viologen was used, there was no difference in activity of aerobically grown GC compared to the respiratory linked NiR activity, while there was a 300-fold increase in total NiR activity in anaerobically grown cells. This strongly suggested two pools of NiR activity, one with access to an internal supply of reducing equivalents, the other without access. The presence of NiR activity in aerobically grown GC indicates that there is an enzyme other than AniA that is responsible for this reductase activity, as there is no detectable AniA protein or *aniA* mRNA transcript in aerobically grown cells (5).

Osmotic shock was used to separate periplasmic proteins from the rest of the cell. The periplasmic fractions from aerobic and anaerobically grown GC showed similar NiR specific activity. Again, as aerobically grown GC do not contain detectable levels of AniA or *aniA* mRNA transcript (5), this similar activity in the two periplasmic fractions is most likely due to a protein separate from AniA.

In order to study the regulation of the *aniA* gene, the *aniA* promoter was cloned into the pLES94 construct and transformed into GC (7). This resulted in an *aniA*::*lacZ* fusion in

single copy in the GC chromosome at a site other than the *aniA* site. RNase protection studies confirmed the pLES940 construct accurately reflects *aniA* promoter activity from both the gearbox and sigma^{70} promoters, as well as accurat _ reflecting the level of *aniA* expression from each individual promoter. This construct was then used to show that under aerobic conditions, GC NiR activity increased 4-fold at the onset of stationary phase while the β-galactosidase reporter under control of the *aniA* promoter did not show any change in level of activity. This differential regulation between NiR activity and β-galactosidase activity underscored the likelihood that a second enzyme was responsible for the increase in NiR activity.

In order to track NiR activity in GC separate from AniA expression, a fusion protein with the FLAG™ peptide covalently bound to the to the C-terminus of AniA was constructed and transformed into GC. Aerobic and anaerobically grown GC containing the fusion were tested for NiR activity and equivalent cell amounts were analyzed by SDS-PAGE and a Western blot probed with anti-FLAG™ monoclonal antibodies. While both groups of cells had NiR activity, there was no detectable FLAG™ peptide in the aerobically grown cells, indicating that a protein other than the AniA::FLAG™ fusion was responsible for the nitrite reduction in these cells.

While at first glance AniA's function appears to be nitrite reduction, further studies into nitrite reduction in GC have led to the conclusion that there is a second enzyme that is responsible for respiration linked nitrite reduction. This is based on the presence of NiR activity in aerobically grown cells while there is no AniA or *aniA* mRNA transcript in these cells, as well as the differential regulation between NiR activity and β-galactosidase activity.

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Gonococcal FrpB: a possible role in siderophore uptake

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FrpB is an iron-regulated outer-membrane protein common to both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. This protein shares homology with the TonB-dependent class of outer-membrane proteins common among Gram negative species (1), the most closely related proteins being CopB of *Moraxella catarrhalis* (71% similarity) (2) and the hemin receptor, HemR of *Yersinia enterocolitica* (48% similarity) (3). Much labor has gone into trying to determine the function of FrpB, but to date, it has remained elusive. Initial experiments indicated that a FrpB-deficient strain of *Neisseria gonorrhoeae* is somewhat impaired in its ability to utilize iron from heme and possibly lactoferrin, but further studies revealed that this decreased ability may actually be due to non-specific membrane perturbations in the mutant strain (1; B. Stone, unpublished data).

During the initial sequencing of *frpB*, the start of an open reading frame (about 100 base pairs) was identified directly downstream. A GCG database search revealed this sequence to share homology with various periplasmic siderophore transporters (Beucher, unpublished data). This encouraged us to sequence further downstream of *frpB* in the hope that some clues about FrpB function could be gained.

Sequencing downstream of *frpB* revealed at least three open reading frames (orfs). Each has sequence similarity to components of siderophore uptake systems, especially the phenolate-siderophore uptake systems. The *orf1* deduced amino acid sequence shares extremely high degrees of sequence similarities with periplasmic phenolate-siderophore transporters, especially CeuE, the *Campylobacter coli* enterobactin periplasmic transporter (4), and FatB, the *Vibrio anguillarum* anguibactin periplasmic transporter (5). Additionally, the *orf1* deduced amino acid sequence contains the "signature sequence" present in all known periplasmic siderophore transporters (6). The *orf2* and *orf3* deduced amino acid sequences share strong degrees of sequence similarities with members of the cytoplasmic siderophore transporter family, including CeuB and C (4), and FatC and D (5). Because of the homologies between the three open reading frames and components of siderophore transport systems, we hypothesize that these open reading frames may play a role in the uptake of iron from siderophores in *Neisseria gonorrhoeae*. The presence of these open reading frames located directly downstream of *frpB* implicates siderophore uptake as a possible role of FrpB.

Although it has long been believed that the *Neisseriaceae* produce no siderophore of their own (7), the gonococcus is able to scavenge iron from the siderophore aerobactin from co-existing bacteria (1), as well as from various human carrier proteins (8-10).

Mutation of *frpB* did not affect aerobactin utilization (1), but other siderophores were not tested carefully. Studies are presently underway in the laboratory to determine whether *frpB* is transcriptionally linked to the downstream orfs and whether FrpB and Orf1, Orf2, and Orf3 play a role in siderophore uptake.

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A phase varying, hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*

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The majority of *in vitro* variants of *Neisseria gonorrhoeae* were unable to use human hemoglobin as the sole source of iron (Hgb-), but a minor population were able to do so (Hgb+). This minor population grew luxuriously on hemoglobin, bound biotinylated hemoglobin and expressed a novel outer membrane protein of 89 kDa. The ability to use hemoglobin for growth apparently was a phase varying phenomenon. Among all strains tested, the Hgb- to Hgb+ variation rate was 0.08 to 2.15 variants per 1000 CFU. The 89 kDa protein was regulated by levels of iron present in the medium and was purified on immobilized hemoglobin.

The N-terminal amino acid sequence of the 89 kDa protein revealed identical amino acids, from position 2 to 16, to HpuB, an 85 kDa iron-regulated hemoglobin-haptoglobin utilization outer membrane protein of *Neisseria meningitidis*. Isogenic mutants of FA19 and FA1090 constructed by allelic replacement using a meningococcal *hpu*::mini-Tn3erm construct (1) no longer expressed the 89 kDa protein. Mutants could not utilize hemoglobin to support growth but still grew on heme. Thus, the gonococcal HpuB homologue is a functional hemoglobin receptor. Using chromosomal DNA from isogenic mutant of FA19, additional insertional mutants were obtained from hemoglobin utilizing variants of several clinical gonococcal strains. These mutants also lost their ability to utilize hemoglobin. The gene for another hemoglobin binding protein, HmbR, has been found in *N. meningitidis* and *N. gonorrhea* MS11 (2, 3). Southern blot hybridization of hemoglobin utilizing FA19 and FA1090 DNA with MS11 *hmbR* demonstrated that these two strains also have the gene encoding HmbR. However, insertional inactivation of the *hmbR* gene did not affect the hemoglobin utilization of either FA19 or FA1090. Thus, while gonococci may have genes for two different hemoglobin binding proteins, the 89 kDa HpuB homologue is essential for the utilization of hemoglobin.

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Diversity, topology, and functional domain mapping of gonococcal transferrin-binding proteins

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The gonococcal transferrin receptor consists of two iron-regulated proteins, Tbp1 and Tbp2. Tbp1 shares homology with a family of outer membrane receptors required for the energy-dependent uptake of ferric siderophores and vitamin B_{12} in *E. coli* (1). Models of membrane topology for these receptors have been generated using a variety of computer algorithms and domain mapping techniques (2, 3). We have generated a hypothetical model of Tbp1 using both computer predictions and sequence heterogeneity observed among three gonococcal sequences and two meningococcal sequences. Conserved stretches of amino acids are expected to be buried in the membrane while variable stretches are localized in exposed loops. We raised polyclonal antibodies against peptides in predicted surface-exposed domains and tested these antibodies for their ability to bind to the gonococcal cell surface, to block transferrin access to the receptor, and to initiate complement-mediated killing of wild-type gonococci. While several antibodies raised against hypothetically exposed loops of Tbp1 reacted against denatured Tbp1 in western blots, only one bound to whole gonococci. The domain to which this antibody was raised is located in the amino-terminal third of the proposed Tbp1 model. In preliminary experiments, this antibody also inhibited access of ferrated transferrin to the receptor and initiated complement-mediated killing of wild-type gonococci. We conclude from these experiments that a domain in the amino-terminal third of Tbp1 is surface-exposed, is important for interaction with ferrated transferrin, and potentially a site for binding of bactericidal antibody.

Gonococcal Tbp2s are more diverse at the predicted protein level than are gonococcal Tbp1s. We have compared five gonococcal Tbp2 sequences (4) with the published meningococcal Tbp2 sequences (5, 6). Several conserved stretches can be identified in an alignment of all 10 Tbp2 sequences; these domains may be important for some aspect of Tbp2 function, localization or stability. By expressing truncated versions of Tbp2, we have delimited a minimal transferrin-binding domain of gonococcal Tbp2, which extends through the amino-terminal half of the protein. Two stretches of conserved residues are contained within the transferrin-binding domain, thus they may be important for optimum ligand binding. Conserved and variable domains of gonococcal Tbp2s will be presented in the context of observations of functional constraints made for Tbp2s from other pathogenic bacteria (7, 8).

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Evidence for a bi-lobed structure for meningococcal transferrin binding protein B

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Members of the Neisseriaceae utilize a cell-surface receptor that binds transferrin (Tf) as an initial step in the iron acquisition process. This transferrin receptor is comprised of two outer membrane proteins, transferrin binding protein A (TbpA) and transferrin binding protein B (TbpB). Comparative analysis of the TbpA sequence predicts an integral transmembrane protein which may act as a gated pore for iron (1). TbpB is predicted to be a peripheral outer membrane lipoprotein anchored to the membrane via a N-terminal-linked fatty acid (1). It has been proposed that there is a conserved mechanism of iron acquisition amongst species containing transferrin receptors which predicts a conserved receptor-ligand interaction. Previous studies have demonstrated that regions of TbpB from *N. meningitidis* and *A. pleuropneumoniae* involved in binding to Tf can be localized to the N-terminal portion of TbpB (2,3). As well, TbpB from human pathogens have been shown to bind to the C-terminal lobe of human transferrin (hTf) (4). However, the concept of a conserved receptor-ligand interaction is difficult to resolve with the demonstration that TbpB from several bovine pathogens binds to the N-terminal lobe of bovine Tf (5).

As part of further elucidation of the mechanism of Tbp-mediated iron procurement from Tf, the specific interactions between the TbpB receptor component of meningococcus and human transferrin (hTf) were investigated. Amino acid sequence alignment of the N-terminal and C-terminal halves of strain M982 TbpB revealed regions of identity, implying that a duplication event gave rise to sequences present in both the N-terminal and C-terminal halves. To further investigate the character of these two regions of TbpB, a series of M982 N- and C-terminal TbpB truncations and N- and C-terminal fusions were utilized to identify regions of TbpB required for interaction with hTf (3).

Recombinant TbpBs expressed in *Escherichia coli* consisted of the N-terminal half of TbpB; the C-terminal half of TbpB; incremental C-terminal deletions of the N-terminal half of the molecule; and maltose binding protein fusions of the N-terminal and C-terminal TbpB halves. After confirmation of expression of the TbpB truncations and fusions by western blot analysis of whole cell lysates, the ability to bind hTf was investigated. Using whole cell lysates as a source of the TbpB truncations, solid phase and affinity isolation assays were performed. These two methods of analysis provided corroborating results where both the N-terminal and C-terminal halves of M982 TbpB were shown to bind hTf. Deletion constructs containing smaller portions of the N-terminal region were unable to bind hTf in either assay. Chimeric hTf/bTf-N/C lobe transferrins were also utilized to localized the binding determinants for these halves of TbpB to the individual lobes of hTf.

The conservation of sequence between the two halves of TbpB, combined with the ability of both halves to bind hTf, provide evidence that TbpB arose from a gene

duplication event. This also has implications regarding TbpB structure. As each half of TbpB can independently bind hTf, TbpB may consist of two lobes, each with a distinct binding region for Tf. This proposed bi-lobed structure of TbpB may be a conserved feature of TbpB from various species, and the ability to demonstrate this may be dependent on the nature of the assay conditions utilized.

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Conserved interactions between heterologous TbpB-TbpA pairs and Tf.

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In response to iron limitation, bacteria in the families *Pasteurellaceae* and *Nesseriaceae* synthesize outer membrane receptor complexes which bind host transferrin (Tf) as the first step in an iron acquisition pathway. The receptor complex consists of Tf binding proteins B (TbpB) and A (TbpA) (1). While both TbpB and TbpA of the human pathogens *Neisseria meningitidis* and *Haemophilus influenzae* bind human Tf (hTf) independently in solid phase binding assays, affinity isolation of TbpB is dependent on the presence of TbpA in an affinity isolation assay using hTf-Sepharose. In addition, both TbpB and TbpA are required for optimal acquisition of hTf-bound iron supplied as a sole iron source in an *in vitro* growth assay (2,3). Therefore, we hypothesized that a specific interaction was occurring between TbpB and TbpA or that a conformational change in hTf resulting from TbpA binding increases TbpB binding to Tf to detectable levels. Furthermore, we hypothesized that this interaction(s) is conserved among the human pathogens *H. influenzae* and *N. meningitidis*.

In an attempt to further investigate this putative TbpB-TbpA interaction, we decided to examine whether it was present when heterologous TbpAs and TbpBs were studied. Thus we affinity isolated TbpA from *N. meningitidis* and *H. influenzae* isogenic mutant strains (2,3) and TbpA from *M. catarrhalis* using selective binding and/or elution conditions (4) and subsequently assessed their ability to facilitate isolation of TbpB from *N. meningitidis* or *H. influenzae*. These studies demonstrated that heterologous TbpAs from human pathogens could facilitate isolation of both TbpBs indicating that this is a conserved interaction.

To further investigate whether this phenomenon was due to TbpA-TbpB interaction or modification of Tf binding to TbpB, we examined the ability of TbpA from *N meningitidis* to affinity isolate TbpBs from the porcine pathogen *A. pleuropneumoniae* and the bovine pathogen *H. somnus* using an hTf-Sepharose affinity matrix. The *H. somnus* and *A. pleuropneumoniae* TbpBs were isolated by the hTf-Sepharose matrix in the presence of the *N. meningitidis* TbpA, but not in its absence. Identical results were obtained using apo-Tf, indicating that the interaction is not dependent on the iron-loaded state, and the corresponding conformation, of Tf. Collectively, these results suggest a TbpA-TbpB interaction is responsible for this phenomenon, rather than modulation of Tf structure by TbpA.

In an attempt to localize regions of TbpBs involved in interactions with TbpA or hTf, the ability of a series of chimeric *A. pleuropneumoniae-N. meningitidis* TbpBs to affinity isolate on hTf in the presence and absence of the *N. meningitidis* TbpA or on pTf in the

presence or absence of the *A. pleuropneumoniae* TbpA was examined. Several chimeric TbpBs were identified that were able to affinity isolate in the presence of the heterologous TbpA. A detailed analysis of these results will be presented and discussed.

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Specificity of the gonococcal heme transport system

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Neisseria gonorrhoeae can utilize free heme as a source of Fe for growth; however, little is known concerning the mechanisms involved in heme transport. We have characterized the binding and accumulation of heme by *N. gonorrhoeae* and defined the specificity of the gonococcal heme receptor. We have also initiated studies to determine if *N. gonorrhoeae* produces a hemolytic-like-activity. In many pathogenic organisms a hemolysin or cytotoxin appears to function in the lysis of erythrocytes in vivo, resulting in the liberation of heme which may then be utilized as an iron source. Our results indicate that whole cell extracts obtained from *N. gonorrhoeae* F62, FA19, and MS11 are capable of lysing sheep red blood cells. Thus, the gonococcus may possess the ability to lyse erythrocytes in vivo resulting in the liberation of heme; the iron from heme could then be transported via the gonococcal heme transport system. Our results indicate that a common receptor which recognizes heme (through the PPIX ring) is involved in binding both heme and hemoglobin. Binding of radiolabeled heme was shown to be inhibited by the addition of heme, hematoporphyrin, or hemoglobin, but not by ferric citrate. Thus *N. gonorrhoeae* may utilize at least 2 receptors for the binding of heme-containing compounds; the putative hemoglobin receptor which binds both heme and hemoglobin, and a second receptor specific only for heme. Following the interaction of heme with the gonococcal hemin receptor(s), ^{59}[Fe] from radiolabeled heme is taken up into the cell at a constant rate by an energy dependent mechanism. We also found that the majority of ^{59}[Fe] from heme was associated with the gonococcal periplasmic ferric binding protein, FbpA. We did not detect the uptake of ^{14}[C] from radiolabeled heme indicating that the PPIX ring is not transported into the cell. Taken together, our results indicate that heme binds to gonococcal outer membrane receptors through the PPIX ring, and following binding, iron is removed and transported into the cell where it is associated with the periplasmic ferric binding protein, FbpA.

Molecular analysis of *lbpAB* encoding the two compor : meningococcal lactoferrin receptor.

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Pathogenic Neisseria are able to acquire Fe from lactoferrin (LF), transferrin (TF), hemoglobin (Hb) and Hb complexed to haptoglobin (Hb-Hp) (1-6). Acquisition of Fe from TF, Hb and Hb-Hp inv
olves the production of a bipartite receptor composed of a specific TonB dependent transport protein and an accessory lipoprotein (7, 8). Acquisition of Fe from LF is associated with the production of the TonB dependent outer membrane protein, LbpA (4).

We cloned and analyzed *lbpA* from *N. meningitidis* DNM2. The predicted amino acid sequence of LbpA suggests that LbpA is an outer membrane protein with a 24 amino acid leader peptide and a signal peptidase I cleavage site. At the amino acid level LbpA shares a high degree of similarity with TonB dependent outer membrane receptors, suggesting that LbpA is a member of this family of high affinity transporters. The LbpA protein of DNM2 appears to be highly conserved sharing 99% identity with IroA (3) and 95% identity with the LbpA identified by Pettersson *et al* (9).

5' to *lbpA* we discovered an open reading frame (ORF) which we designated *lbpB*. The predicted amino acid sequence of the putative LbpB is highly homologous to Tbp2, the lipoprotein component of the TF receptor, and may encode the lipoprotein component of the LF receptor.

Fe-availability controls the expression of proteins required for acquisition of Fe. Classically Fe repression occurs at the level of transcription and is mediated by the transcriptional repressor, Fur. A Fur homologue has been identified in the Neisseria (10) and RNA dot blot analysis indicates that expression of *lbpA* is regulated by Fe at the level of transcription. RT-PCR analysis confirms that regulation of *lbpA* occurs at the level of transcription and suggest that *lbpA* and *lbpB* are co-transcribed on a polycistronic mRNA.

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Transcriptional regulation of pilC2 of *Neisseria gonorrhoeae*: Response to oxygen availability and evidence for growth phase regulation in *E. coli*

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The type 4 pilus of *Neisseria gonorrhoeae* is a predominant surface antigen which facilitates adhesion to host target cells (1), an essential event in gonococcal infection. *pil*C2 encodes a 110 kDa pilus tip-located adhesin (2) that is involved in pilus-mediated adherence to human epithelial cells in culture, pilus assembly and natural competence for DNA transformation (3). Luciferase activity directed from a chromosomal *pil*C2::*lux*AB transcriptional fusion was reduced approximately 3-fold when cells were grown anaerobically versus aerobically. We observed a concomitant reduction in gonococcal piliation, by electron microscopy, and reduction in their ability to adhere to ME-180 human epithelial cells when bacteria were grown in the absence of oxygen. Additionally, we present evidence for growth phase regulation of the gonococcal *pil*C2 gene in *E. coli*, determining that all sequences necessary for growth phase regulation are contained on a 118 bp *pil*C2 fragment. Expression from the minimal *pil*C2 fragment fused to *lacZ* in single-copy in *E. coli* was induced 2-fold as cells exited exponential growth. Surprisingly, induction was independent of an intact *rpo*S gene which encodes the starvation induced sigma factor RpoS. In conclusion, we have demonstrated that *pil*C2 is both positively and negatively regulated at the level of transcription, with an overall change in expression of approximately 6-fold. This regulation is most likely relevant to physiological conditions within the human host which influence gonococcal infections.

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Sequence analysis of the structural *tbp*A gene: protein topology and variable regions within neisserial receptors for transferrin iron acquisition

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The meningococcal transferrin receptor is comprised of two transferrin-binding proteins, Tbp1 and Tbp2. Tbp1 is a 98 kDa transmembrane protein with only small variations in its molecular weight among strains, and was originally thought not to be exposed (1). Bishop et al. (2) raised murine monoclonal anti-Tbp1 antibodies, one of which showed bactericidal activity and recognized a conformational epitope. This data suggests that Tbp1 is surface-exposed and immunogenic in humans and animals, and antibodies to their native structure could be bactericidal to homologous and heterologous strains. In order to assess at the molecular level the conservation of the Tbp1 protein, we obtained the complete sequence of the *tbp*A gene of the strain B385. The predicted B385 protein sequence was aligned with sequences with identities ranging from 43 to 93 percent including the closely related neisserial Lbps.

Variable Regions. The analysis of the multiple sequence alignment revealed large highly conserved areas and several well defined, smaller regions of sequence variability. Most differences among the meningococcal Tbp1s were localized to five regions of the mature protein, called VR1 (199-287), VR2(306-381), VR3(480-546), VR4(618-651) and VR5(681-708). The VRs may be associated with receptor specificity, since they show the highest sequence divergence between Tbps and Lbps, contain variable (and thereby thought to be exposed) regions, and also contain well conserved segments. By correlative analysis we were able to detect an overall 0.5 frequency of specific residues against a 0.3 frequency present in less variable segments which points out the contribution of such regions to the receptor specificity. The highest variability between Tbps and Lbps was found at VR1. We were able to detect a pair of well conserved cysteines (241 and 249) within this region (VR1), separated by a short and highly variable heptapeptide that is present in the Tbps but not in the Lbps.

VR3 resulted the most variable among Tbps. This region was also noticeable from the correlative analysis since we found that the Lbps have a deletion of 8-10 amino acids in this region compared to Tbps.

Topology model: We constructed a topology model for Tbp1 by applying the same principles that have been recognized for the structure of *E. coli* outer membrane proteins (3,4). The protein is thought to span the membrane 28 times, thereby exposing 14 hydrophilic loops to the outer surface, with VRs in the fifth, sixth, eighth, tenth and eleventh loops respectively.

Like FepA, and perhaps the other Ton-B dependent receptors (3), we postulate a similar gated channel model for Tbp1. As in the proposed topology model for Lbp (5) we propose large exposed loops, but we locate an additional loop in the outside layer of the membrane by assuming two additional membrane spanning sequences: a new one (14-21) at the N-terminus of the mature protein, and by placing two b-strands in the region 183-198. Another major discrepancy refers to the sequence 113-SGAINEIEYEN-124, which we do not believe to form a transmembrane section, but to be included in a highly important extracellular loop (between b5 and b6), well conserved by structural or functional constraints in Tbp as well as in Lbp. We strongly believe that our model fits the Lbp as well, regarding the showed differences in some of the exposed regions.

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Molecular characterization of FrpB, the 77 kDa iron-regulated OMP

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Under iron limitation *Neisseria meningitidis* produces several extra outer membrane proteins (OMPs). To gain insight in the mechanism of iron acquisition by *N. meningitidis,* we are studying these iron-limitation inducible OMPs. The proteins are also studied to assess their vaccine potential. This report deals with the 77 kDa FrpB protein, the major iron-regulated OMP.

Monoclonal antibodies (mAbs) were raised against the FrpB protein of two different strains. The antibodies showed only a very limited cross-reactivity with various meningococcal strains (1). Most antibodies were bactericidal. The genes encoding FrpB from three different strains were cloned and sequenced. Comparisons between these *frpB* genes and the published gonococcal one (2) revealed that especially the region from amino acid residue 350 to 390 displays pronounced sequence variability. A topology model was constructed for FrpB, based, amongst others, on sequence comparisons. In this model, the most variable part corresponds to loop 7, the longest of the 13 predicted surface-exposed loops. By using synthetic peptides we could demonstrate that the epitopes of bactericidal mAbs are located within this loop. From five additional meningococcal strains, the parts of the *frpB* genes corresponding to this region were cloned and sequenced. The sequences can be divided into three groups, with a much higher similarity within the groups than between them.

We are investigating the possibility to direct the immune response away from the variable, immunodominant loop 7, towards other, more conserved loops. Mouse antisera were raised against synthetic peptides corresponding to the 13 loops predicted in the model and tested for binding to whole cells expressing FrpB. The highest binding was observed for antibodies directed against loops 6, 7 and 12 showing that in addition to the variable loop 7, other surface-exposed epitopes exist in this protein. An *frpB* mutant with a deletion of 38 amino acid residues in loop 7 was constructed and expressed constitutively in *N. meningitidis* strain H44/76 by placing it behind the *porA* promoter. The mutant protein was present in the outer membrane in amounts comparable to those of wild-type FrpB expressed from the same promoter. The deletion led to a strong increase of the binding of the mouse anti-peptide sera directed against loops 5, 6, and 12. This demonstrates that the immunodominant loop 7 does indeed shield other, more conserved loops. The ability of the *frpB* deletion mutant to induce antibodies against these other loops is now being investigated.

The FrpB protein showed homology to CopB of *Moraxella catarrhalis* and to the hemin-binding protein of *Yersinia enterocolitica*. Studies were performed to determine the function of the FrpB protein with the help of a knock-out mutant. The mutant showed no significant difference from its parental strain in iron acquisition from transferrin, lactoferrin, aerobactin, hemin or citrate. A minor effect on serum resistance was observed. The function of the protein is thus still unknown.

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Co-localization of the meningococcal transferrin binding proteins (Tbp1 and Tbp2) and evaluation of their relative roles in binding human transferrin.

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Apo-transferrin and holo-transferrin were separately conjugated to 15nm colloidal gold. Iron-restricted *Neisseria meningitidis* strain SD (B:15:P1.16) bound up to three-fold more holo-hTf than apo-hTF ($P<0.001$). The ability of a meningococcal mutant lacking either Tbp1 or Tbp2 to discriminate between apo and holo-transferrin was also investigated. There was no significant difference between the amount of gold-labeled apo-transferrin bound by the isogenic Tbp1 mutant (expressing Tbp2) and the parent strain ($p = 0.18$), whereas an isogenic Tbp2 mutant (expressing Tbp1) was significantly less effective at binding gold-labeled apo-transferrin ($P<0.001$). The isogenic Tbp1 and Tbp2 mutants and the parent strain all bound significantly more holo-hTf than apo-hTf, whereas the double "knock-out" mutant, failed to bind hTf irrespective of the iron-loading. In the isogenic mutants, Tbp2 was more effective in binding either apo or holo-transferrin than Tbp1.

Monoclonal antibodies against Tbp1 and Tbp2 were used to co-localize the transferrin-binding proteins on strain SD (B:15:P1.16). The ratio of Tbp1:Tbp2 was approximately 1:1. Tbp1 was occasionally observed in close proximity to Tbp2, but the two proteins were generally quite separate indicating they do not usually form a single transferrin receptor.

Study of human transferrin binding sites within the transferrin binding protein Tbp2 from *N. meningitidis* M982 using the pMAL expression system

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N. meningitidis M982 strain (B:9,P1.9) expresses a transferrin binding protein 2 (Tbp2) of 88 kDa [1]. The minimal human transferrin (hTf) binding domain was demonstrated to be localized in the N-Terminal part of the molecule (aa 34-350) [2]. A comparison between the N-terminal (aa 1-351) and the C-terminal (aa 352-691) regions of the Tbp2 amino acid sequence from four M982-like strains indicated that some stretches were repeated in the molecule suggesting that there may exist a symmetry in Tbp2 [3]. This observation implied that an hTf binding site may be present in the Tbp2 C-Terminal half but this remained to be demonstrated. The pMAL expression system (Biolabs) was used to verify this hypothesis as it allowed the purification of truncated forms of rTbp2 on amylose via the maltose binding protein (MBP) followed by the hTf binding analysis in non denaturing conditions. To validate the system, the full length Tbp2 (aa 2-691) was expressed as a fusion with MBP into the pMAL-c2 (cytoplasmic targeting) and pMAL-p2 (periplasmic targeting) vectors; no difference was observed in terms of hTf binding capacity. The PCR products encoding the N- and C-Terminus of M982 Tbp2 were cloned into pMAL-c2 and the MBP-rTbp2 fusions were characterized relative to their antigenicity and hTf binding ability. By using a chemiluminescent kit (Amersham), the C-Terminus fusion was shown to bind human transferrin on Western Blot; using the purified fusion proteins and the same sensitive detection, we demonstrated by dot blot that the hTf binding capacity of the C-Terminus was much more reduced than that observed with the N-Terminus and that the hTf binding activity located in the N-terminal half was also less than that of the full length Tbp2. Based upon this observation and along with the M982 Tbp2 sequence symmetry, ten other constructs were produced and characterized to localize more precisely the binding site(s) within the C-Terminus. The presence of this other binding site was further localized to the central region of the molecule and no hTf binding was visualized within the distal region as defined by this kind of expression system. The involvement of the C-terminal peptide repeated in the N-Terminus of M982 Tbp2 and common to the same homologous peptide in Tbp2 from *A. pleuropneumoniae* [5] was examined. Furthermore, the role of the three major hypervariable stretches within the Tbp2 central region (as previously described [4]) on the hTf binding ability of the full length molecule was investigated using deletion variants (Δ aa 388-396, Δ aa 418-476, Δ aa 499-521). Preliminary data suggested that a particular fragment around the aa 388-396 region may be involved in positioning correctly the C-Terminus and the N-Terminus into the full length Tbp2 for ensuring an

efficient human transferrin binding and therefore it may perhaps function as an hinge between the two domains.

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Characterization of the meningococcal lactoferrin receptor

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Efficient iron acquisition is an important virulence factor for pathogenic bacteria, since the concentration of free iron in the human host is low. To overcome this problem, many bacteria produce small, iron-chelating compounds, called siderophores, which are able to scavenge iron very efficiently from the environment. The pathogenic *Neisseriae,* however, do not produce siderophores, but are able to acquire iron directly from host iron-binding proteins, such as transferrin in serum and lactoferrin on mucosal surfaces (1). Under iron limitation , the synthesis of several outer membrane proteins (OMPs), including receptors for transferrin and lactoferrin, is induced. We are studying the molecular mechanism of iron-acquisition from lactoferrin by *N. meningitidis.*

By screening a λgt11 gene library with monoclonal antibodies, we have cloned the *lbpA* gene, encoding the 102 kDa iron limitation-inducible lactoferrin receptor. Expression of this gene in *E. coli* allowed these cells to bind lactoferrin, but not to use lactoferrin as an iron source (2). Sequencing of the gene revealed that this lactoferrin receptor is highly homologous to TbpA, one of the two transferrin-binding proteins in the neisserial outer membrane. Moreover, some homology to TonB-dependent siderophore receptors of *E. coli* was found, suggesting the involvement of a TonB homologue in iron-acquisition from lactoferrin (3). The presence of homologues of TonB and its accessory proteins ExcB and ExcD in *N. meningitidis* could be demonstrated in Southern blotting experiments, and the corresponding genes were cloned.

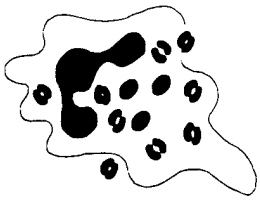
Upstream of *lbpA,* an open reading frame was identified, tentatively designated *lbpB.* A band, probably corresponding to LbpB, could be detected with peroxidase-conjugated lactoferrin after blotting of outer membrane proteins of *Neisseria meningitidis* from SDS-polyacrylamide gels to nitrocellulose paper. The putative LbpB protein displays homology to TbpB, the second transferrin-binding protein in the neisserial outer membrane (4). Therefore, we assume that iron-acquisition from lactoferrin requires two distinct lactoferrin-binding proteins. At the moment we are cloning and sequencing the part of *lbpB,* corresponding to the N terminal part of the protein.

Immunological cross-reactivity studies and sequencing of the *lbpA* gene of another strain revealed a high degree of conservation. The variation was mostly restricted to a few cell surface-exposed loops in a proposed topology model. The protein is supposed to traverse the outer membrane 26 times in a beta-sheet conformation, exposing the most variable and hydrophilic parts to the outer surface (4). The variability of the most immunogenic

loop, containing the epitopes of the available monoclonal antibodies, was studied in further detail after PCR amplification of the corresponding DNA fragment of several strains. Presently, we are verifying the proposed topology model for LbpA experimentally.

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Structure of Neisserial Proteins

Characterization of the relative conformational stability of *Neisseria meningitidis* porins

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Porins are water filled channels which play important roles as molecular sieves in the outer membrane of gram-negative bacteria. The major porins from *Neisseria meningitidis* are the (PorB) class 2 and (PorB) class 3 proteins, which are mutually exclusive among meningococcal strains (1). Both proteins have been overexpressed, isolated, and refolded from *E. coli* inclusion bodies (2), and characterized by a combination of functional and physicochemical techniques. Our investigation has focused primarily on the trimeric assembly and conformational stability of these neisserial porins in the native state. The resultant data have been interpreted on the basis of x-ray crystallographic structures determined for other bacterial porins (3,4) in conjunction with topology models proposed for neisserial porins (5,6).

Our studies indicate that recombinant (PorB) class 2 and (PorB) class 3 proteins refold and assemble as trimers, thereby retaining the overall structural features of their native counterparts. Preliminary observations regarding the SDS-resistant properties of these trimeric structures prompted us to investigate the relative conformational stabilities of both proteins and correlate specific differences with sequence and topology variations described in the literature (5,6). Comparative studies on the chemical and thermal denaturation of neisserial porins monitored by UV, circular dichroism, and fluorescence spectroscopy facilitate elucidation of specific domain interactions which govern the stability of these proteins. Experiments have been performed on porins isolated from strain M986 [(PorB) class 2], strain M981 [(PorB) class 3], a mutant strain 44/76 D1/D4 [(PorB) class 3], and their respective recombinant counterparts. The conformational properties of a native (PorA) class 1 protein obtained from mutant strain 44/76 D3/D4 have also been investigated.

Spectrophotometric studies conducted in the presence of SDS or Gdn•HCl have revealed increases in both tyrosine and tryptophan exposure accompanying the detergent- and chemical-induced unfolding processes, respectively. Significantly, (PorB) class 3 protein exhibits a transition over the range of 0.25 - 0.5 % SDS, whereas only minor changes are observed for (PorB) class 2 protein at comparable SDS concentrations. Unfolding transitions involving the concomitant disruption of secondary and tertiary structure occur at midpoints of 2.5 M and 4.75 M Gdn•HCl for (PorB) class 3 and (PorB) class 2, respectively. These results clearly indicate a significant correlation between the SDS-resistant properties of (PorB) class 2 protein and its enhanced stability with respect to chemical denaturation (7). Reversible thermal unfolding transitions are observed for (PorB) class 2 and (PorB) class 3 proteins, although the relative stabilities and pathways

are quite distinct. Specifically, (PorB) class 3 exhibits a biphasic melting profile with transitions at 62.5 °C and 90.0 °C, while (PorB) class 2 unfolds via a single cooperative transition at a midpoint of 85.5 °C. The observed differe s in thermal stability may be correlated with the relative susceptibility of both porins to chemical denaturants.

Envisioning the possibility that heterotrimers of class 1/2 or class 1/3 may exist *in vivo,* we recently extended our studies on the conformational stability of meningococcal porins to include (PorA) class 1 protein. Analysis of wild type strains expressing both PorA and PorB genes indicate that those expressing class 1/2 (strain M986) contain predominantly SDS-resistant species, whereas those expressing class 1/3 (strain 44/76) are comprised exclusively of SDS-sensitive species. Consequently, it is conceivable that the presence of either class 2 or class 3 within heterotrimers in wild type strains is the limiting factor that renders these oligomeric structures inherently more (class 1/2) or less (class 1/3) stable.

In summary, functional and physicochemical studies of (PorA) class1, (PorB) class 2, and (PorB) class 3 proteins provide significant insight into *Neisseria meningitidis* porins, particularly structure-stability relationships within the porin superfamily.

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Studies on the PorA protein of *Neisseria meningitidis* by X-ray crystallography and NMR.

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The antigenic variability of the PorA protein of the meningococcus has attracted study as a result of the use of this protein as a target for serosubtyping and in vaccines (1,2,3,4). Most of the antigenic variability of this protein resides in two loops (loop I or VR1 and loop IV or VR2) protruding from its proposed β-barrel porin structure (5,6). The variability of these loops is not continuous and there are a number of distinct families of peptide sequence in both VR1 and VR2. In addition, minor variation also occurs in these families (7). In order to investigate the structural consequences of the antigenic variability of PorA, we have instigated a systematic analysis of the three-dimensional structures of a range of linear peptide antigens in complex with antibodies against different serosubtype variants. Such an investigation will reveal whether different serosubtype variants are related in structure, despite the absence of significant sequence homology between many variants.

We now have crystals of two Fab fragments in complex with linear peptide antigens derived from VR1 and VR2 sequence variants. A complete data set of reflections has been collected from crystals of a P1.7 serosubtype variant in complex with Fab fragment to a resolution of 2.9 Angstroms (R_{merge}= 3.1% for all reflections from 30 to 2.9 Angstroms resolution; 91% complete). Data has also been collected from crystals of a second Fab fragment in complex with a P1.10 linear peptide antigen, although diffraction is not yet to a sufficiently high resolution to permit a determination of the structure. We are currently investigating solutions of both these structures by molecular replacement methods. Both crystal complexes contain a single domain from Streptococcal protein G, which binds exclusively to the C_H1 domain within Fab: inclusion of protein G was required to obtain satisfactory crystals, and may assist in the solution of the structure of the complex by molecular replacement (8).

Nuclear magnetic resonance offers an alternative approach to the structural analysis of antibody-antigen complexes. We have synthesised a linear peptide antigen corresponding to the P1.7 serosubtype, incorporating ^{15}N into all alanine and glycine residues and studied the complex of this peptide with Fab fragment from a mouse monoclonal against serosubtype P1.7. Heteronuclear single and multiple quantum coherence methods were employed to distinguish proton resonances originating from protons bound to ^{15}N nuclei, thus removing interfering proton signals from the Fab fragment. Small but significant changes in amide proton chemical shift were found on binding of the peptide to the Fab fragment. A two-dimensional ^{15}N-filtered NOESY

conducted on the Fab-peptide complex demonstrated that several NOEs could be detected from the peptide amide protons to adjacent Cα protons. In addition, three NOEs were also visible to methyl protons, presumed to be from the three alanine residues in the peptide. Experiments are currently in progress to assign each NOE to a specific residue, and thus determine the conformation of the P1.7 peptide in its bound state.

By collating the results derived from NMR and protein crystallography experiments we hope to build up a detailed description of the molecular basis for immune recognition of meningococcal antigens. It should be readily apparent from the results why particular monoclonal typing antibodies cross-react with some serosubtype variants, but not others. This approach will also provide more general information about the three-dimensional structural relationships between different serosubtype variants.

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High resolution model of the *Neisseria* pilus fiber

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The atomic resolution structure of *Neisseria gonorrhoeae* pilin (1) was used along with published biochemical, genetic and biophysical data to model the pilus fiber. Five pilin molecules form each 4.1 nm repeat of the helical fiber. The long conserved N-terminal alpha helices pack against each other on the interior of the fiber, wrapped by continuous beta sheet. The hypervariable C-terminal region is exposed on the surface.

The fiber model facilitates rational design of further experiments to probe the function and structure of the fiber. For example. new anti- peptide antibodies can be raised against small structural motifs such as turns, and used to verify the exposed or buried nature of these regions in the fiber. Furthermore, peptides could be chosen which are conserved but partially accessible in the assembled fiber, with the goal of eliciting an antibody response that would play a role in immunity. Mutations have been designed to investigate the structural importance of the hypervariable region, and to add reactive sites for metal labeling to use in electron microscopy.

This model also allows us to interpret previous experimental results. in the context of the pilus. For example, epitopes encompassing residues 94-108 and 37-56 are known from immunoelectron microscopy experiments to be exposed on the ends of fibers (2) and are indeed located at the ends of the proposed fiber model, buried by longitudinal contacts within the fiber. The covalently linked saccharide (1,3) is exposed on the fiber surface, where it could affect antigenic variation, adhesivity or target cell specificity.

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Noncapsular Vaccines, Poster

Opsonophagocytosis responses to meningococcal antigens adsorbed to beads

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In order to identify serogroup B meningococcal structures that mediate antimeningococcal opsonophagocytosis, *in vitro* flow cytometry (FCM) and chemiluminescence (CL) assays evaluating functional antigen-specific opsonophagocytic responses were developed. Serogroup B meningococcal outer membrane vesicles (OMV) (1) were adsorbed to fluorescent latex beads (OMV-beads) of similar size to that of meningococci (1μm in diameter) and opsonized with acute phase and convalescence sera from patients with serogroup B meningococcal disease (MCD). Phagocytosis of these "meningococci-mimicking" beads with selected bacterial components and subsequently OMV antigen-specific opsonins exposed on the surfaces, depended on both the amount of adsorbed antigen and on the concentration of opsonizing sera. FCM cytograms and histograms gated for phagocytosing cells confirmed and visualized the antigen-specific nature of the opsonophagocytosis responses.

OMV-beads opsonized with 5% serum from a patient recovering from MCD, caused 97% of the donor monocyte- and polymorphonuclear leukocyte population to phagocytose an average of 15.8 beads per cell with a CL response of 46,550 mVs, whereas opsonized control beads coated with bovine serum albumin were phagocytosed by 19% of the cells with 1.1 beads per cell and a CL response of 53 mVs (initial bead:phagocyte ratio of 20:1). Opsonization with MCD convalescence serum induced higher phagocytosis than acute phase serum from the same patient, indicating that increased amounts of anti-OMV opsonins are induced during infection. When a pneumococcal antigen preparation was adsorbed to beads and opsonized with the same acute and convalescence MCD patient sera, the difference in opsonophagocytic effects between the sera was abolished. Heat-inactivation of sera and replacement of MCD patient sera with sera from non-infected patients with hypogammaglobulinemia reduced the phagocytosis.

Opsonized OMV-beads elicited phagocyte responses of similar magnitude to those of opsonized, fluorescein isothiocyanate-labelled whole meningococci. Serial confocal laser scanning microscopy sections (z-series) were generated through the incubated leukocytes and merged into one image to visualize intraphagocyte location of fluorescent particles.

We conclude that epitopes on the meningococcal outer membrane seem to be recognized by patient anti-meningococcal opsonins in these functional phagocytosis assays, which provide a basis for evaluation of various purified meningococcal components as mediators of human opsonophagocytic responses and hence future vaccine constituents.

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Noncapsular Vaccines, Poster

Serum bactericidal activity elicited by two outer membrane protein serogroup B meningococcal vaccines among infants, pre-school children, and adults in Santiago, Chile

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Background: Two serogroup B vaccines have been developed by Finlay Institute (FI) in Cuba and the National Institute of Public Health (NIPH) in Norway (1,2). Each vaccine is based on specific isolates from epidemics in Cuba (CU385, B:4:P1.15) and Norway (44/76-SL, B:15:P1.7,16). Vaccine efficacy (VE) has been demonstrated among children and young adults 11 to 16 years of age in randomized, double-blind controlled trials (RDCT) using 2-dose regimens of the FI-produced vaccine in Cuba, and the NIPH-produced vaccine in Norway (estimated VE, 83% and 57%, respectively). However, VE has not been demonstrated in children <5 years of age (1-3). In 1993, Santiago had a clonal, serogroup B epidemic; 60% of cases occurred among children <5 years of age (4). Because the Chilean epidemic strain (#539, B:15:P1.3) was different than the strains used to produce the FI and NIPH vaccines, an immunogenicity study was conducted as a potential correlate for VE.

Methods: Standardized SBA elicited by the FI-produced and NIPH-produced vaccines, and a non-meningococcal control vaccine was determined in a RDCT among infants <1 year of age (N=187), pre-school children 2-4 years of age (N=183), and adults 17-30 years of age (N=173). Participants received 3 doses of vaccine, 2 months apart; blood samples were obtained prior to dose 1 and dose 3, and 4-6 weeks following dose 3. Response was defined as a $\geq$4-fold rise in titer compared with prevaccination. All sera were tested against the Chilean outbreak strain. Based on these results, additional assays were performed; adult sera were tested against the Cuban vaccine type strain and infant sera were tested against the Norwegian vaccine type strain.

Results: Local and systemic reactogenicity profiles for both the FI-produced and the NIPH-produced vaccines were satisfactory and consistent with previous studies (1,2). Among infants, there was no significant difference in the proportion of responders against the heterologous Chilean epidemic strain between those vaccinated with the control vaccine (bleed 2, 1.8%; bleed 3, 5.6%) and either the FI-produced (bleed 2, 1.9%; bleed 3, 9.6%) or the NIPH-produced vaccine (bleed 2, 5.8%; bleed 3, 11.5%). Among pre-school aged children, recipients of the FI-produced vaccine (bleed 2, 14.3%; bleed 3, 30.6%), and recipients of the NIPH-produced vaccine (bleed 2, 22.2%; bleed 3, 34.5%) were more likely than control vaccine recipients (bleed 2, 1.8%; bleed 3, 5.3%) to be responders ($p<0.05$ vs. control). Similarly, adult recipients of the FI-produced

vaccine (bleed 2, 26.9%; bleed 3, 36.5%), and adult recipients of the NIPH-produced vaccine (bleed 2, 49.9%; bleed 3, 60.0%) were more likely than adult control vaccine recipients (bleed 2, 1.9%; bleed 3, 3.8%) to be responders ($p<0.05$ vs. control). Among adults, the proportion of responders was significantly higher among recipients of the NIPH-produced vaccine when compared to the FI-produced vaccine at bleed 2 ($p<0.04$) and bleed 3 ($p<0.03$). SBA geometric mean titers revealed similar response patterns across all three age groups.

Among adult sera tested against the Cuban type strain, there was no significant difference in the proportion of responders between those vaccinated with the FI-produced vaccine (bleed 2, 30.4%; bleed 3, 65.2%) and the NIPH-produced vaccine (bleed 2, 24.0%; bleed 3, 48.0%). Against the Norwegian type strain, infant recipients of the NIPH-produced vaccine (bleed 2, 100%; bleed 3, 100%) were significantly more likely than control vaccine recipients (bleed 2, 2%; bleed 3, 2%) to be responders ($p<0.001$).

Conclusions: SBA results for infants and pre-school aged children suggest that the FI-produced and NIPH-produced vaccines may not confer protection against the Chilean outbreak strain. Among pre-school aged children and adults, a third dose of either the FI-produced or NIPH-produced vaccine was associated with a higher proportion of responders than two doses. However, the proportion of responders among pre-school aged children after two or three doses of either vaccine was relatively low. Among adults, SBA results against the Chilean strain were consistent with VE data for the NIPH-produced vaccine. Among adult recipients of the FI-produced vaccine, the proportion of SBA responders against the Cuban type strain was less than expected based on VE data for the FI-produced vaccine. Although SBA against the Chilean outbreak strain among infants vaccinated with the NIPH-produced vaccine was disappointingly low, the 100% SBA response rate against the Norwegian type strain among NIPH-vaccinated infants was surprising, suggesting that the NIPH-vaccine may confer protection against the homologous strain in this age group. Although SBA may not be an ideal serologic correlate for VE for serogroup B meningococcus, a high proportion of SBA responders to the circulating strain is likely to predict VE in the effected population.

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