Host-pathogen interactions
In vivo experiments with Neisseria gonorrhoeae

Myron S. Cohen, Susan Isbey, Larry Charniga, Ann Jerse, Leesa Whicker, H. Steven Seifert and Janne G. Cannon

Departments of Medicine and of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA and Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, IL, USA.

A variety of animal models for gonococcal infection have been developed, but each has limitations. Gonococcal exudates from infected patients and/or gonococci grown in a laboratory setting have been used to create urethral infection in male volunteer subjects. Experiments with volunteer subjects have been used to describe phenotypic variation of the organism in vivo, to test vaccine candidates, and to examine virulence factors. We have inoculated sixty-six subjects in eighteen iterations; thirty-one subjects developed clinical infection. Swanson and coworkers previously examined Opa (1) and pilin (2) variation in vivo using experimental subjects. We have extended these observations using a larger number of subjects and samples (3). We observed more Opa (4) and pilin (5) variation in vivo than occurs during growth in vitro over the same period of time. Schneider and coworkers (6) reported evolution of lipooligosaccharide expression during growth in vivo of gonococcal strain MS11, with the implication that such changes were required for the full expression of natural infection. We observed no changes in the LOS of the FA1090 variant we used; however, the FA1090 inoculum employed included organisms expressing the LOS epitope (which binds the 3F11 antibody) selected in strain MS11. We have also examined semen specimens from four experimental subjects and nineteen clinic patients. Clinic patients had a larger number of organisms (approximately $10^6$) in the ejaculate than experimental subjects ($10^{3-4}$).

We have begun to examine gonococcal virulence factors through use of isogenic mutants of strain FA1090. RecA is required for gonococcal DNA repair, transformability, and pilin variation. Given the degree of pilin variation observed during the course of experimental infection (2,5), it seemed possible that such change was required. A recA FA1090 mutant was generated using a procedure that did not require insertion of an antibiotic resistant marker. Permission to inoculate the mutant organism was obtained from UNC’s Institutional Biosafety and Human Subjects Committees. Six out of eight subjects inoculated with $10^6$ colony forming units of FA1090 recA developed infection whereas 9/11 subjects inoculated with the same number of RecA+ Fa1090 became infected. Time to infection was slightly delayed
and fewer subjects infected with FA1090 recA developed a urethral discharge, relative to those infected with FA1090 wild type. Gonococci in the exudate harvested from infected subjects had the recA phenotype and no detectable pilin variation. We conclude that pilin variation is not required for gonococcal urethral infection in male subjects.

In summary, we have expanded studies using the human challenge model of Neisseria gonorrhoeae. Our work suggests that the model can be used safely and efficiently, with minimal trauma to the subjects. Consistent with earlier studies (1-3) variation in pilin and Opa phenotype in vivo were considerably greater than in organisms passed in vitro during the same period of time. The basis for these changes and their importance for infection and/or transmission of the organism are not known. We believe, however, that the challenge model lends itself to a better understanding of the gonococcal biology. The challenge model can also be used for rapid and efficient testing of a gonococcal vaccine.

References
Association of gonococcal lipooligosaccharide phenotype with virulence

H. Schneider, R.A. Kuschner, J.W. Boslego, A.S. Cross and C.D. Deal

Walter Reed Army Institute of Research, Washington, DC, 20307-5100 USA.

In a previous study of the pathogenesis of gonorrhea (1) male volunteers were inoculated intraurethrally with a strain of *Neisseria gonorrhoeae*, MS11mkA, (mkA) which expresses a single 3.6 kD lipooligosaccharide (LOS), and whose estimated ID$_{50}$ is $10^5$ gonococci. As infections among volunteers progressed to urethritis, they shed decreasing numbers of mkA gonococci, and in their stead increasing numbers of a variant gonococcus, MS11mkC (mkC), which supplanted the original mkA inoculum. The mkC variant no longer expressed the 3.6 kD LOS of mkA but did express four new LOS, all larger than the mkA LOS. The epitope of one LOS of 4.8 kDa is shared by the terminal lacto-n-neotetraose of human paragloboside, a precursor of blood group substances. The mkC variant occurs in vitro among mkA gonococci at a frequency of $10^{-3}$, and 100 were presumably in the ID$_{50}$ mkA inoculum used to infect the volunteers. We have conducted a series of human challenge experiments to confirm the hypothesis that the mkC LOS variant is of greater virulence than the mkA variant, and to test the hypothesis that as few as $10^2$ mkC gonococci are sufficient to infect male volunteers.

The gonococcal strains used here and their growth have been described (1). Volunteers were infected with 0.2 ml of broth suspensions of gonococci using a pediatric catheter fitted to a tuberculin syringe inserted ca. 4-5 cm into the urethra. Serial log dilutions of sediments from urine specimens submitted at 12 h intervals after inoculation were cultured on GCDC agar (2) containing VCN antibiotic mixture. Colonies were enumerated and scored for colony type, and monoclonal antibodies (Mab) were used to distinguish LOS variants in colony lifts.

In our first experiment we compared the infectivity of the variants by inoculating two groups of five volunteers each with ca. 40,000 of either mkA or mkC gonococci. All of volunteers receiving mkC developed gonococcal urethritis while only 2/5 (40%) receiving mkA became ill. Mab analysis of gonococci recovered from urinary sediments of the latter two individuals showed a transition from mkA phenotype colonies to increasing numbers of mkC phenotype colonies as their infections progressed from dysuria to urethral discharge. This was an indication that infections following mkA challenge were due not to the mkA gonococci, but to the 0.1%, ca. 40, mkC variants present in the mkA challenge. Incubation time for these infections ranged from 50 h to 86 h (median = 74 h).
We tested the infectivity of the mkC variant in three other experiments where we inoculated groups of six or seven volunteers with small numbers of gonococci. We found that 8/20 (40%) volunteers became infected following inoculation with from 250 to 1600 mkC gonococci, confirming the hypothesis that the ID$_{40}$ for mkC is #250 gonococci and that 10$^2$ gonococci could constitute infectious dose. Incubation times for these eight infections ranged from 50 h to 146 h (median = 82 h), and were independent of dose; the two longest, 122 h and 146 h, followed inoculation with 1250 and 1600 mkC gonococci.

In every instance where challenge resulted in an infection we found that the gonococci shed by the infected volunteers switched from the challenge Opa colony type to Opa$^+$ colony type. The transition was rapid and encompassed most, if not all of organisms. In 10 of 15 infections we detected the Opa$^-$ to Opa$^+$ transition 12 h to 24 h before the onset of symptoms. In two infections it coincided with the onset of dysuria, and in three we observed the transition 36 h to 54 h before symptoms were evident.

In their study of the epidemiology of naturally acquired gonorrhea by sailors on shore leave in the Philippines, Holmes, et al. (3) estimated the sailors had a 22% risk of becoming ill with gonorrhea following a single unprotected encounter with an infected hostess. The data from our first experiment comparing the infectivity of mkA to mkC suggests that the 40 mkC gonococci (0.1%) present in the 40,000 mkA challenge were responsible for the two infections among the five mkA inoculated volunteers. This number, 40 gonococci, constitutes the mkC ID$_{40}$, and that a lesser number, i.e. 20 gonococci, are sufficient for natural transmission.

The courses presented by our experimentally infected volunteers have varied considerably. We have seen brief and extended incubation times, erratic shedding of gonococci during the course of infections, and acute and coincidental onset of both dysuria and discharge, as well as dysuria for periods prior to the onset of dysuria. The various presentations of disease, particularly following challenge with small doses of virulent gonococci suggests that the experimental gonorrhea infection model we have described here may in part mimic naturally acquired infections.

References
Studies on gonococcal pilin antigenic variation

H. Seifert\textsuperscript{1}, L. Wainwright\textsuperscript{1}, J. Frangipane\textsuperscript{1}, B. Howell\textsuperscript{*}, M. Cohen\textsuperscript{2,3} and J. Cannon\textsuperscript{2}

\textsuperscript{1} Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611 USA. \textsuperscript{2}Department of Microbiology and Immunology. \textsuperscript{3}Department of Medicine, University of North Carolina Medical School, Chapel Hill, NC 27514.

Antigenic and phase variation of the gonococcal pilus has evolved to promote avoidance of immune surveillance and alter pilus receptor binding (4,5). Pilin antigenic variation occurs when a variant pilin DNA sequence transfers from one of several silent loci to the expression locus in a nonreciprocal, RecA-dependent manner (3). We have determined that the conserved \textit{Sma}/\textit{Cla} repeat present in all pilin loci is required for efficient antigenic variation (7). Deletion of this repeat from the expression locus lowers recombination between \textit{pilS1} and \textit{pilE}, but the rate of phase variation is not similarly lowered. The phase variation observed results mainly from alternative means that are independent of antigenic variation. Two or more proteins have been detected in cell lysates that specifically bind to \textit{Sma}/\textit{Cla}. The DNA sequences within \textit{Sma}/\textit{Cla} required for protein binding are being determined, and general properties of the DNA binding proteins are also being investigated. We hypothesize that these proteins express biochemical activities that allow for high frequency DNA recombination between the silent loci and the expression locus, producing antigenic or phase variation.

We are also studying the process of pilin antigenic variation during experimental infection in male volunteers (14). Our initial studies have shown that pilin antigenic variation occurs at a high apparent rate during infection (6). This could result from a stimulation of DNA recombination and/or from selection for new pilin variants. A \textit{recA} variant of strain FA1090 was created by transformation with a \textit{PacI} linker-generated, frame-shift mutation (\textit{recA5}) that avoids the use of antibiotic resistance. Antigenic and phase variation is blocked in this strain. Inoculation of male volunteers with the \textit{recA5} strain allows the same level of infection as the wild type strain and elicited clinical signs of disease. Reisolated \textit{recA5} clones express the same pilin gene as the inoculum showing that antigenic variation is not required for the establishment of infection. These observations suggest that a high rate of antigenic variation occurs \textit{in vivo} that provides a large number of antigenic variants to aid in infection and transmission to new hosts.
References


Host-pathogen interactions

A low Mr moiety released from blood cell fractions which enhances CMP-NANA sialylation of LPS in gonococci

N.J. Parsons, C. Constantinidou, J.A. Cole and H. Smith

School of Biochemistry and the Medical School, The University of Birmingham, Birmingham, B15 2TT, UK.

Serum resistance of gonococci in most patients is due to sialylation of a Galβ1-4GlcNAc group in a conserved 4.5 kDa LPS component by host CMP-NANA catalysed by a gonococcal sialyl transferase. This sialylation is enhanced by one or more low Mr factors, first demonstrated by experiments with the enzymes in gonococcal extracts but now shown to occur in live gonococci and hence to have a probable role in pathogenicity. Enhancing activity is released in diffusates from both dialysis of blood cell sonicates at 18-20°C and, like CMP-NANA, from high Mr fractions purified from sonicates dialysed at 4°C. These diffusates absorbed maximally at about 260nm but relevant UDP sugars had no enhancing activity. The enhancing moiety in the diffusates has been separated from CMP-NANA by HPLC in fractions that also increase the induction of serum resistance in gonococci by CMP-NANA.
Dynamics of the interaction between gonococci and epithelial cells

J.P.M. van Putten, S.M. Paul, B.D. Robertson and E.T. Schwan

Max-Planck-Institut für Biologie, Infektionsbiologie, Spemannstrasse 34, 72076 Tübingen.

Neisseria gonorrhoeae is able to attach and enter cultured mammalian cells provided that the appropriate bacterial phenotype is established. Characteristics of the 'invasive' gonococcal phenotype are a non-sialylated form of LPS and the expression of a distinct member of the opacity protein outer membrane protein family. In search for the molecular mechanisms underlying the bacterial adherence and entry process, we further investigated the interaction of neisserial LPS and opacity protein with host cells. Construction of defined LPS mutants indicated that the terminal lacto-\(\text{N}\)-neotetraose and the digalactoside moiety of LPS are not required for bacterial attachment and entry, while inner core mutants were defective in entry. Analysis of host cell determinants for bacterial binding proteins resulted in the identification of a putative opacity protein receptor. This molecule, which appears to be present in all epithelial cell lines, particularly recognizes the gonococcal opacity protein associated with tight adherence and entry, but hardly other members of the opacity protein family. Purified receptor and receptor analogues totally block opacity protein-mediated bacterial attachment to eukaryotic cells in vitro, suggesting that the identified structure is an essential component in the adherence process. The function of this binding site in the bacteria-induced endocytosis process is currently under investigation.
Immunogenicity and evolutionary variability of IgA1 protease from *Neisseria meningitidis*


Max-Planck Institut für molekulare Genetik, Ihnestrasse 73, D-14195 Berlin, Germany.

IgA1 protease is highly immunogenic in humans during carriage and infection (1). Immune sera containing 250 μg/ml of IgG are not unusual. Antibodies to other antigens stimulated in Gambian children by immunization (to A capsular polysaccharide) or by carriage (to Opc protein) decreased rapidly. In contrast, antibodies to IgA1 protease stimulated by carriage continued to increase over a 4 year period after epidemic disease. B cell epitopes within meningococcal IgA1 proteases recognized by murine monoclonal antibodies (Mabs) were subdivided into epitopes 1 - 5 by subcloning the *iga* gene (2) and by reactivity with peptides synthesised on pins (Geysen technique). Epitope 4, localized to a 104 amino acid region, could not be mapped by the Geysen technique and probably represents a discontinuous epitope. A synthetic 104mer peptide which spans this region reacts with the murine Mabs and with human antibodies and is highly immunogenic in mice whereas smaller peptides (30mers, 64mers) do not react with the Mabs and are not as immunogenic.

The DNA sequence of that part of the *iga* gene encoding the mature IgA1 protease (3 kb) has been determined for 4 meningococci (3 serogroup A, 1 serogroup C). 6% of the nucleotides, scattered over the C-terminal 2 kb showed some sequence variation, reflecting the mosaic gene structure of *iga*. The nucleotide segments characteristic of individual mosaics were present in both meningococci and gonococci but certain sequences were species-specific. Within the epitope 4 region, only 4 clustered amino acids distinguished IgA1 proteases which reacted with murine Mabs from those that did not react.

IgA1 protease epitopes were uniform within serogroup A subgroups over decades with one exception (2). The subgroups differed from each other in respect to epitopes 3 - 5 and in DNA sequence as indicated by T-tracks of the variable parts of *iga*. Within subgroup III, bacteria isolated prior to the Mecca outbreak of 1987 were slightly variable according to pulsed field gel electrophoresis (PFGE) and uniform according to T-tracks. During the Mecca outbreak, 2 novel and distinct PFGE subclones were isolated, one of which expressed the original IgA1 protease and the
other of which had acquired an unrelated iga gene by recombination. Only derivatives of the latter (recombinant) subclone were isolated during subsequent spread to Africa. In different countries, different minor PFGE variants were isolated, indicating continued microevolution.

References
Structure and biological function of the components of the IgA protease polyprotein precursor

T.F. Meyer¹, K. Otzelberger¹, U. Wölk¹, B.T. Eger², U. Langenberg¹, D. Lorenzen¹, E.F. Pai² and J. Pohlner¹

¹Max-Planck-Institut für Biologie, Tübingen, Germany. ²University of Toronto, Faculty of Medicine, Department of Biochemistry, Canada.

The iga gene of the pathogenic Neisseria species encodes a large exported polyprotein consisting of four distinct domains, i.e. the protease domain (Igaₚ), the ( -peptide (Iga₁), the " -protein (Iga₄) and the membrane embedded $ -protein (Iga₅), which are separated from each other post-secretionally by autoproteolytic events (2,4). Sequence analyses of various neisserial iga genes, comprising genetic mosaics generated by horizontal exchange (1), led us to identify conserved motifs indicating distinct structure/function relationships. Likewise, each allele of the " -proteins harbours at least one motif characteristic of a eucaryotic nuclear location signal (NLS). We demonstrate consistently the efficient uptake of extracellular " -proteins by a distinct subset of human primary cells and the association of the " -protein with distinct sub-nuclear structures (3). Our data further suggest the combined uptake of " -protein and IgA protease and a complex function of the polyprotein inside infected and non-infected human cells. Work involving human T-cells indicate an immuno-modulatory function of the Iga polyprotein with potentially severe implications for individuals with systemic and even localized neisserial infections. We have therefore started to determine the three-dimensional structure of the mature form of IgA protease and obtained crystals from polyethylene glycol solutions. Their X-ray diffraction pattern is consistent with space group P2 and extends to a resolution of approximately 2.5 A. Data sets from native crystals as well as from some prospective heavy-atom derivatives have been collected and are being processed.

References
Clinical aspects of *Neisseria meningitidis* infections

P. Brandtzaeg

Departments of Pediatrics and Clinical Chemistry, Ullevål University Hospital, 0407 Oslo, Norway.

Quantitative measurements of lipopolysaccharides (LPS) and host derived inflammatory mediators have enabled us to describe different clinical presentations of systemic meningococcal disease (SMD) at a molecular level.

Meningococcal meningitis without shock and multiple organ dysfunction is the most common clinical presentation. On admission our patients revealed a comparatively low graded bacteraemia and endotoxinaemia. We found <1 cfu/ml (median, range 1-240 cfu/ml, n=12) in whole blood and LPS levels in plasma <3 pg/ml[0.03 EU/ml] by LAL (median, range <3-260 pg/ml, n=44). After penetration to the subarachnoid space increasing bacterial proliferation is reflected by increasing CSF levels of LPS which often are 100-1000 x higher than measured in simultaneously collected plasma samples. Various proinflammatory cytokines (TNF-α, IL-1, IL-6) are generated in CSF at a much higher levels than in plasma reflecting the differences in LPS levels. Production of IL-8 (a chemotactic protein for neutrophils) is not compartmentalised to the same extent as TNF-α and IL-1 in CSF. The levels of protein were quantitatively associated with the levels of LPS (r=0.50, p=0.01, n=26) in CSF reflecting altered endothelium barrier function. The glucose CSF/blood ratio was inversely correlated with LPS in CSF (r=0.62, p=0.0005, n=28) indicating altered brain cell metabolism. The number of leucocytes did not correlate significantly with the levels of LPS in CSF (r=0.33, p=0.07, n=30). The median disease onset hospital admission time was significantly longer than for patients with fulminant septicemia (22 vs 12 h) in 45 patients studied consecutively. Death and sequelae are related to brain edema and hypoxia reflecting tissue damage induced by the inflammatory response.

Fulminant meningococcal septicaemia is characterised by vascular collapse, kidney and lung dysfunction, disseminated intravascular coagulation (DIC), muscular infarction, extensive skin and adrenal haemorrhage with minimal pleocytosis. The onset admission time is short. All our patients with LPS plasma levels >700 pg/ml (7EU/ml) developed a persistent septic shock. The median LPS level was 10500 pg/ml (range 170000-1000 pg/ml) among 15 nonsurvivors and 1900 pg/ml (range 12000-10 pg/ml) among 16 survivors. The case fatality rate among 90 SMD patients related to septic shock increased with increasing plasma levels of LPS i.e.<1000 pg/ml 0% (n=64), 1000-5000 pg/ml 38% (n=13), 5000-10000 pg/ml 67% (n=3),
10000-15000 pg/ml 80% (n=5), >15000 pg/ml 100% (n=5). LPS levels in CSF were low (<160 pg/ml) although bacteria was present in 50% of the cases. The bacterial proliferation is primarily compartmentalised to the circulation. Owing to the short time span a significant inflammatory response and pleocytosis has not yet developed.

LPS induce an intravascular inflammatory response through a very complex interplay of mediators. Cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10) are all involved. Bioactive TNF-α was detected in 12 of 13 patients with LPS >700 pg/ml whereas non of 25 patients with lower LPS levels had detectable TNF-α. Circulating IL-10 but not IL-4 and transforming growth factor β (TGF-β) appears to function as antiinflammatory cytokines in vivo. The massive activation of the complement system - primarily through the alternative pathway - is associated with circulating levels of LPS and may contribute to the vascular collapse. Up-regulation of tissue factor on circulating monocytes and possibly endothelial cells appears to activate the coagulation system via coagulation factors VIIa, X,V, and prothrombin. The fibrinolytic system is down-regulated through the release of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells inducing an imbalance between coagulation and fibrinolysis facilitating clot formation. During the initial leucopenic phase which is caused by margination of leucocytes to the vessel wall, the neutrophils appear to release large amounts of antiparasitic principles (among others elastase, calprotectin). These molecules may influence the integrity of the endothelium as reflected by release of E-selectin (an adhesion molecule) and endothelin (a vasoactive peptide). Induction of increased NO-synthesis may be an important final mechanism contributing to vasodilation and cardiac dysfunction.

In cases of mild systemic meningococcal disease i.e. invasive disease without vascular collapse or meningitis, the bacteraemia / endotoxinaemia do not reach a critical shock level. This is reflected by low levels of circulating LPS, cytokines and other inflammatory mediators.

Reference
Enhanced immune response to meningococcal antigens in C5 deficient mice

J. Andreoni and P. Densen

Department of Internal Medicine, VA Medical Center and University of Iowa College of Medicine, Iowa City, Iowa 52242.

Investigation of the striking association between neisserial disease and late complement component deficiency (LCCD) in humans has identified unique features of the interaction of \textit{Neisseria} with host defenses (1). Studies have consistently demonstrated an increased antibody response to meningococcal (Mg) antigens (especially LOS) in convalescent LCCD as compared to convalescent complement sufficient sera (2,3). This antibody is broadly cross-reactive with LOS from non-serogroup A meningococci as well as gonococci and is bactericidal for serogroup B meningococci. This IgG does not appear to be subclass restricted and recognizes the N-lactotetraose moiety (gal-glc NAc-gal-glc) present in gonococcal 1291 LOS, regardless of whether or not the terminal gal is sialylated. The epitopic specificity of this antibody appears to involve the glc NAc component of this moiety. Because this antibody is bactericidal for serogroup B meningococci and because the N-lactotetraose moiety is the fundamental repeating unit in the capsular polysaccharide on type 14 \textit{Streptococcus pneumoniae}, which is often poorly immunogenic, we have been interested in the basis for the enhanced immune response to this moiety in LCCD persons following natural meningococcal infection. The existence of a congenic strain of C5 deficient/sufficient mice provided an opportunity to examine the mechanisms underlying this phenomenon and the role of the terminal complement components in the immune response to \textit{Neisseria}. Possible factors contributing to the enhanced antibody response in LCCD persons include: a greater antigenic load due to increased bacterial proliferation and/or altered clearance by the reticuloendothelial system resulting in prolonged exposure to Mg antigens.

Initial experiments were designed to determine the effect of the complement deficiency on the immune response to a fixed amount of antigen. In order to avoid differences in Mg replication, outer membrane blebs were harvested from log phase Mg and injected IV into four dose groups consisting of five mice each for both C5 deficient and sufficient animals. Mice were bled via the tail vein prior to and at 3, 6 and 8 weeks after injection. Preinjection serum had no detectable Western blot activity to an SDS-PAGE separation of the Mg bleb antigens. A dose dependent response in specific IgG to Mg LOS was seen in both deficient and sufficient mice by 3 weeks. However, a distinct enhancement in the antibody response to Mg LOS was observed for each deficient group when compared with the parallel complement sufficient
group. These results were confirmed in replicate experiments and indicated that the LOS specific antibody reacted with the same LOS band as did its human counterpart. Quantitation of the antibody response by ELISA, using purified LOS as the antigen, demonstrated that the C5 deficient mice consistently produced $\geq 2x$ more antibody than sufficient mice.

Recent studies employing confocal laser microscopy have demonstrated that the distribution and persistence of LPS following IV injection into normal mice differs depending on whether or not it is presented in its native state bound to the organism cell wall or as the phenol water purified molecule. In the former situation LPS was persistently detected primarily in the spleen, whereas in the latter the liver served as the main site of clearance (4). Thus, in a second set of experiments we assessed the effect of splenectomy on the immune response of normal and congenic C5 deficient mice to IV injected meningococcal outer membrane blebs. Preliminary assessment three weeks following injection indicates that intact C5 deficient mice exhibited the typical ($\geq 2x$) enhanced antibody response to meningococcal LOS compared to C5 sufficient animals as judged by ELISA. This response was not observed in splenectomized C5 deficient animals. Additional antibody quantitation at later points in time are necessary to determine whether the antibody response in splenectomized deficient and sufficient animals differs and whether it differs from intact sufficient mice.

Taken together these results support the following conclusions. First, like their human counterparts, C5 deficient mice exhibit an enhanced antibody response to meningococcal LOS. Second, the complement deficiency itself, not antigen load, is the major determinant of this heightened immune response. Third, an intact spleen is critical for the development of this enhanced antibody response. The distribution and persistence of LOS within the spleen as well as the cellular identify and molecular events that constitute the mechanism by which LCCD influences the quantitative and qualitative aspects of the antibody response to meningococcal LOS remain to be determined.

References
Host-pathogen interactions

Phagocytosis of *Neisseria meningitidis* in complement deficient individuals depends on the C3 deposition onto its surface and the IgG receptor (Fc( R)) allotypes of the granulocytes

Fijen C.A.P., Kuijper E.J., Bredius R.G.M.¹, de Haas M.², Daha M.R.³, van de Winkel J.G.J.⁴, Out T.¹ and Dankert J.

Dept Medical Microbiology and ¹Clinical Immunology Laboratory, University of Amsterdam. ²CLB, Amsterdam. ³University Hospital, Leiden. ⁴Dept Immunology, University Hospital, Utrecht, the Netherlands.

Host defence against meningococcal infection depends on complement and antibody mediated phagocytosis and bactericidal activity. Individuals with a complement deficiency have an increased risk for developing meningococcal disease (1). Phagocytosis is the main defence mechanism against meningococcal disease both in late (C5-9) complement component deficient (LCCD) persons, lacking serum bactericidal activity and in properdin deficient persons without bactericidal antibodies. IgG receptors (Fc( RIIa and IIIb) (2), in conjunction with the complement receptors on PMN are involved in phagocytosis. We questioned whether certain PMN Fc( R) allotypes are associated with an increased risk to develop meningococcal disease.

In 15 LCCD and in 20 properdin deficient patients with or without previous meningococcal disease the distribution of Fc( RIIa and IIIb) allotypes on PMN was studied. In LCCD patients the Fc( RIIa-R131, Fc( IIIb-NA2 phenotype was strongly associated with previous meningococcal disease (Odds ratio 13.9, Fisher test p=0.036), whereas such an association was not present in the properdin deficient group. In vitro phagocytosis experiments with Fc( R typed control PMNs were done to clarify the role of Fc( R polymorphisms and of complement C3 deposition onto meningococci in the host defence against this bacterium. Two strains (serogroup B and W135) were used. PMNs with the Fc( RIIa-H131 allotype phagocytosed in the presence of specific IgG2 antibodies significantly (p<0.05) more than PMNs with Fc( RIIa-R131 allotype. PMNs with Fc( IIIb-NA1 allotype phagocytosed both strains more efficiently (p<0.05) in the presence of specific IgG1 than PMNs with the Fc( IIIb-NA2 allotype. Phagocytosis of W135 meningococci in the presence of 5% properdin deficient serum containing anti-meningococcal antibodies was less than in the presence of this serum reconstituted with properdin. C3 deposition onto the W135 strain exposed to 1% properdin deficient serum was low, as measured in whole cell ELISA. Exposure to properdin deficient serum after reconstitution with purified properdin (or agamma globulinaemic serum) increased C3 deposition. The effect of properdin suppletion was significantly less (Fisher test p<0.05) in control
sera. We concluded that in LCCD individuals phagocytosis of meningococci is highly associated with the Fc( R allotype on PMN, but not in properdin deficient individuals. In properdin deficient sera phagocytosis of meningococci, irrespective the Fc( R allotype of the PMNs is less effective due to a lower C3 deposition onto the meningococcal surface.

References
Effect of Gc LOS sialylation on antibody deposition and complement activation

T. Zaleski and P. Densen

Department of Internal Medicine, VA Medical Center and University of Iowa College of Medicine, Iowa City, Iowa 52242.

Gonococci (Gc) that cause symptomatic local disease become sensitive to complement dependent killing upon repeated culture in vitro. Killing is mediated by an IgM antibody that is present in most normal sera and is specific for gonococcal lipooligosaccharide (LOS). The terminal galactose present in the LOS on freshly isolated Gc or Gc grown in the presence of CMP-NANA is sialylated and these organisms are resistant to IgM initiated complement-mediated killing (1,2). Although previous studies have shown that sialylation results in decreased IgM binding to Gc LOS on Western blots and a decreased ability to remove bactericidal IgM from serum by absorption with intact organisms, the mechanism by which killing is inhibited has been incompletely explored since IgM binding is not completely eliminated by LOS sialylation (1-3). In addition, in other systems sialic acid has been shown to downregulate the complement cascade by enhancing factor H binding which in turn accelerates C3 convertase decay, decreases C3 deposition, and promotes C3 inactivation, thereby reducing complement dependent hemolysis or killing (4,5). The current studies sought to clarify the effect of Gc LOS sialylation on the contribution of both antibody and complement binding as determinants of the inhibition of Gc killing.

Resistance to killing and sialylation of LOS were readily detectable when Gc were grown to log phase in broth containing 2 \( \times \) g/ml CMP-NANA and were complete at 20 \( \times \) g/ml. Sialylation had no effect on the total binding of radiolabeled IgM to intact gonococci. The effect of sialylation on LOS specific IgM binding, as assessed by ELISA using LOS purified from Gc grown in the absence or presence of CMP-NANA, was variable. However, Gc grown in the presence of different concentrations of CMP-NANA demonstrated a progressive increase in the concentration of IgM required to produce half maximal Gc killing. This finding suggests that one consequence of LOS sialylation is to alter the functional consequence of bound IgM.

Sialylation increased total \(^{125}\)I factor H binding to intact Gc in a dose dependent manner that mirrored its effect in reducing killing. Maximal factor H binding increased 1.6 fold and was not influenced by the presence or absence of serum or bound IgM. Sialylation also altered the kinetics and magnitude of C3 binding, reducing maximal
binding by 82%. In addition, the cleavage of C3b to iC3b was accelerated, as judged by autoradiography of SDS-PAGE separated preparations of serum treated Gc following their dissolution in hydroxylamine to release ester linked C3.

Taken together these results indicate that LOS sialylation exerts a significant impact on the qualitative, if not quantitative, binding of bactericidal IgM to Gc. This effect contributes to a major reduction in complement (C3) binding. Moreover, on sialylated Gc C3 that is bound in the form of C3b is rapidly cleaved to its hemolytically inactive form thereby further reducing the probability that a given gonococcus will be killed. In summary, although LOS sialylation inhibits the contribution of both IgM and complement to Gc killing, its effect on IgM binding appears to be of greater magnitude in this process.

References
Deficiency of the late acting complement components (LCCD) is associated with increased susceptibility to recurrent meningococcal disease (5, 7). It has been suggested that these individuals suffer milder disease than complement sufficient patients (6). This, however, is difficult to confirm from epidemiological studies. If it is true it might be because certain pathological processes do not occur with the same severity when the terminal complement pathway cannot be activated (4,6). Alternatively LCCD subjects may become clinically infected with less virulent organisms. There is certainly evidence in Europe that they become infected with organisms that rarely cause disease in complement sufficient subjects (3). In addition, it is not always possible to determine whether recurrent infections in LCCD subjects are relapses of existing infections, or whether the patients suffer repeated new infections.

In order to study meningococcal strains that caused disease in LCCD patients, 16 Neisseria meningitidis strains recovered during 1985-1990 from cases with complement component C6 deficiency (C6D) in the Western Cape Province, South Africa, were compared with 124 routine case isolates from the same area. Serogrouping of the strains from deficient subjects revealed that the common serogroups, particularly serogroup B, predominated. However, the prevalence of rare serogroups was significantly higher than in the control group. Sero-subtyping showed no significant difference between the two groups of isolates. Multilocus enzyme electrophoresis (2) of the 140 isolates revealed the same degree of genetic diversity existed in isolates from both groups. However, the ET-5 complex, which is known to be associated with epidemic disease (1), was found in 22 (18%) of the routine isolates but in none from the C6D subjects. This was marginally significant. What was highly significant was the finding that eight of the 16 isolates from C6D subjects were in one of the six ET clusters, cluster F, which comprised a total of 20 isolates.

Two C6D subjects had had two episodes of infection within a few months of each other. Serogrouping alone was non-informative as to whether these were relapses.
However, sero-subtyping, and multilocus enzyme electrophoresis established that each patient had suffered two different infections. One patient had two group C infections and serial measurements of his anti-serogroup C polysaccharide antibodies established that he suffered his second serogroup C infection despite a good anti-capsular polysaccharide antibody response to his first infection.

Our results also emphasise the susceptibility of LCCD patients to new infections rather than relapses. Moreover they show a difference in the clonal composition of strains that infect LCCD subjects as opposed to those causing clinical infection in the population at large. This supports the concept that LCCD individuals may become infected with less virulent organisms which could at least partially explain differences in clinical picture they present.

References
**Host-pathogen interactions**

**Immune responses to meningococcal outer membrane antigens induced by tonsillopharyngeal carriage of *Neisseria meningitidis***

E. Rosenqvist\(^1\), E. Wedege\(^1\), E.A. Høiby\(^2\), D.A. Caugant\(^2\), G. Bjune\(^1\) and L.O. Frøholm\(^2\)

Departments of \(^1\)Vaccines and \(^2\)Bacteriology, National Institute of Public Health, N-0462 Oslo 4, Norway.

Natural immunity against *Neisseria meningitidis* in children and youngsters is gradually increasing as a function of age, probably induced by asymptomatic carriage of meningococci in throat (1-3). Heist *et al.* (4) found in 1922 that blood from more than 95% of normal individuals was bactericidal to meningococcal strains obtained from asymptomatic carriers. He postulated that most cases of meningitis occur among those few individuals who lack bactericidal activity. Dr Heist, whose own blood was non-reacting against meningococci, unfortunately later died from meningococcal meningitis.

We have studied the effects on the antibody levels of natural acquisition of meningococci in the nasopharynx in different groups of volunteers, whether they served as placebo controls or received the vaccine during clinical phase II trials with a group B meningococcal vaccine in Norway 1987-88 (5). The antibody responses to individual outer membrane antigens in the meningococci were studied by immunoblotting and ELISA with purified antigens and in bactericidal assays. The carrier rate of meningococci was about 50% among the military recruits in phase II-2 (age 19-21 years), about 15% among the medical students in phase II-3 and about 9% among secondary school students in phase II-6 (age 13-14 years). The percentage of strains that have been responsible for most of the systemic meningococcal disease in Norway in the 1980s (ET-5 complex and ET-37), varied from 2.3% to 10.2% in the different groups.

A significantly higher level of antibodies was observed in the prevaccination sera from carriers than from non-carriers, but after two doses of the vaccine this difference disappeared. Eighteen individuals in the placebo groups were identified as new carriers of meningococci during the studies. We found examples showing that colonization with non-groupable and non-subtypable carrier strains induced bactericidal antibodies against a systemic B:15:P1.7,16 strain. The immune responses against the B:15:P1.7,16 strain were mainly directed against class 5 proteins (Opa/Opc), the H8 antigen and lipopolysaccharides, but also other outer membrane proteins cross-reacted in immunoblots. A strong positive correlation was observed between
bactericidal activity and IgG antibodies against Opc (7). These results show that carriage of non-pathogenic meningococci in tonsils and pharynx may induce high levels of bactericidal antibodies.

References
The use of murine-human heterohybridomas to produce gonococcal specific human isotype antibodies

M.D. Cooper¹, G.A. Jarvis² and R. Kirkpatrick¹

¹Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, Springfield, IL 62794 and ²Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, CA 94143.

Introduction: The availability of human antibody directed against specific components of the gonococcus has been difficult to achieve in quantity. Antibodies which had a mucosal origin have been particularly difficult to obtain. These locally produced antibodies are important to study since they are the first encountered after the attachment and invasion of the mucosal surface. In order to obtain an immortal cell line which had the capability to be a continuous producer of human antigonococcal antibodies we used a unique fusion of a murine-human heterohybridoma.

Results and Discussion: Using a unique double fusion technique we have developed stable mouse-human heterohybridomas which produce human monoclonal antibodies (3). Mouse myeloma P-63 cells were initially fused with unimmunized enriched peripheral blood B cells. These 8-azaguanine resistant hybridomas were then fused with B-cell enriched fallopian tube lymphocytes immunized with whole killed gonococci (Rice-2). Immune lymphocytes were identified by ELISPOT assays (2.1) prior to fusion for production of gonococcal specific human IgG, IgA and/or IgM. Clones from the second fusion producing gonococcal specific monoclonal antibodies were identified by ELISPOT and ELISA assays. Flow cytometric analysis of the monoclonal cells revealed that these hybridomas were capable of multiple isotype expression (IgG, IgA, and IgM). This was confirmed by reisolation of the clones by limiting dilution and picking only those clones which arose from single cells. The morphological characterization of these cells was done using flow cytometry techniques. Isotype expression was confirmed by ELISPOTS, IMMUNOBLOTS, and ELISA assays. We chose one of these clones, 1H11, for further characterization and study because of its multiple isotype expression. This clone showed monoclonal antibodies directed against a 45,000 MW protein of an outer membrane complex (OMC) (4) isolated from a PID isolate (Rice-2). The IgG and IgA was purified from the culture fluids using Protein G and jacalin. The fact that IgA bound to Jacalin indicates that it is of the IgA1 subclass. Both the IgG and IgA were directed against the 45,000 MW protein. Functionally the purified IgG was bactericidal against the homologous isolate with about a 35% kill. Although the IgA1 was not lytic in the bactericidal assays (as expected) it did block the IgG mediated lysis. This was
observed when the purified IgA1 was added to the IgG mediated bactericidal assay thus indicating that the IgA1 could function as a blocking antibody. Other IgG monoclonal antibodies were isolated from other clones and also participated in bactericidal activity from 27-40%. Both the IgG and IgA1 isolated from the 1H11 clone blocked adherence of the Rice-2 strain to HEC-1-B cells. The 1H11 IgG blocked adherence by approximately 71% while the IgA had a 47% inhibition of adherence.

These studies indicate that it is possible to produce several human monoclonal antibodies from a mouse-human heterohybridoma which have specificity for gonococcal epitopes. These clones are stable and have been producing IgG, IgA1, and IgM isotype antibodies for at least two years. It is intriguing that these clones are coexpressing multiple immunoglobulin isotypes with apparent specificity against the same gonococcal epitope.

References
Relationship of the onset of symptoms and dysuria to opacity protein (protein II) expression in experimental gonorrhea


Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington D.C., 20307-5100, USA.

In five iterations, 36 human male volunteers were challenged by intraurethral inoculation of *Neisseria gonorrhoeae* MS11mkC, and 5 with MS11mkA. Inocula varied from 250 to as many as 44,000 transparent (Opa\(^-\)) cells (~0.2% Opa\(^+\)). Opa\(^+\) colonies present in the original inocula from the fifth iteration were screened by western immunoblot to determine which opacity proteins were expressed. We detected 5 different opacity proteins ranging in size from 29 kDa to 36 kDa. Eighty percent of the isolates expressed the 30 kDa protein.

Nineteen of 36 (53%) volunteers inoculated with Opa\(^-\) MS11mkC and 2 out of 5 (40%) inoculated with Opa\(^-\) MS11mkA developed dysuria and discharge. During the initial phase of the infection, volunteers shed only challenge Opa\(^-\) organisms. There was, however, a rapid transition from Opa\(^-\) to Opa\(^+\) in most if not all of the organisms shed by the 19 volunteers who became infected. Transition from Opa\(^-\) to Opa\(^+\) occurred within 24 hours prior to onset of symptoms in 15 volunteers. The remaining 6 volunteers shed Opa\(^+\) cells from 36 to 87 hours prior to onset of symptoms. There was no apparent relationship between challenge dose and time of transition to Opa\(^+\), or to the onset of symptoms. Opa\(^+\) colonies from 6 infected subjects in our last experiment were screened at every time point to determine which opacity proteins were expressed. During the course of their infections, the organisms shed by each of the volunteers expressed a number of opacity proteins which varied from subject to subject and specimen to specimen. While 11 different opacity proteins were detected, the 30 kDa protein predominated.
Specific unresponsiveness to LPS during infections by Neisseria gonorrhoeae

R. Demarco de Hormaeche, L.M. Chamberlain and C.E. Hormaeche

University of Cambridge, Department of Pathology, Division of Microbiology and Parasitology, Tennis Court Road, Cambridge, CB2 1PQ.

Although infections by Neisseria gonorrhoeae leave no apparent post-infection immunity, they induce a powerful antibody response. Antibodies to various gonococcal antigens have been found in patients with gonorrhoea but they appear to play little if any role in the resolution of the infection or prevention of re-infection (1). So far no specific antibody response has been clearly associated with immune effective mechanisms. However, antibodies directed to the LPS and to the outer membrane protein PI are bactericidal in vitro (10). LPS is a major target for bactericidal antibodies but it has been demonstrated that it is involved in immune evasion both through antigenic variation (5,8) and host mimicry. The latter is represented by both the presence in the LPS of epitopes which share specificity with human cell epitopes (6) and, most importantly, by in vivo sialylation (9,7). Gonococcal LPS acquires sialic acid from the host during infection, thus the organisms appear coated by material of host origin (4) which prevents them from being seen as non-self at least during short lived infections.

This work was undertaken to analyze the antibody response and the bactericidal activity of the antibodies elicited during prolonged gonococcal infection. Gonococcal infections were provoked in the guinea pig subcutaneous chamber model using strains MO1 (wild type of reduced virulence), JKD298 (an aroA mutant of strain MS11, ref.2) and the three LPS variants of strain Gc40 D1, D2 and D4 of decreasing LPS molecular mass and virulence (3). Kinetics of infection was monitored by viable counts on samples of fluid obtained from an infected chamber in each animal at intervals during infection. Antibodies to proteins and purified LPS were measured by ELISA (2) in samples of fluid obtained from a contralateral non-infected chamber.

As described (3), infections by the LPS variants D1, D2 and D4 of strain GC40 had different evolutions: infections by D1 were severe and ended with rejection of the chambers after 2 weeks while infections by D2 and D4 were mild and resolved by spontaneous clearance after 1 and 3 weeks respectively. Infections with strains MO1 and JKD298 cleared spontaneously at about 3 weeks after inoculation.
All animals mounted antibody responses to proteins that increased during infection. Antibodies reached maximum values at 28-36 days after inoculation. The level of antibodies did not correlate with the amount of live bacteria present in the chambers at any given time. However, clearance of the infection was followed by a sharp increase in the amount of antibodies detected in each animal. Antibodies continued to increase weeks after the infections had cleared.

Antibodies specific to LPS, in contrast, were only detected briefly during the initial stage of infections by any of the strains. Anti-LPS antibodies did not attain high levels and lasted only a few days and then dropped markedly, without showing any increase after resolution of the infections. Animals apparently became specifically unresponsive after continuous stimulation with LPS released during infection. The similarity of responses seen with several strains of different LPS specificity, suggests that specific unresponsiveness to the LPS during infection may be a phenomenon common to all gonococci. Six animals infected with JKD298 were tested for production of antibodies to sialylated LPS. With one exception, all animals had similarly low ratios of antibodies to sialylated and non sialylated LPS extracted after incubation of JKD298 with or without CMP-NANA as described (4). Experimental infections by the virulent strain D1 did not clear spontaneously. The production of antibodies directed to non-LPS antigens was not sufficient to clear the infection.

Dilutions of chamber fluid from guinea pigs infected with GC40 D1 during the early stages of infection were tested in parallel for antibodies against D1, D4 and D2 LPS of decreasing molecular mass (3). All animals gave higher readings with the more truncated LPS types D4 and D2 than with the more complex LPS of the infecting variant D1. These results suggest that the anti-LPS antibodies formed during infection may be directed to antigenic sites of the common core rather than to the specific LPS sites of the infecting type.

Bactericidal antibodies produced during and after infection were investigated in all animals using sialylated and non sialylated gonococci of the homologous strain in a standard bactericidal assay (8). Fresh normal guinea pig serum was used as complement source diluted as to contain 2 haemolytic doses.

No bactericidal activity was found in any guinea pig at any time during or after infections with any of the strains used. This may indicate that the antibodies elicited by infection have no bactericidal activity or that blocking antibodies are formed in sufficient amount to suppress their effect. However, as LPS is the major antigen in the bactericidal reaction, LPS-specific unresponsiveness may explain the lack of bactericidal activity observed.
References


Immune responses to capsular polysaccharides of *Neisseria meningitidis* in two C2-deficient sisters: Role of the alternative pathway in serum bactericidal reactions

B. Selander, A.G. Sjöholm, E. Holmström, L. Truedsson, H. Käyhty, E. Wedege and C. Söderström

Departments of Medical Microbiology and Infectious Diseases, Lund University Hospital, Sölvegatan 23, S-223 62 Lund, Sweden. National Public Health Institute, Helsinki, Finland. National Institute of Public Health, Oslo, Norway.

Hereditary deficiencies of terminal complement proteins, C5-C9, and of the alternative activation pathway, C3, factor B, factor D and properdin, are known to be associated with disseminated neisserial infection. In addition, systemic disease caused by polysaccharide encapsulated bacteria including *N. meningitidis* has been documented in about 25% of patients lacking C2, a component of the classical activation pathway, C1q, C1r, C1s, C4 and C2 (1). The present study concerned a C2-deficient girl with meningitis caused by *N. meningitidis* serogroup W-135 at the age of 14, and her C2-deficient elder sister, who was clinically healthy. Both sisters were vaccinated with tetravalent meningococcal vaccine (Mencevax ACWY). About one year later they were revaccinated. Sera were obtained before and four weeks after the vaccinations. Antibody responses to each of the polysaccharide antigens were measured with enzyme-linked immunosorbent assay.

The bactericidal activity in C2-deficient sera was examined at serum concentrations between 25% and 50%. The patient showed low antibody levels and no bactericidal activity for serogroups A, C, W-135 and Y in prevaccination sera and responded poorly to the first vaccination. However, revaccination resulted in marked antibody responses and acquisition of bactericidal activity against serogroups W-135 and Y. By contrast, her healthy sister demonstrated good antibody responses to serogroup A, C and W-135 polysaccharides after the first vaccination. These responses were associated with bactericidal activity for serogroups C and W-135. Possible impairment of antibody responses to polysaccharide antigens in the patient as compared with antibody responses in her sister could not be ascribed to differences of IgG allotypes (4). A surpising finding was that the healthy sister had lost her bactericidal activity for serogroup W-135 by the time of the second vaccination in spite of persisting anticapsular antibody concentrations. However, the serum showed opsonic activity for serogroup W-135 in phagocytosis by neutrophil granulocytes. The presence of a suspected blocking factor remained after revaccination.
For further investigation, the C2-deficient serum was depleted of C1q, factor D and properdin (5). By reconstitution with purified proteins it was shown that the serum supported alternative pathway-mediated killing of serogroup W-135 and that the activity was blocked in the presence of C1q. The blocking activity remained after IgA depletion of the serum, which excludes that it was due to IgA class antibodies (2). Judging from Western blot analysis, the serum did not contain antibodies to class 4 outer membrane protein, another known type of blocking factor (3). Experiments with serogroup C and C2-deficient serum depleted of C1q, factor D and properdin demonstrated that the "C2-bypass" mechanism (6) enhanced bactericidal activity without being necessary for the reaction. The findings show that some anticapsular antibodies can recruit the alternative pathway in defense against \textit{N. meningitidis} and that vaccination could be meaningful in persons with classical pathway deficiencies. However, improved vaccines are desired since antibody responses may be impaired.

References


Susceptibility to meningococcal disease: Association with rare complement C7 variants?

G. Dewald, C. A.P Fijen, E. J. Kuijper and J. Dankert

1Department of Medical Microbiology/Reference Laboratory for Bacterial Meningitis, University of Amsterdam/RIVM, Amsterdam. 2Department of Human Genetics, University of Bonn.

It is well established that hereditary deficiencies of components of the terminal complement pathway (C5,C6,C7,C8,C9) predispose to systemic infections with Neisseria meningitidis (2). Among the numerous case reports on an association between C7 deficiency and meningococcal disease there is also one report on a patient with partial C7 deficiency and recurrent meningococcal infection (4).

A significantly decreased C7 level has been reported to be associated with the presence of C7*3, a hypomorphic C7 allele (3,5). The C7 3 allotype is one of the genetically determined charge variants of human C7 which have been identified by isoelectric focusing of serum samples followed by specific detection of C7 bands (3). C7 1 is the most common allotype in Caucasians (1,3). The allotypes differing from C7 1(i.e. C7 2 or C7 3) are found only in approximately 1% of a Caucasian population (1,3). We speculated on an increased frequency of C7 3 and possibly other rare C7 allotypes in patients with meningococcal disease.

Using isoelectric focusing of neuraminidase-treated serum samples and an immunoblotting procedure we performed C7 typing on serum of 214 patients who had survived meningococcal disease (54 x serogroup B, 82 x serogroup A or C, 78 x the uncommon serogroups W135, X, Y, Z, 29E or NG). Sera of healthy blood donors (n=102) have been used as controls; no uncommon C7 allotypes were encountered. In contrast, uncommon C7 allotypes were found in about 3% of the patients with meningococcal disease. At least 5 different uncommon C7 allotypes were observed. The one non-Caucasian patient with an uncommon C7 variant was excluded from the calculations. The present data show a significantly increased frequency of uncommon C7 variants among patients with infection due to serogroup W135 (Fisher exact test; p=0.028). Among patients with meningococcal disease due to serogroup A or C an association with the C7 3 allotype seems likely (Fisher exact test; p=0.08). A further characterisation/classification of the encountered variants is in progress.

References


Serum factors affecting the sensitivity to *Neisseria meningitidis* lipopolysaccharide

A.B. Petrov¹, J.A. Hazelzet², L. Aarden³ and J.T. Poolman¹

¹ National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. ² Sophia Children’s Hospital, Erasmus University Rotterdam, The Netherlands. ³ Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Septic shock is the major clinical syndrome leading to lethality during meningococcal infection. We studied *in vitro* the sensitivity of human or pig whole blood and *in vivo* pigs for septic shock induced with *Neisseria meningitidis* endotoxin. LPS from strain H44/76 in a form of outer membrane vesicles was more active than phenol extracted LPS or outer membrane complex (10 and 1,000 times respectively) in *Limulus* lysate assay and to stimulate IL-6 or TNF-α production in human whole blood. The sensitivity of pig whole blood showed 10 fold individual difference to produce IL-6. This phenomenon was correlate with the inhibitory activity of pig sera to suppress endotoxin induced accumulation of cytokines *in vitro* in human whole blood.

The capacity of the sera to inhibit endotoxin action was thermostable (up to 75°C 10 min) and was not related to the background level of anti-LPS IgG or IgM antibodies. Only one of four anti-LPS murine monoclonal antibodies suppressed the IL-6 accumulation *in vitro*. *In vivo* development of septic shock syndrome (hemodynamic and metabolic disorders) was very closely related to the inhibitory activity of plasma *in vitro*. The amount of endotoxin detected in sera after intravenous infusion of 10⁸ g/kg in pigs was not correlated with the clinical manifestation of septic shock but corresponded to the IL-6 and TNF-α level. The mechanism of the sensitivity to *Neisseria meningitidis* endotoxin will be discussed.
Molecular and genetic analysis of an adhesion- and invasion-deficient Neisseria meningitidis serogroup B mutant

E.M. Ribot¹, N.J. Raymond², K.A. Birkness¹, D.S. Stephens² and F.D. Quinn¹

¹Centers for Disease Control and Prevention, Atlanta, GA 30333 and ²Emory University School of Medicine, Atlanta, GA 30322.

Although Neisseria meningitidis has received a great deal of attention, little is still known of the genetics and cell biology of the host-parasite interaction. Understanding the mechanism by which N. meningitidis attaches and invades human epithelial and endothelial cells is critical to our knowledge of meningococcal disease. Among meningococci there are three major capsular serogroups associated with disease; A, B and C. The nature of the capsule in serogroups A and C has led to the development of useful vaccines against these serogroups. However, the serogroup B capsular polysaccharide does not induce protection. Other membrane factors currently under study by different groups as serogroup B vaccine candidates include lipooligosaccharide (LOS) and a number of outer membrane proteins (OMP) (3,4), such as class 1, class 2 and class 3. The identification of alternative serogroup B antigens, especially those involved in virulence, could lead to new diagnostic probes and to new vaccine candidates as well as contribute to an overall understanding of the pathogenesis of this organism.

The use of tissue culture models for the study of attachment and invasion have shed some light on the pathogenesis of the disease. However, the molecular mechanisms involved in N. meningitidis attachment and invasion are still poorly understood. The recent development of a transposon mutagenesis system and the use of relevant cell lines provide the opportunity to gain new insight into meningococcal meningitis. The main focus of this study was to examine the mechanism of attachment and invasion of N. meningitidis at the cellular level and to identify the bacterial genes involved in this interaction. The creation of a N. meningitidis mutant library by insertion of transposon Tn916 into the chromosome of serogroup B strain NMB has been described (1,2). Part of this library was screened for mutants that showed an altered ability to attach and invade a human endometrial tissue culture cell line (HEC-1-B). Using this approach, we have identified a mutant (VVV6) which showed a significant decrease in its ability to attach \(10^{-1}\) and invade \(10^{-2}\) HEC-1-B cells compared to the parent strain (NMB) and to an additional well characterized capsule deficient mutant (M7) (2). Transmission electron microscopy studies suggested morphologically thinner pili present in reduced numbers. Homologous recombination
experiments of the wild-type parent strain with VVV6 DNA produced transformants having the mutant phenotype in the tissue culture.

The decreased ability to attach and invade is likely linked to the disruption of a gene(s) encoding for a factor(s) necessary for recognition of the host cell. Southern hybridization, PCR and DNA sequence data indicates the presence of a single intact copy of transposon Tn916 (Class I insertion) in the VVV6 chromosome. Meningococcal nucleotide sequence flanking the transposon was obtained and subjected to computer analysis and GenBank database comparison. Preliminary results indicate that the sequence contains part of two open reading frames (ORFs). One of the ORFs shows significant homology, approximately 60%, to an E. coli gene with no known function. The other ORF shows no significant homology to any gene in GenBank. Further cloning and sequencing is still underway in an effort to attribute a possible function of the identified gene(s). Identification of this gene(s) might shed additional light on the mechanism of attachment and invasion of N. meningitidis.

References
The influence of capsule and LPS phenotype on the invasion potential of a serogroup B strain expressing Opc and pili

M. Virji, I. Peak, K. Makepeace, M. Jennings, D.J.P. Ferguson and E.R. Moxon

Department of Paediatrics, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, UK.

*Neisseria meningitidis* isolates from disseminated infections are invariably capsulate and commonly express pili. Additionally, their LPS may be sialylated (1). Many of these strains also express outer-membrane opacity proteins Opa and Opc. Opc was shown to increase bacterial interactions with eukaryotic cells using spontaneously arising mutants in a serogroup A strain C751 (2). Since this strain is non-piliated, the additional influence of pili could not be determined. In the current study, we have investigated the influence of capsule, pili and sialylated LPS on the functional properties of Opc in a serogroup B strain MC58 (B:15:PI.7,16b).

**Isolation of mutants and variants with altered expression of surface structures:** The strain MC58 expresses capsule, pili, Opc as well as an Opa protein. Its transformation to a non-capsulate phenotype was carried out by deletion/insertion mutation in the sialyl transferase gene within the *cap* locus. A spontaneous Opa- colony arising in a culture of cap- mutants was used to derive a panel of variants and mutants with further altered expression of Opc and pili. Initial studies showed that non-piliated, non-capsulate phenotype was not adherent even though it expressed large amounts of Opc. Since studies on the serogroup A strain C751 had shown that sialylation of LPS resulted in the inhibition of Opc-mediated interactions (3), it was possible that in strain MC58, the LPS phenotype was not permissive for Opc function. Immunochemical investigations on the LPS of MC58 showed that it was related to L3 immunotype and may be expected to be sialylated. Other variants of the strain MC58 arising at a frequency of <0.5% expressed LPS of lower Mr (by SDS-PAGE) and concurrently lost the ability to react with the monoclonal antibody (mAb) SM82. The low Mr LPS (LPS<sub>L</sub>) was resistant to sialylation when CMP-NANA was provided in the culture medium. Using the property of non-reactivity with mAb SM82, a further panel of variants was derived that expressed non-sialylated LPS<sub>L</sub> (Table).

**Studies on cellular interactions:** Phenotypic characteristics and host-cell association of derivatives are shown in the table. Capsulate bacteria require the expression of pili for increased adherence has been reported previously (4). Loss of sialylation in capsulate phenotype (A8) did not affect bacterial interactions significantly (Table). However, in non-capsulate mutants, LPS phenotype had a significant
influence both in bacterial association as well as uptake. Important observations can be summarised: (a) In non-piliated non-sialylated LPS phenotype, the expression of Opc enabled bacteria to interact with Huvecs in large numbers (Å11 vs Å4) and invasion of host cells was increased significantly. The role of Opc was confirmed by the use of variants and mutants lacking the protein. In addition, mAb against Opc inhibited bacterial interaction with Huvecs. (b) The expression of pili in addition to Opc resulted in synergistic effect on association and uptake (Å2 vs Å4). By TEM, many more bacteria were observed within membrane bound vacuoles in Å2 compared to Å4. (c) Sialylated LPS phenotype inhibited Opc-mediated interactions with Huvecs and abolished uptake (Å4 vs Å5). (d) The expression of pili appeared to be an essential requirement for bacterial interactions when LPS was sialylated, the presence of Opc was not sufficient (Å5 vs Å3). Thus sialylation of LPS mimics capsulation status in meningococci.

Some phenotypic characteristics and interactions of derivatives of serogroup B strain MC58 with endothelial cells

<table>
<thead>
<tr>
<th>Der</th>
<th>Cap</th>
<th>LPS</th>
<th>Pili</th>
<th>Opc</th>
<th>A</th>
<th>Der</th>
<th>Cap</th>
<th>LPS</th>
<th>Pili</th>
<th>Opc</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>58*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Å8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Å3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>Å2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+ +++</td>
</tr>
<tr>
<td>Å5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>Å4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Å1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>Å9</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Å1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Der: Derivative. '58*' represents the parental phenotype
LPS: '+' represents sialylated L3 immunotype. '-' represents non-sialylated LPS.
The expression of pili was confirmed by electron microscopy.
A: Relative association of bacteria with human endothelial cells ('-' represents <2 bacteria per cell; '+++' represents >200 bacteria per cell).

In summary: These studies confirm previous observations that Opc protein mediates bacterial interactions with human cells and show that in diverse serogroups the phenotype most suited for adherence and invasion of host cells is non-capsulate, lacking in sialic acid on LPS and in addition requires a surface expressed adhesin such as Opc. In such phenotypes, additional expression of pili enhance bacterial interactions mediated by Opc. Therefore, modulation of capsule and LPS are prerequisite for the realisation of functional potential of outer membrane proteins in

261
bacterial virulence for host cells. Since capsule and LPS sialylation confer resistance to host's immune mechanisms (5,6,7), dynamic modulation of surface polysaccharides and or selection may play an important role in virulence determination under different environmental conditions.

References
Meningococcal outer-membrane protein Opc mediates interactions with multiple extracellular matrix components

M. Virji, K. Makepeace and E.R. Moxon

Department of Paediatrics, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, UK.

The precise route/s of meningococcal entry into the host from its preferred colonisation site of the respiratory epithelium have not been elucidated. Bacterial adhesion to host cell surface as well as to subcellular matrix adhesion components that become exposed on cellular damage may allow bacteria to gain entry into the host from the nasopharynx. Colonisation of the vascular compartment and further dissemination may also require translocation across extracellular matrix (ECM). Studies on bacterial interactions with ECM components are therefore important in investigations on virulence mechanisms of meningococci.

We have analyzed the ability of meningococcal phenotypes with altered expression of capsule, LPS type, pili, Opa and Opc proteins to adhere to ECM synthesised by cells in culture. Bacterial adherence to matrigel, an ECM derived from murine sarcoma cells (1), to matrix derived from cultured Huvecs as well as to several purified proteins derived from ECM or plasma was investigated.

Interactions of variants of serogroup A and B strains with matrigel: Studies using capsule-deficient, non-piliated variants of C751 (serogroup A) showed that the presence of opacity proteins (OpaB or Opc) increased meningococcal interactions with matrigel significantly and Opc-expressing bacteria adhered in numbers which were 5 fold greater compared to bacteria without the opacity proteins. Antibodies against Opc decreased the adherence of Opc-expressing variants to matrigel. The influence of capsule, LPS immunotype (L3, L8), Opc and pili in meningococcal adherence to matrigel was determined using variants of a serogroup B strain MC58. Opc-expressing, acapsulate phenotype with non-sialylated LPS (L8) adhered in large numbers to matrigel. The expression of capsule or of sialylated LPS (L3) inhibited this interaction. Additional presence of pili in capsulate or acapsulate phenotypes did not increase bacterial interactions significantly.

Bacterial adherence to ECM derived from cultured Huvecs: Huvecs synthesise several adhesive components which may be incorporated into their ECM (2, 3). Some components in cultured cells may also be derived from foetal calf serum used in the growth medium. Fibronectin, thrombospondin (TSP) and von Willebrand factor
(vWF) could be detected in matrix recovered after non-enzymic detachment of Huvecs grown to confluency. Adherence of bacteria to Huvec ECM was examined using immunofluorescence techniques after removal of cells from culture dishes. These studies used capsule-deficient Opc- and OpaB-expressing variants of strain C751. Interactions of OpC- or OpaB-expressing bacteria were significantly greater (4-8 fold) than of bacteria lacking these proteins. In addition, their adherence to wells containing Huvec matrix was considerably greater compared to wells coated with BSA alone.

**Bacterial adherence to purified plasma and matrix components:** Purified fibronectin, vitronectin, laminin, collagen type IV, TSP and vWF were immobilised on plastic plates and relative interactions of Opc- or OpaB-expressing variants were investigated. Opc-expressing bacteria adhered in significantly increased numbers to several but not all proteins (2-10 fold increase) compared to bacteria lacking the expression of Opc or Opa proteins. Minimal or no interactions were observed with TSP and vWF. In contrast, interactions of OpaB- expressing bacteria increased only when plates were coated with fibronectin.

**In conclusion:** Our investigations have shown the potential of meningococcal opacity proteins to adhere to ECM components and imply that the molecular mechanisms of bacterial invasion of host tissues may involve adhesive proteins of the host. Such interactions may result not only in bacterial adherence to exposed ECM but also provide an alternative mechanism for bacterial interactions with host cell surface via receptors such as the integrin family of cell surface glycoproteins that bind to adhesive and matrix proteins (4).

**References**


Interactions of *Neisseria meningitidis* expressing Opc and Opa proteins with human phagocytes

G. McNeil, M. Virji and E.R. Moxon

Department of Paediatrics, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, U.K.

**Introduction:** In order to investigate the pathogenesis of meningococcal disease, work has focused on the bacterial adhesins which mediate attachment to human mucosal epithelial cells and, recently, endothelial cells (1). Bacterial surface structures which may influence adhesion include pili, capsule, and outer membrane opacity proteins (Class 5, Opa and Opc). Investigations into the interactions of *Neisseria meningitidis* (Nm) with human mononuclear (MN) and polymorphonuclear (PMN) phagocytes are limited to studies on serum opsonisation and phagocytosis (2,3,4). Our previous studies have examined the interactions of Nm with human monocytes. The present study investigates the effect of Opc and Opa proteins, together with capsule and pili on interactions with PMN and MN using chemiluminescence (CL) assays. In addition, the effect of LPS phenotype was examined using variants of serogroup A and B strains.

**Results and Discussion:** The effects of pili, capsule and LPS phenotype on Opc-mediated interactions with PMN and MN were investigated using variants of the capsulate, piliated strain MC58 (serogroup B). Comparison of acapsulate Opc⁺ variants expressing or lacking pili demonstrated similar levels of PMN CL, suggesting that pili are not required for association. This observation was also true of MN, confirming earlier studies using piliated and non-piliated variants of different serogroups (5). Capsulate Opc⁺ bacteria induced a 4-fold lower CL response in PMN as well as MN, compared with acapsulate Opc⁺ bacteria. Similarly, Opc⁺ variants with sialylated LPS (immunotype L3) induced lower CL responses compared to variants with non-sialylated LPS (immunotype L8).

Meningococcal isolates from the nasopharynx are often down-regulated in capsule expression (6). As such, Opc-mediated interactions of Nm and phagocytes in the nasopharynx may represent an important step in meningococcal pathogenesis. In order to study the potential of Class 5 proteins, a panel of variants expressing Opa proteins (A,B,D) or Opc were selected from a capsule-deficient, non-piliated serogroup A strain C751. This strain produces LPS of immunotype L9 but is not sialylated intrinsically. The highest level of CL in PMN was induced by Nm expressing Opa proteins B and D. OpaA variants also induced significant responses, while
bacteria expressing Opc failed to induce a significant response. By comparison, MN CL responses to bacteria expressing Class 5 proteins decreased in the order: Opc > OpaB > OpaD > OpaA. Results from CL assays parallel those observations from phagocytic killing assays.

As indicated above for MC58, and observed previously with epithelial and endothelial cells for C751 (7), sialylation of LPS inhibits Opc-mediated interactions. In addition, sialylation has been implicated in resistance to neutrophil killing of gonococci (8,9). We therefore investigated the effect of LPS sialylation further on Opa- and Opc-mediated interactions with PMN and MN, using variants of strain C751 grown on cytidine monophosphate N-acetylneuraminic acid (CMP-NANA). Sialylation of both OpaB- and Opc-expressing bacteria reduced interactions with MN by approximately 27% and 35% respectively. With PMN, sialylation of OpaB+ bacteria reduced interactions by approximately 47% and that of Opc+ by 60%. Therefore it would appear that sialylation inhibits interactions of Opa- and Opc-expressing bacteria with phagocytic cells, with a greater effect on Opc.

Conclusions: The expression of certain Class 5 proteins on the surface of bacteria may increase their association with PMN and MN, however, only when capsule is down-regulated. Opc, which has been implicated in the invasion of epithelial cells, endothelial cells (10) and monocytes (5) by Nm, had comparatively little effect on PMN CL. By contrast, OpaB-, OpaD- and OpaA-expressing bacteria all induced high CL responses by PMN. LPS sialylation decreased Opc- and OpaB-mediated interactions with PMN and MN, with a greater effect on Opc, while the presence of pili had no significant effect.

References


Opc interactions at the apical surface of polarised endothelial cells require serum factors that bind to the integrin family of cell surface receptors

M. Virji, K. Makepeace and E.R. Moxon

Department of Paediatrics, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, UK.

Previous studies have shown that Opc- or OpaB- expression in a capsule-deficient strain with non-sialylated LPS correlates with increased association of bacteria with eukaryotic cells of different origins (1,2,3). Compared to OpaB, Opc imparted greater ability to invade several epithelial and endothelial cells and Opc-expressing bacterial invasion of human endothelial cells was considerably more than any other cell types investigated. In the present study, we have investigated the molecular mechanisms of Opc mediated interactions with human umbilical vein endothelial cells (Huvecs). Huvecs produce polarised cells in culture and the use of these cells facilitate studies on interactions of bacteria at apical and basal surfaces. The studies have used capsule-deficient, non-piliated meningococcal variants either expressing or lacking opacity proteins. The variants were derived from strains of serogroup A (C751) and serogroup B (MC58).

Apical interaction of Opc-expressing bacteria is serum dependent: It was observed that in the absence of serum, there was a marked reduction (>90%) in the association of Opc-expressing bacteria with Huvecs. In addition, whilst low concentration of serum increased bacterial association with host cells, higher concentrations were inhibitory. This is consistent with the notion that serum contains factor/s that mediate Opc interactions at low concentrations and competitively inhibit at high concentrations. Further investigations showed that pre-exposure of Opc+ bacteria to serum was sufficient to obtain increased adherence. The presence of monoclonal antibody (mAb) B306 against Opc during incubations with serum alone inhibited the subsequent interactions of serum-exposed bacteria to Huvecs. Bacterial viability was not essential for these interactions since gentamicin-killed bacteria exposed to serum adhered to Huvecs. In contrast, adherence of OpaB-expressing bacteria to Huvecs was not serum-dependent.

Investigations on Huvecs non-polarised by brief EDTA treatments showed that re-organisation of basally distributed receptors enabled bacteria to interact with Huvecs in the absence of serum; suggesting that distinct mechanisms of interactions may operate at apical and basal surfaces of polarised cells.
Adherence to the apical surface of Huvecs is inhibited by peptides containing the motif Arg-Gly-Asp (RGD): Bacteria were incubated with confluent Huvec monolayers in the presence of serum-supplemented media with the addition of RGDS or RGES peptides. Immunofluorescence analysis of cultures showed that there was a striking inhibition of bacterial adherence to the host cell surface in the presence of RGDS but not RGES. These experiments suggested that bacteria treated with serum may bind to adhesion factors present in the serum and interact with one or more of the RGD-recognising sub-family of integrins which include $3\ $1, $5\ $1, $v$1, $v$3 and $v$5 (4).

Identification of integrin/s involved in apical interactions of serum-treated Opc-expressing bacteria: Several monoclonal antibodies known to inhibit integrin binding to their respective ligands ('blocking' mAbs) were used in inhibition assays. In addition, a non-blocking mAb against $1$ integrins and a mAb against $2$ integrins were used, these bound to the endothelial cells but were not inhibitory. Overall, blocking mAbs against $v$3 complex (Vitronectin receptor) consistently appeared to be the most effective in inhibiting serum dependent endothelial adherence of Opc-expressing bacteria at the apical surface suggesting that the principal integrins on Huvecs involved in interactions with Opc-expressing meningococci may belong to the vitronectin receptor family.

Vitronectin mimics serum-dependent interactions of Opc-expressing bacteria with Huvecs: One of the mediators of bacterial interactions with Huvecs may be vitronectin (Vn) was suggested by the above experiments. In order to test the hypothesis, we used purified Vn in experiments to study its role in mediating bacterial interactions. Vn was found to be a potent mediator of bacterial adherence to Huvecs. In addition, Vn-mediated adherence of Opc-expressing bacteria was inhibited by mAb B306 against Opc and by RGDS but not RGES peptides. Blocking mAbs against $v$3 inhibited Vn-mediated adherence of Opc-expressing bacteria but this interaction was not affected by mAbs against other integrins.

In summary: These data suggest that a sequence of molecular events resulting in trimolecular complexes at the apical surface of endothelial cells may drive neisserial invasion of Huvecs. The expression of Opc appears to enable bacteria to utilise the normal signal transduction mechanism of host cells via ligands in serum that adhere to endothelial cell integrins.

References


Investigations into the molecular basis of meningococcal toxicity for human endothelial cells: the role of LPS and pili

K.L.R. Dunn, M. Virji and E.R. Moxon

Department of Paediatrics, University of Oxford, Oxford, U.K.

*Neisseria meningitidis* harmlessly inhabits the oropharyngeal mucosa of a subset of the population but occasionally disseminates in susceptible individuals and becomes potentially one of the most rapid causes of death in humans. One common clinical manifestation of meningococcal disease is the appearance of petechiae, small haemorrhages in the skin, or purpura, larger haemorrhagic lesions in which meningococci have been identified (1,2), and analysis of autopsy material from diseased humans has revealed widespread vascular damage, including damage to endothelial cells (3). In contrast *Haemophilus influenzae* septicaemia and meningitis are not normally associated with these characteristic skin lesions. Using an *in vitro* model of toxicity we have shown that *Neisseria meningitidis* causes damage to cultured human umbilical vein endothelial cells, in contrast to the lack of cytotoxicity exhibited by *Haemophilus influenzae* type b (4), reflecting the situation *in vivo*. We have used this model to study the mechanisms of toxicity.

**The role of LPS in meningococcal toxicity**: Various bactericidal treatments failed to abolish meningococcal toxicity; in particular the toxin was shown to be heat-stable. In addition to filter-sterilised meningococcal culture supernatants, meningococcal outer membrane vesicles (OMV) were able to mimic toxicity for human endothelia suggesting that the toxin is a component of the outer membrane. Purified neisserial LPS was shown to possess toxicity for human endothelia and in further studies, polymyxin B, an inhibitor of the biological actions of LPS (including the toxicity of gonococcal LPS for human epithelial cells (5)), was used to investigate the possible role of LPS in toxicity. It was found that polymyxin B had a significant inhibitory effect on the toxicity of purified LPS, meningococcal OMV and live meningococci, suggesting that LPS is the major toxic factor (table).

**The relationship between pilus-mediated adherence and toxicity**: Previous results showed that whilst pili are not necessary for toxicity, piliated bacteria exerted a toxic effect more rapidly than nonpiliated bacteria (6). It was suggested that this could be due to their increased adherence to endothelial cells, thus providing a means of delivering toxin to the cell surface. The contribution of pili to toxicity was assessed using piliated variants of strain
Effect of polymyxin B on toxicity of meningococcal OMV for human endothelial cells

<table>
<thead>
<tr>
<th>Dose OMV (μg) protein</th>
<th>% Toxicity</th>
<th>% Toxicity with 10 μg/ml PMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>40.2</td>
<td>8.3</td>
</tr>
<tr>
<td>0.5</td>
<td>13.7</td>
<td>-6.9</td>
</tr>
<tr>
<td>0.1</td>
<td>1.3</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

C311 which displayed different degrees of adherence to various endothelial and epithelial cells. A piliated variant which displayed a high degree of adherence to human endothelial and epithelial cells exhibited the highest level of toxicity for these cells, whilst the parental piliated variant and the nonpiliated variant displayed lower levels of toxicity corresponding to their relative levels of adherence to these cells. The piliated variants did not display increased adherence to endothelia of non-human origin (7) and did not cause increased damage to these cells. Thus the degree of toxicity reflected the relative level of adherence of the piliated variants to the different cell types.

Bacteria expressing the Opc protein were also used to study the relationship between adherence and toxic damage. In the presence of diminished capsule expression, the Opc protein was associated with increased adherence to endothelial cells in strain C751 (8), however when the LPS of capsule-deficient, Opc-expressing bacteria was sialylated this adherence was reduced (9). The adherence of an Opc-expressing variant with native C751 LPS is therefore greater than that of a sialylated Opc-expressing variant and that of a variant which lacks the Opc protein. It was found that there were no significant differences between these variants with respect to the degree of damage caused to human endothelial cells. In addition data showed that damage is mediated to a similar extent whether or not LPS is sialylated. These data show that pilus-dependent but not Opc-dependent adherence causes an increase in toxicity, suggesting that pili have an effect on host cells, in addition to mediating adherence, which contributes to the overall damage.

References


Quantitative analysis of the interaction of Neisseria meningitidis with human nasal epithelium in vitro

R.C. Read¹, A.J. Fox², K. Miller³, T. Gray⁴, R. Borrow², D.M. Jones², N. Jones⁵ and R.G. Finch¹

Depts of Microbial Diseases¹ and Pathology³, City Hospital, Depts of Pathology⁴ and Surgery⁵, University Hospital, University of Nottingham and Manchester Public Health Laboratory, Withington Hospital, Manchester², UK.

The interaction of Neisseria meningitidis with the epithelial surface of the nasopharynx may be a critical step in establishment of nasopharyngeal colonisation. We quantitatively studied this interaction by infecting explants of nasal turbinate mucosa with two wild meningococcal strains: a fully capsulate blood culture isolate, and an epidemiologically related nasopharyngeal isolate which is non-capsulate but otherwise phenotypically identical. After 4h of infection, infected explants displayed increased discharge of mucus from goblet cells and morphological change of epithelial cells from columnar towards cuboidal (n=7, P<0.05). After 24h of infection there was significant cytotoxicity of infected explants compared with controls on morphometric analysis (n=9, P<0.05). Both strains adhered to surface non ciliated mucin secreting cells after 4h of infection and to all cell types after 24h of infection, when there was maximum cellular disruption. By 24h there was penetration between cells to localise intercellularly with basal cells but no organisms were identified within or below the basement membrane. There was no quantitative difference between the two strains with regard to these features. Endocytosis into surface non ciliated cells occurred only with the capsulate strain but was rare (in 2/9 explants infected for 24h); such cells contained huge coalescing vesicles but retained normal structure otherwise. Phagocytosis by polymorphonuclear leucocytes was observed only with the non capsule strain but was also rare (in 2/9 explants infected for 24h).

Mechanisms by which meningococci might achieve stable colonisation of ciliated nasopharyngeal mucosa include toxin epithelial damage, surface cell adhesion and penetration between disrupted epithelial cells. The presence of capsule may be permissive to further mechanisms, including formation of large aggregates of surface associated organisms and replication to large numbers within epithelial cells.
Inhibitory effects of saliva on binding of meningococci to epithelial cells

A.A. Zorgani¹, J. Stewart¹, C.C. Blackwell¹, R.A. Elton² and D.M. Weir¹

Departments of ¹Medical Microbiology and ²Public Health Sciences, University of Edinburgh, Scotland.

Introduction: Individuals who are non-secretors of their ABO blood group antigens are over-represented among patients with invasive meningococcal disease (4). During an outbreak of meningococcal disease in a secondary school, carriage of the causative strain of Neisseria meningitidis (B:4:P1.15) was associated with the non-secretor phenotype (5). Previous analysis of serum and secretory antibodies obtained from these children found that non-secretors had significantly lower levels of anti-meningococcal salivary IgM (8). In this study we used flow cytometry to assess the effects of salivary components on binding of the outbreak strain to buccal epithelial cells.

Subjects and methods: In addition to the outbreak strain, Neisseria lactamica and the following meningococcal isolates were used in these studies: NG:4:-;C:4:-.

Two pools of saliva, one from secretors (n=120) and one from non-secretors (n=120) were prepared from material collected in the survey following the school outbreak (5). Each pool was divided into 4 aliquots: one was not treated; one was absorbed with the meningococcal strains used in the experiments; one was absorbed with meningococci then filtered to remove any bacterial debris; one was used for preparation of affinity purified IgA and IgM.

Binding of the bacteria to buccal epithelial cells (BEC) from healthy donors was assessed by flow cytometry with fluorescein-labelled bacteria by the method described previously (7). The quantitative ELISA method described previously was used to measure total IgA and IgM and each of these isotypes specific for meningococci (8). Fresh saliva was obtained from each of the donors and used in the inhibitory studies in comparison with the pools.

The results were analyzed by the Student’s t-test to calculate confidence intervals.

Results: N. lactamica and the non-capsulate strain bound in greater numbers to the cells compared with the two capsulate strains.
Binding of the outbreak strain to BEC was significantly inhibited by either fresh saliva (P = 0.0003) or the pooled material which had been stored at -20°C (P=0.0006). The inhibitory effect was greater for pooled (P = 0.49) or fresh (P = 0.0001) saliva from secretor donors compared with results obtained with non-secretor saliva.

Although no antibody activity was detected in the absorbed pools, there was still significant inhibitory activity. Following filtration to remove any bacterial debris, the inhibitory effect was less marked, but the effect was still significant for both secretor (P = 0.018) and non-secretor (P = 0.005) saliva preparations.

Purified IgA (P =0.02) and IgM (P = 0.03) were equally inhibitory. A panel of saliva from which either IgA, IgG or IgM had been absorbed retained significant inhibitory activity.

**Discussion:** Colonization of mucosal surfaces by bacteria is partly determined by the capacity of the host to block attachment of adhesins on the microorganism that bind specific receptors on epithelial cells. In addition to immunoglobulins, primarily secretory IgA and IgM, there are high molecular mass glycoconjugates present on mucosal surfaces that prevent interactions between bacterial adhesins and their receptors (3,6).

These studies were initiated to examine the differences observed previously; there were higher levels of salivary IgM specific for neisseriae in specimens obtained from secretors compared with non-secretors in the school population. The results of the present study suggest that the higher levels of IgM in saliva of secretors cannot account for the greater inhibitory activity. Depletion of the antibodies by absorption with meningococci significantly reduced the inhibitory activity; however, even in the absence of detectable antibodies, the saliva was still inhibitory compared with the controls. There was no difference in the inhibitory activity associated with purified IgA compared with IgM. It is increasingly accepted that salivary IgA might play a minor role in saliva-mediated aggregation of bacteria (1,2).

**References**


Virus infection and meningococcal disease

M.W. Raza¹, M.M. Ogilvie¹, C.C. Blackwell¹, R.A. Elton² and D.M. Weir¹

Departments of ¹Medical Microbiology and ²Public Health Sciences, University of Edinburgh, Edinburgh, Scotland.

Introduction: Coincident upper respiratory tract infections with viruses or mycoplasma have been reported to be risk factors for epidemic disease due to serogroup A Neisseria meningitidis (6), and there is recent evidence for an increase in meningococcal disease following epidemics of influenza A (2). One of the ways in which viral infections are thought to contribute to susceptibility to invasive bacterial disease is enhancement of colonization on mucosal surfaces. By flow cytometry, we have demonstrated that compared with uninfected HEp-2 cells, respiratory syncytial virus (RSV) infected HEp-2 cells bind significantly greater numbers of Neisseria meningitidis, irrespective of serogroup, serotype or subtype of the bacteria tested (7). Two hypotheses were proposed to explain the enhanced binding: 1) glycoproteins F (fusion) or G (attachment) coded for by the virus and expressed on the surface of the infected cell can act as new receptors for the bacteria; 2) RSV infection upregulates host surface antigens that can be used as receptors by the bacteria.

Materials and methods: Three strains of meningococci isolated from patients with meningitis were examined: C:2b:P1.2; P:2b:P1.10; B:2b:P1.10.

The flow cytometry methods for measuring binding of fluorescein-labelled bacteria to HEp-2 cells and RSV-infected HEp-2 cells (7) and detection of cell surface antigens with monoclonal antibodies (8) have been described previously. Monoclonal antibodies to the following antigens were used in this study: the RSV-encoded glycoprotein G (Prof. P. Watt, Southampton University); CD14 (Scottish Antibody Production Unit, SAPU); CD15 (Lewis³) (Serotec and SAPU); CD11a (Serotec); CD11b (Serotec); CD11c (Serotec); CD18 (Serotec); and CD29 (Serotec). Since CD14 is a lipopolysaccharide (LPS) receptor, binding of sheep red blood cells coated with endotoxin purified from Salmonella typhimurium or meningococci to Hep-2 cells in monolayers and inhibition of binding by anti-CD14 was analyzed.

Results: There is evidence to support both hypotheses. Enhanced binding of the bacteria appears to be associated with the G glycoprotein but not the F glycoprotein. Infection of Hep-2 cells with RSV enhanced expression of CD11a, CD14, CD15, CD18 and CD29. CD11b and CD11c were not detected on either uninfected or virus-infected cells.
We have demonstrated that several strains of meningococci have surface antigens that bind to Lewis\(^a\) and the structurally related Lewis\(^x\) (CD15) cell surface antigens (3,9). Although enhanced expression of CD15 antigens has been detected on RSV-infected cells, monoclonal anti-CD15 did not significantly alter the bacterial binding. Binding was significantly inhibited by prior treatment of uninfected and RSV-infected HEP-2 cells with anti-CD18 \((P < 0.05)\) but not with antiCD-14 or anti-CD29. For RSV-infected cells, there was significant inhibition of bacterial binding following treatment of the cells with anti-CD14 \((P < 0.001)\) and anti-CD18 \((P <0.01)\) but not with anti CD29. Pretreatment of the Hep-2 cells with anti-CD14 also inhibited binding of sheep red cells coated with LPS purified from \textit{S. typhimurium} or meningococci.

**Discussion:** Despite laboratory evidence that RSV-infected cells bind more meningococci, virus infection in general does not enhance colonization. In surveys of Greek and Scottish school children and Greek military recruits (in both summer and winter), we have not found an association between symptoms of upper respiratory infection and isolation of meningococci (1,4,5). The effect of viral infection might not be apparent among these older children and young adults if secretory antibodies to meningococcal surface components play an important role in prevention of colonization. Longitudinal surveys of infants are presently underway to determine if viral infection is associated with carriage of meningococci or \textit{Neisseria lactamica} in a population in which levels of antibodies specific for neisseriae should be low.

The appearance of glycoprotein G on the surface of RSV-infected cells along with enhanced expression of host cell antigens CD14, CD18 and CD29 to which meningococci bind might be responsible for the increased binding of these bacteria to RSV infected cells (7). The CD18 antigen is associated with integrin molecules involved in recognition between phagocytic and endothelial cells; and we are not aware of other reports of these on epithelial cell lines. In addition, these studies provide further examples of bacteria “hijacking” host cell recognition molecules for colonization of mucosal surfaces.

**References**


Exposure to cigarette smoke and colonization by *Neisseria* species

O.R. El-Ahmer¹, D.A.C. Mackenzie¹, V.S. James¹, C.C. Blackwell, M.W. Raza¹, A.T. Saadi¹, R.A. Elton², M.M. Ogilvie¹ and D.M. Weir¹

Departments of ¹Medical Microbiology and ²Public Health Sciences, University of Edinburgh, Scotland.

**Introduction:** Although there is no direct association between disease due to *Neisseria meningitidis* and active smoking, there is a significant association between cigarette smoking and isolation of meningococci from the nasopharynx which appears to be greater among heavier smokers (1,2,9). There is an increased risk of meningococcal carriage among individuals who live with smokers and also an increased risk of meningococcal disease among children who live with smokers (8). The increased risk of carriage might be due to the greater chance of acquiring meningococci from close contact with smokers and not due to the direct effect of passive exposure to smoke. Studies among Greek school children found a higher proportion of carriers among those from households where the mother or other carer smoked compared with those from non-smoking households. Fathers’ smoking was not associated with isolation of meningococci from the children (4).

In this study we examined the hypothesis that, as we have found for *Staphylococcus aureus* (7), cells from smokers would bind greater numbers of meningococci compared with cells from non-smokers.

**Subjects and methods:** Flow cytometry methods for measuring binding of fluorescein-labelled bacteria to buccal epithelial cells and detection of cell surface antigens with monoclonal antibodies have been described previously (6). Two isolates of meningococci were used in these studies, the B:4:P1.15 strain responsible for an outbreak in a Scottish secondary school (2) and a P:2b:P1.10 strain isolated from a Greek child with meningitis (10). Three strains of *Streptococcus pneumoniae* with serotypes associated with meningitis (F7, 12F, and 18C) provided by Dr. L.E. Smart, Meningococcal and Pneumococcal Reference Laboratory, Glasgow, Scotland were also tested as was a strain of *Haemophilus influenzae* type b.

Cells from smokers and non-smokers were examined for expression of the following antigens we have previously found to be receptors for bacteria (6, Raza et al., these proceedings): Lewis⁺; Lewis⁺⁺; CD14; CD18.

Results for the binding assays were assessed by Wilcoxon’s Rank Sum Test.
**Results**: There was no significant difference in binding of the two meningococcal strains tested to cells obtained from 8 pairs of smokers and non-smokers. For all experiments with pneumococci, cells of smokers (n=8) bound more bacteria than cells from non-smokers (n=6) (P <0.001). Similar results were obtained for *H. influenzae* with cells from 5 smokers and 3 non-smokers (P < 0.05).

There was no difference in detection of Lewis\(^a\) or Lewis\(^x\) antigens on cells of smokers and non-smokers. The amounts of CD14 and CD18 detected were insignificant and not subjected to analysis.

**Discussion**: Although cells from smokers bound significantly more staphylococci (7), haemophilus and pneumococci that those of non-smokers, this pattern was not observed for meningococci. This indicates that although smokers are more likely to be colonized, density of colonization might not differ from that of non-smokers. The increased binding of staphylococci, haemophilus and pneumococci to cells of smokers cannot be explained by differences in expression of the Lewis antigens to which many strains of staphylococci and haemophilus bind (3,6). Other host antigens such as fibrinogen and fibronectin to which bacteria have been reported to bind are being investigated at present.

Preliminary analysis of the data from a longitudinal survey of healthy infants and their mothers on factors affecting carriage of potentially pathogenic bacteria found there were four pairs from whom meningococci were isolated from either the infant or mother or both and each of the mothers smoked. There was a significant association between isolation of *S. aureus* (P <0.01), *H. influenzae* (P < 0.01) and *N. lactamica* (P < 0.001) from both mother and infant. There were also significant associations between parental smoking and isolation of *S. aureus* (P <0.05), *H. influenzae* (P < 0.01) and *N. lactamica* (P < 0.05) from infants but no association between isolation of these bacteria and socioeconomic group (5). Studies are underway to determine if *N. lactamica* binds in greater numbers to cells of smokers.

**References**


Are monocytes the “Trojan horses” of meningococcal disease?

Department of Medical Microbiology, University of Edinburgh, Edinburgh, Scotland.

Introduction: There is evidence that mononuclear phagocytes can cross the intact blood brain barrier (BBB) (6); and it has been suggested that if bacteria ingested by these cells were not killed, this might be one means by which they could enter the central nervous system (8). In the presence of antibodies to their surface antigens, bacteria are usually phagocytosed via the Fc receptor and killed by the oxidative burst within the monocyte. In non-immune hosts, some microorganisms that are phagocytosed via the CR3 receptor do not switch on the oxidative burst and survive within the monocyte (4). In this study we assessed this “Trojan horse” hypothesis for Neisseria meningitidis. The objectives of the study were: to assess survival of opsonized and unopsonized meningococci within monocytes; to determine to which receptors on the surface of monocytes unopsonized meningococci bound (e.g. Lewisα, Lewisx, CR3); to compare the oxidative burst in monocytes exposed to opsonized and unopsonized meningococci.

Materials and methods: The method of Leijh et al. (5) was used to assess freshly isolated peripheral blood monocytes and the human monocytic leukaemia cell line (THP-1) for their abilities to kill the following strains of meningococci: P:2b,P1.10; B:2b,P1.10. The former has an adhesin that binds Lewisα detected by agglutination with an anti-idiotypic reagent produced by immunization of mice with monoclonal antibody to the Lewisα antigen. The latter strain was not agglutinated by the reagent (1).

Binding of meningococcal strains to monocytes was assessed by flow cytometry by the method adapted from studies of bacterial binding to epithelial cells (7). Cell surface antigens were detected by flow cytometry using monoclonal antibodies (anti-Lewisα, anti-Lewisx, anti-CD11b) (Scottish Antibody Production Unit and Serotec) and fluorescein isothiocyanate-labelled antibodies to the monoclonal reagents (Sigma) (7). The oxidative burst was measured by flow cytometry with the commercially available “Burst Test” kit (Orpegen) and also by freshly prepared dihydrorhodamine (Molecular Probes). Four strains of meningococci either unopsonized or opsonized with pooled sera or a 1/1000 dilution of convalescent serum from a patient who had meningitis due to a B:2b:P1.10 strain were used to assess the oxidative burst: P:2b:P1.10; B:2b:P1.10; B:4:P1.15; NG:15:NT.
Results: Meningococci opsonized with pooled human serum were phagocytosed and killed by both freshly isolated human monocytes and the THP-1 cells. Unopsonized bacteria were phagocytosed; however, they survived and multiplied within both types of cells up to 5 hr after ingestion. Binding of meningococci to monocytes could be inhibited by pre-treatment of the cells with monoclonal anti-Lewis\(^a\), anti-Lewis\(^x\) (CD15) and anti-CD11b which binds to the CR3 receptor. Inhibition of binding with anti-Lewis\(^a\) or anti-Lewis\(^x\) was much greater for the strain which was agglutinable by the anti-idiotype reagent. Stimulation of the peripheral blood monocytes with endotoxin enhanced expression of Lewis\(^a\) and CD11b; and stimulated cells bound nearly twice the number of bacteria compared with resting cells. Compared with the oxidative burst measured with unopsonized bacteria, the results for opsonized bacteria were greater in 7/9 experiments with P:2b:P1.10, 9/9 with B:2b:P1.10, 4/10 with B:4:P1.15, and 8/10 with NG:15:NT.

Discussion: The results indicate that bacteria which bind to and are ingested by monocyte receptors such as Lewis\(^a\), Lewis\(^x\) or CR3 are able to survive for significant periods of time in the protected intracellular environment. The role of opsonizing antibodies in protection against invasive meningococcal disease has not been examined as extensively as that of bactericidal antibodies. This is probably because the methods for analysis of phagocytosis and measurement of the oxidative burst are less easily standardized. Serum opsonins are low in early stages of meningococcal disease but increase rapidly (3). The broad range of opsonins suggest they are directed against targets unrelated to serogroup or serotype (2), an observation that complements our studies on the distribution of adhesins that bind Lewis\(^a\) on meningococci (1). It has been suggested that opsonizing antibodies are present at lower concentrations than bactericidal antibodies; and, these opsonins might be responsible for protection of the majority of individuals from invasive disease when exposed to an epidemic strain. If the “Trojan horse” effect is an important means by which the bacteria survive in the blood stream or cross the blood brain barrier, induction of opsonins to components that bind these bacteria to the CR3 receptor or Lewis antigens on monocytes in non-immune individuals might be of considerable protective value.

References


Neisserial porins induce upregulation of B cell co-stimulatory ligand, B7-2:
Possible mechanism behind their adjuvant activity

L.M. Wetzler

The Maxwell Finland Laboratory for Infectious Diseases, Boston City Hospital, Boston University School of Medicine, Boston, MA, 02118, USA.

The neisserial porins, protein IA (PIA) and protein IB (PIB) from the gonococcus and class 1, 2 or 3 from the meningococcus are the major protein constituent of the outer membrane and function as porins (2). These proteins have been demonstrated to be immunogenic without the addition of adjuvants (9). In addition, they have been used to augment the immune response to other poorly immunogenic substances, i.e. meningococcal capsular polysaccharide (8), *H. influenzae* PRPP capsule (3), human gangliosides (5), malarial peptides(6), etc. Furthermore, the porins have been shown to induce antibodies to these poorly immunogenic substances in a T cell dependent manner (3,5,8) as demonstrated by an apparent booster response and immunologic memory. This suggests that it is possible that the porins could be recruiting or activating T lymphocytes in order to induce a T cell dependent antibody response towards normally T cell independent antigens. To explore this hypothesis, the ability of the neisserial porins to affect T-B lymphocyte interactions and T lymphocyte co-stimulation were investigated. T lymphocyte co-stimulation is necessary for T lymphocyte activation and requires two sets of signals between the antigen presenting cells or B lymphocytes and the T lymphocytes. In current T lymphocyte co-stimulation models (7), signal 1 is mediated by the interaction of the Major Histocompatibility Complex (MHC) with a peptide (T cell epitope) occupying its major groove on the antigen presenting cell and the specific T cell receptor. In experimental studies signal 1 is successfully mimicked by CD3 crosslinking. The co-stimulatory signal, signal 2, is mediated by the interaction of the co-stimulatory ligands, B7-1 or B7-2 (4), expressed only on activated B lymphocytes, and their counter-receptors on T lymphocytes, CD28 and possibly CTLA-4. Signal 2 is necessary for T cell activation, otherwise T cell hypo-responsiveness can be induced (7).

Murine B lymphocytes were isolated from lipopolysaccharide (LPS) non-responsive mice (strain C3H/HeJ) (1). Neisserial porins, PIA, PIB, class 1 and class 3, were purified from strains lacking the reduction modifiable proteins, protein III or class 4, and in the case of class 1, also lacking the class 3 protein and in the case of class 3, also lacking the class 1 protein (9). These porins were formed into proteosomes (purified protein micelles) to remove all detergent (9) which otherwise would adversely affect the lymphocytes. The isolated B lymphocytes were incubated with...
various concentrations of the porin proteosomes (0.1-50 \( \mu \) g/ml). Incubation with Dextran/LPS was used as a positive control and incubation with plain cell culture media or LPS alone was used as a negative control. After 2 days of incubation, the surface expression of B7-1, B7-2 and Class II MHC was measured by flow cytometric analysis. B7-1 expression increased equally on B lymphocytes incubated with the neisserial porins, Dextran/LPS or media, as compared to B7-1 expression on freshly isolated B lymphocytes. However, B7-2 expression was much greater on B lymphocytes incubated with the neisserial porins or dextran/LPS as compared to B7-2 expression on B lymphocytes incubated with plain media or freshly isolated B lymphocytes. The same pattern was noted for the expression of class II MHC, consistent with the activation of the B lymphocytes. Increased B7-2 surface expression was detected on B lymphocytes incubated with concentrations PIB as low as 1-2 \( \mu \) g/ml.

To measure the B lymphocyte co-stimulatory ability, B lymphocytes incubated with the neisserial porins, Dextran/LPS, media or LPS alone were fixed with paraformaldehyde and incubated with CD3 crosslinked T lymphocytes isolated from murine strain C3H/HeJ. After 3 days, the cell cultures were pulsed with tritiated thymidine. T lymphocyte activation was measured by quantitating the uptake of the tritiated thymidine after an additional 18h incubation. T lymphocytes were activated (co-stimulated) by the B lymphocytes incubated with the neisserial porins and Dextran/LPS, but not by B lymphocytes incubated with media or LPS. In addition, B lymphocytes incubated with concentrations of PIB as low as 1-2 \( \mu \) g/ml were able to co-stimulate T lymphocytes. These data demonstrates that neisserial porins can induce T lymphocyte co-stimulatory ability on B lymphocytes. This is most likely due to the increased surface expression of B7-2, and is not mediated by LPS. It is possible that the mechanism of Neisserial porin immuno-stimulation and adjuvant activity is related to this ability to induce upregulation of B7-2 and subsequent B lymphocyte mediated T cell co-stimulation.

References


Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression and phagocytosis, but prime neutrophils to increase their oxidative burst

R. Bjerknes¹, H-K. Guttormsen², C.O. Solberg² and L.M. Wetzler³

¹Department of Pediatrics and ²Medical Department B, Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway. ³The Maxwell Finland Laboratory for Infectious Diseases, Boston City Hospital, Boston University School of Medicine, Boston, MA, 02118, USA.

Neutrophils and serum opsonic antibodies are important for the effective host defense against infections with N. meningitidis (9) and N. gonorrhoeae (8). A group of the neisserial major outer membrane proteins, Protein IA (PIA) and IB (PIB) of the gonococcus and class 1, 2 and 3 proteins of the meningococcus constitute the majority of the surface proteins of these organisms and function as porins (3,6). They are capable of inserting into the membranes of eukaryotic cells (6). To explore the possible effect of these porins on neisserial pathogenesis, the in vitro effects on neutrophil function of the Class 1 and 3 proteins from N. meningitidis and Protein IB (PIB) from N. gonorrhoeae were investigated. Isolated human neutrophils were incubated with purified neisserial porins and the various parameters of neutrophil function were measured by flow cytometric analysis. These parameters included actin polymerization, primary and secondary granule release, Fc receptor expression, complement receptor expression, leucocyte oxidative burst and phagocytosis of opsonized meningococci. Negative controls included incubating the neutrophils with plain PBS and PBS with 0.001% Empigen BB, equivalent to the concentration of Empigen BB in the porin preparations.

Incubation with the porins inhibited human neutrophil FMLP-induced actin polymerization in a concentration dependent manner (2). In addition, degranulation (as measured by chemoattractant-stimulated upregulation of granule associated molecules) of both primary (marker: CD63) and secondary (marker: CD11b) granules was inhibited by incubation with the porins (1). The neutrophil expression of Fc(RII (CDw32) and Fc(RIII (CD16), as well as the activation-dependent downregulation of Fc(RIII, were reduced by incubation with the porins. Incubation with the porins impaired the upregulation of complement receptors 1 (CD35) and 3 (CD11b) in response to FMLP. To determine if these alterations might affect the anti-meningococcal opsonophagocytic function of neutrophils, neutrophils were incubated with the porins and the uptake of meningococci (strain 44/76) in the presence of patient serum containing known amounts of IgG against meningococcal

290
porins was measured by flow cytometric analysis (5). Following incubation with the porins, mean number of meningococci per phagocyte dropped 40-50% as compared to the phagocytic capacity of neutrophils untreated with the porins. Interestingly, the porins primed neutrophils to increase their H2O2 production in response in response to FMLP, whereas no such priming was observed if the neutrophil protein-kinase C was stimulated directly with PMA (2). Even though there were differences between the functional parameters tested, the meningococcal class 1 protein and PIB tended to alter neutrophil functions more than the meningococcal class 3 protein. All changes in neutrophil function mentioned above were found to be significant by the students t-test (p < 0.05).

Lipopolysaccharide (LPS) can be a minor contaminant of any bacterial protein preparation, however LPS contamination of these porin preparations have been shown to be less than 0.01% by silver staining of SDS-PAGE gels. Despite this, saturating amounts of a monoclonal antibody (mAb) to the LPS ligand on neutrophils, CD-14, was added as a control in the above mentioned experiments (except for the phagocytic assay) (7). This mAb inhibited LPS mediated effects on the neutrophil but did not alter any of the porin induced effects. LPS increases the FMLP induced upregulation of CD11b on neutrophils (10) and increases neutrophil actin polymerization and phagocytosis (4). In our these experiments, the neisserial porins decreased the FMLP induced upregulation of CD11b and decreased neutrophil actin polymerization and phagocytosis. Therefore, we believe that the neisserial porins effect on neutrophils is not due to LPS contamination of the protein preparations.

Thus, the neisserial porins inhibited neutrophil actin polymerization, degranulation, opsonin receptor expression and phagocytosis, but primed the neutrophils to increase their oxidative burst, and that these effects are not due to LPS contamination. It is possible that these in vitro observations reflect a neisserial anti-neutrophil strategy which may influence the virulence of the bacteria and impair the protective capacity of neutrophils against _N. meningitidis_ and _N. gonorrhoeae_.

References


The effects of pro- and anti-inflammatory cytokines on the neutrophil oxidative burst response to pathogenic and nonpathogenic strains of *Neisseria meningitidis*

P. Kragsbjerg\(^1\) and H. Fredlund\(^2\)

The Departments of Infectious Diseases\(^1\) and Clinical Microbiology and Immunology\(^2\), Örebro Medical Center Hospital, Örebro, Sweden.

**Introduction:** Cytokines are one group of soluble mediators that have been shown to modulate PMNL function in experimental models (1). In previous studies, the proinflammatory cytokines TNF-\(\alpha\), IFN-\(\gamma\), and G-CSF have been shown to increase the oxidative burst in PMNL after stimulation with bacterial as well as nonbacterial stimulants (2-6). The concentrations and kinetics of inflammatory cytokines in serum and cerebrospinal fluid (CSF) from patients with invasive meningococcal disease (MCd) have been studied and high levels of TNF-\(\alpha\), IL-6, and IL-8 have been correlated to fatal outcome of the patients (7-9). It is therefore of interest to study the effects of these cytokines on the PMNL functions when stimulated with different strains of meningococci.

**Materials and methods:** A chemiluminescence (CL) procedure (10) was used to study the effects of human recombinant TNF-\(\alpha\), IL-8, IL-6, IFN-\(\gamma\), LIF, IL-10, G-CSF, and IL-1\(\beta\) on the PMNL oxidative burst response to the meningococcal strains of the serogroups B and 29E (MCB and MC29E). For each cytokine the effects of increasing concentrations were studied. The effects of some of these cytokines were also studied by phagocytic killing experiments (10).

**Results:** With the proinflammatory cytokines TNF-\(\alpha\), IFN-\(\gamma\), and G-CSF a dose response effect on the CL responses was demonstrated. TNF-alpha, IFN-gamma, and G-CSF increased the CL response more when the PMNL were stimulated with MCB compared to MC29E strains. With IL-8 there was a similar increase in the CL response for both the MCB and the MC29E strains. There was no significant effect on the CL response with IL-6, LIF, or IL-10, but with IL-1\(\beta\) a decrease of the CL response was found. Phagocytic killing experiments showed an increased killing of the MCB strain with the highest concentration of TNF-\(\alpha\).

**Discussion and conclusions:** In this *in vitro* model TNF-\(\alpha\), IFN-\(\gamma\), IL-8 and G-CSF increased the PMNL oxidative burst response to the pathogenic meningococcal serogroup B strain. This is in accordance with previous studies in which the effects of these cytokines were studied on PMNL's stimulated with other
pathogens or stimuli (2, 4-6). TNF-α increased the PMNL oxidative burst response to the nonpathogenic meningococcal serogroup 29E strain but to a lesser extent. No change in the CL response was seen with IL-6, IL-10, or LIF. In appropriate concentrations the proinflammatory cytokines TNF-α, IFN-γ, IL-8, and G-CSF may have a positive influence on the eradication of meningococci by PMNL in meningococcal disease.

References
The pattern of early neutrophil and platelet activation in an *ex-vivo* model of meningococcal bacteraemia

N.J. Klein¹, R.S. Heyderman², C.A. Ison³, M. Peakman⁴ and M. Levin²

Molecular Immunology Unit, Institute of Child Health, London, UK¹. Paediatric Infectious Diseases Unit² and Department of Microbiology³, St Mary's Hospital Medical School, London, UK. Immunology Dept, KCHMS, London, UK⁴.

*Neisseria meningitidis* colonises the mucosa of the upper respiratory tract in up to 25% of healthy individuals and in a small proportion, particularly in children, colonisation is followed by invasion. This frequently leads to clinical disease, the most common forms of which are meningitis and overwhelming sepsis. Despite the availability of antibiotics, the mortality from this infection remains at 10%, rising to 40% in patients presenting in severe shock. At the present time, it is unclear why some patients develop fulminant sepsis whilst others develop less severe forms of the disease. However it seems likely that the nature of the specific interactions which occur between host defences and the invading pathogen plays a major part in determining the spectrum and severity of clinical disease.

Platelet consumption and a low circulating neutrophil count are widely regarded as important poor prognostic indicators in the assessment of meningococcal sepsis. These changes are thought to reflect the complex interrelationship between the haemostatic imbalance and the more widespread inflammatory process central to the pathogenesis of the disease. The aim of this study was to identify the early surface and secreted markers of neutrophil and platelet activation that determine this interaction following invasion of the meningococcus into the circulation.

Using an *ex-vivo* model of meningococcal bacteraemia, marked changes in neutrophil expression of CD11b, L-selectin, and release of elastase were observed after as little as 30 minutes in citrated whole blood inoculated with type strains of *N. meningitidis*. In contrast, in the context of either viable or rapidly dying organisms, and evidence of neutrophil degranulation, enhanced platelet activation was not detected by either the expression of platelet surface glycoprotein GPIb, activated GP IIb/IIIa, P-selectin or b-thromboglobulin release.

The degree of neutrophil activation observed after exposure to *N. meningitidis* did not appear to be directly related to bacterial killing, phagocytosis or TNF release. Furthermore, neither neutrophil activation or bacterial killing were sufficient to trigger platelet activation in this system. The host's inflammatory reaction to *N. meningitidis*
is thought to be a major determinant of disease severity. The results from this study provide insight into the host/bacterial mechanisms which may influence the outcome of patients with meningococcal disease.

References
Assessment of candidate anti-inflammatory treatments in an *in vitro* whole blood model of meningococcal sepsis

B. Chan, P. Kalabalikis, N. Klein, R. Heyderman and M. Levin

Academic Department of Paediatrics, QEQM Wing, St. Mary's Hospital Medical School, South Wharf Road, London W2 1NY.

Meningococcal septicaemia remains an important cause of mortality and morbidity in both adults and children. Despite the availability of an increasing array of antibiotics and advances in intensive care, the mortality from meningococcal disease remains at 10% overall, increasing to 40-60% in patients presenting with meningococcal septic shock (6). In the past decade, evidence has accumulated that the severity of the disease is related to the plasma levels of endotoxin which in turn are related to the intensity of the host inflammatory response. Levels of cytokines such as TNF" as well as activation of complement and the coagulation pathways correlate with disease severity. It is unlikely that improved prognosis can occur with more effective antibiotic therapy. A major focus of current research is to develop other modalities of treatment aimed at reducing the host inflammatory response and the resultant tissue and organ damage initiated by the bacteria.

While there are a wide range of possible anti-inflammatory therapies which might be of benefit in children with meningococcal disease, a major obstacle to the introduction of such agents into clinical use, and to the choice of the appropriate agent is the current lack of any *in vitro* or animal model of the disease which may accurately reflect the clinical situation. In order to study the early inflammatory events that occur on entry of the bacteria into the blood stream, we have devised an *in vitro* whole blood model which we suggest may mimic the *in vivo* clinical situation and have used it to assess candidate anti-inflammatory treatments (1).

Using elastase-" α1-antitrypsin (elastase-" α1-AT) (4) and TNF" (5,7) production as markers of neutrophil and macrophage activation respectively, known concentrations of heat and penicillin killed meningococci were incubated with whole blood and the time course of activation was determined. Plasma levels of elastase-" α1-AT (2) and TNF" (1) were measured by enzyme-linked immunosorbent assay (ELISA) technique and were found to increase in a dose-dependant manner in response to the bacteria. Elastase-" α1-AT was detected early on with most release occurring between 15-30 min whereas TNF" was detected later, between 120-180 min. The early release of elastase suggests that neutrophil activation is rapid and that any intervention designed to prevent their activation would have to be undertaken very early in the
inflammatory process to be effective. The model was used to evaluate the effect of candidate anti-inflammatory agents in reducing neutrophil and macrophage activation. We studied the effect of dexamethasone, a corticosteroid which has been shown to be a potent inhibitor of cytokine biosynthesis in LPS stimulated whole blood in vitro; prostacyclin, a strong physiological inhibitor of platelet aggregation, and pentoxiphylline, a methylxanthine which has been shown to inhibit neutrophil and macrophage function. These pharmacological inhibitors were chosen for the study as they are readily available for clinical use. We also investigated the effect of HA-1A, a human IgM anti-lipid A monoclonal antibody, which is currently undergoing trials in children with meningococcal sepsis (3).

Dexamethasone, prostacyclin and pentoxiphylline added to whole blood 5 min after the addition of meningococci caused a significant (p<0.01) dose dependant inhibition of TNF\(^\text{a}\) release but had no effect on elastase release. HA-1A had no significant effect on either TNF\(^\text{a}\) or elastase release. We suggest that our model may be useful in selecting candidate anti-inflammatory agents for future evaluation in clinical trials.

References


Passage of *Neisseria meningitidis* through a tissue culture bilayer mode

K.A. Birkness\(^1\), B.L. Swisher\(^2\), E.G. Long\(^1\), E.H. White\(^3\), E.P. Ewing, Jr.\(^2\) and F.D. Quinn\(^1\)

\(^1\)Pathogenesis Laboratory, Division of Bacterial and Mycotic Diseases and \(^2\)Scientific Resources Program, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333, USA.

Meningococcal disease continues to be a serious world health problem having caused recent epidemics in South America, Africa and the Middle East and remaining a principal cause of morbidity and mortality in young children in developing countries where the disease is endemic (1). Pharyngeal carriage of *Neisseria meningitidis* is common, but the mechanism by which the organism penetrates the mucosal surface and enters the blood stream is still largely unknown. Several animal models have been used to study aspects of meningococcal pathogenesis including monkeys, chicken embryos, mice and guinea pigs. While these models have been useful in answering many experimental questions, the fact that *Neisseria meningitidis* is an exclusively human pathogen limits the relevance of animal models in the study of its pathogenesis.

The nasopharyngeal organ culture system developed by Stephens *et al.* (2) permits study of the interaction between bacteria and the intact mucosal surface as it would occur in a natural infection. However, these tissues are difficult to obtain, are variable from donor to donor, require the initial use of antibiotics, and have limited viability. Human buccal epithelial cells, used by many researchers to study attachment of meningococci, are readily available, but vary greatly in age, size and viability. Mammalian tissue culture monolayers have also been used extensively in the study of neisserial pathogenesis. Studies using HeLa human cervical carcinoma cells, HeclB human endometrial carcinoma cells, Chang conjunctival cells, HEP-2 larynx carcinoma cells and human umbilical vein endothelial cells have added much to current knowledge about meningococcal attachment and invasion. However, when infecting the human host, the bacterium is required to react with more than a monolayer of cells.

We have recently developed an artificial tissue system incorporating epithelial and endothelial monolayers on a microporous membrane to examine the process of attachment and passage which must occur as the meningococcus makes its way from the mucosal surface through the epithelial cells and into the vascular system. The model allows us to observe and quantify the passage of bacteria through the multiple layers and to study the mechanisms by which the bacteria make this passage.
Layering of one cell type over another allows the cells to communicate and interact as they might in vivo. In preliminary studies we examined attachment and invasion by meningococci using several epithelial cell monolayers including HeclB, HEp-2 and HeLa cells. While the bacteria were found to invade all epithelial cell types, larger numbers of bacteria were found to attach to and invade the HeclB cells. Based on these data we chose to use HeclB cells, an endometrial carcinoma cell line frequently used to study gonococcal attachment and invasion, as the epithelial layer. We chose HMEC-1 cells, a human microvascular endothelial cell line, as the second layer in our model. Using this system we have examined a variety of meningococcal strains including epidemic and sporadic case and carrier isolates, strains with and without pili or capsule and other spontaneous and transposon-induced mutants. We have found marked differences among the strains in terms of their ability to pass through the bilayer which may correlate with differences in virulence. We have also seen microscopic evidence that the bacteria are passing from the apical surface through the layer of epithelial cells and through the membrane to the endothelial layer below without causing damage to the epithelial cells. This resembles what one observes in the human host where extensive tissue damage in the nasopharynx is rare. We have also examined several other genera of bacteria whose interaction with eucaryotic cells has been well characterized; bilayer assay results among strains of Haemophilus influenzae type b, Salmonella typhimurium, Shigella flexneri and Yersinia enterocolitica showed differences but were consistent with previously published invasion and attachment results.

This model is adaptable to a wide variety of microbial pathogens and can be modified by substituting any physiologically relevant eucaryotic cells for the component layers. The system's makeup of cells of human origin and its reproducibility give it advantages over animal and primary organ culture models, while the added complexity of multiple layers allowing cell-to-cell communication makes it a more realistic human tissue model than standard cell monolayers for the study of the attachment and invasion factors contributing to meningococcal pathogenesis.

References
Erythrocytal latex agglutination test and its modification

A.A. Demina, and Yu.V. Martynov

Central Research Institute of Epidemiology, Novogireevskaja, ul., 3a, Moscow 111123, Russia.

The advantages of the latex agglutination (LA) test over other express methods are well known: high sensitivity, stable activity of the preparation, economic efficiency, absence of need to use costly equipment and specially trained personnel. The simplicity of the test and registration of results makes it affordable to use in an admitting office, at any level of patient medical care provision, and also in field conditions. LA is used extensively for the etiological diagnosis of several diseases including meningococcal infection (MI) (1,4). When LA was used for practical application, we faced some difficulties, the elimination of which would have increased the effectiveness of this technique.

The purpose of our investigations are: 1. Improvement of LA technique by using its microvariant in capillaries 2. Elimination of false-positive reactions especially in blood samples 3. Use of blood erythrocytes from patients in LA diagnostics.

1. The quantity of pathological material to be analyzed is sometimes very small, especially as regards children. We developed a very economical method of LA, namely its microvariant in capillaries. For this purpose we used capillaries 10cm long and 0.8cm in diameter. The capillary is filled with the material to 3/5 of its length and to another 1/5 with latex particles (LP) without any air bubbles between them. After swaying the capillary from side to side 5-10 times, it is kept in a horizontal position. The agglutination reaction usually develops within 3 to 5 min and may persist for several days. This method allows one to reduce by half the quantity of pathological material to be studied and the LP's by fourfold (3).

2. Falsely positive results obtained from CSF or blood samples may be due to presence of tissue protein or rheumatoid factors. In these cases the results of the control LA may be negative or doubtful. Preliminary treatment of 0.5ml of the sample was with 0.05ml of trypsin (concentration 1mg/ml in 0.15M NaCl) with shaking for 5min, followed by inactivation of the trypsin with 0.05 ml trypsin-inhibitor with 5min of shaking, and then perform the LA. This procedure destroyed proteins but did not affect the activity of specific polysaccharides.
3. The usual material to be studied is blood serum of a patient while erythrocytes are discarded. The idea to use erythrocytes from patients as material for LA was based on the specificity of the pathogenesis of some clinical forms: meningococcemia, pneumococcal and *Haemophilus* bacteraemia. High adsorbability of the erythrocytal surface led us to the idea that causative agent antigens adsorb on their stroma and can be analyzed using LA. To verify this hypothesis we carried out experiments with erythrocytes from animals (mice) and humans, which confirmed both high adsorbability of erythrocytes and the possibility of using them in LA (2). Testing erythrocytal latex agglutination (ELA) in samples from patients confirmed in principle the possibility to use the erythrocytes from patients to detect antigens using LA. For this purpose blood samples (0.5ml) from patients were centrifuged for 10 min at 1000rpm, supernatant (serum) was removed and studied separately using other reactions. The pellet (erythrocytes) was washed 3 times in 0.15M NaCl. This pellet was used to prepare 5% suspension of erythrocytes in 0.15M NaCl. This suspension is the source for ELA. The ELA findings are analyzed visually. In the case of positive results, conglomerates of latex particles and erythrocytes are somewhat bigger than usual LA, and are slightly rosy in the centre because of erythrocytes. The control and negative results obtained using ELA do not differ from those in conventional LA. The advantage of ELA is not only in the fact that erythrocytes are an additional source for detecting the causative agent, but also is reflected in the absence of non-specific reactions which otherwise could be possible in serum samples.

References


