Capsules and lipopolysaccharides

Chemically modified *Neisseria meningitidis* capsules as vaccines

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Despite the success of vaccines composed of the meningococcal groups A, C, W-135 and Y capsular polysaccharides, they still have serious limitations. First, all the polysaccharides induce an inadequate immune response in infants (1), and second, although group B *N. meningitidis* is responsible for a significant amount of meningococcal meningitis, its capsular polysaccharide is precluded from the above vaccine due to its poor immunogenicity in adults (2). Serological evidence accumulated from animal experiments has indicated that the first limitation can probably be overcome by the covalent coupling of the polysaccharides to protein carriers to form highly protective conjugate vaccines (3). However, this technique alone does not overcome the second limitation of the poor immunogenicity of the group B meningococcal capsular polysaccharide (GBMP), because even when conjugated to tetanus toxoid (TT) it failed to produce a significant response in mice (3). This is probably attributable to structural mimicry between the GBMP and human tissue antigens (4). Antibodies specific for the GBMP, recognise an epitope found only on the polysaccharide when it is in an extended helical conformation in which there are at least ten residues per helical turn (5,6) and the same epitope has also been identified on fetal N-CAM and other human tissue (4).

The failure of the GBMP-TT conjugate to provide satisfactory levels of protective antibody prompted interest in further chemical modification of the GBMP prior to its conjugation. One modification that could be made without disturbing the extended helical epitope was to replace the *N*-acetyl groups of the sialic residues of the GBMP with other N-acyl residues. The modified polysaccharides were then conjugated to tetanus toxoid and the conjugates were used as experimental vaccines in animals. Only the *N*-propionylated (NPr) and *N*-butylated (NBu) conjugates were able to induce protective (bactericidal) antibodies in mice when administered with Freunds complete adjuvant. The NPr-GBMP-TT conjugate produced two distinct populations of antibodies, one which (minor population) cross-reacted with the GBMP. Of significance was fact that the major population of antibodies was only specific for NPr-GBMP and yet contained all the bactericidal activity (7). This together with other serological evidence, indicates that the NPr-GBMP mimics a different epitope on the surface of group B meningococci than is presented by the GBMP (8). Therefore NPr-GBMP-TT is an ideal vaccine candidate, although its success as a vaccine would

depend on whether it would break tolerance, and even if it did, what if any the consequences would be.

The bactericidal and protective activities of murine IgG NPr-GBMP-specific antibodies were evaluated. It was found that IgG_1 was not bactericidal, presumably, due to the fact that it fixes complement poorly, but was still able to passively protect mice challenged with live group B meningococci. The isotypes IgG_{2a} and IgG_{2b} were both bactericidal and passively protective. An isotype switch from IgG_1 to IgG_2 could be achieved by adjuvanting the NPr-GBMP-TT. While alum still induced mainly IgG_1, antibodies, both Freunds complete adjuvant and RIBI adjuvant were able to considerably augment the population of IgG_2 antibodies.

References

1. **Gotschlich, E.C., Goldschneider, I., Lepow, M.L., and Gold, R.** 1977. The immune responses to bacterial polysaccharides in man. In "Antibodies in Human Diagnosis and Therapy" (E. Haber and R.M. Krause, eds.), pp. 391-402. Raven Press, New York.
2. **Wyle, F.A., Artenstein, M.S., Brandt, B.L., Tramont, E.C., Kasper, D.L., Altieri, P.L., Bermian, S.L., and Lowenthal, J.P.** 1972. Immunologic response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. **126:**514-521.
3. **Jennings, H.J., and Lugowski, C.** 1982. Immunochemistry of group A, B and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. **127:**1011-1018.
4. **Troy, F.A.** 1992. Polysialylation from bacteria to brains. Glycobiology **2:**5-23.
5. **Jennings, H.J., Roy, R., and Michon, F.** 1985. Determinant specificities of the groups B and C polysaccharides of *Neisseria meningitidis*. J. Immunol. **134:**2651-2657.
6. **Brisson, J.-R., Baumann, H., Imberty, A., Perez, S., and Jennings, H.J.** 1992. Helical epitope of group B meningococcal "(2-8)-linked sialic acid polysaccharide. Biochemistry **31:**4996-5004.
7. **Jennings, H.J., Roy, R., and Gamian, A.** 1986. Induction of meningococcal group B polysaccharide-specific IgG antibodies in mice by using an N-propionylated B polysaccharide-tetanus toxoid conjugate vaccine. J. Immunol. **137:**1708-1713.
8. **Jennings, H.J., Gamian, A., and Ashton, F.E.** (1987). N-propionylated group B meningococcal polysaccharide mimics a unique epitope on group B *Neisseria meningitidis*. J. Exp. Med. **165:**1207-1211.

Molecular basis and biological significance of encapsulation and capsule modification in meningococci

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Capsule expression of meningococci is directed by a gene cluster located in the chromosomal *cps* locus. The steps for expression of the poly-"2,8 sialic acid capsule of group B meningococci includes first, the synthesis of the polymer by four gene products of a central DNA fragment, termed region A. The biosynthesis steps for poly-"2,8 sialic acid synthesis includes synthesis of ManNAc, presumably by epimerization of GlcNAc, condensation of ManNAc and phosphoenolpyruvate to form NeuNAc, activation of NeuNAc resulting in CMP-NeuNAc and finally polymerization of the activated precursors by the "2,8 polysialyltransferase (1). These enzymatic activities have been assigned to the four gene products encoded by region A. Biosynthesis of the polysaccharide occurs in the cytoplasm. Before being transported to the cell surface the polysaccharide chains are substituted with a phospholipid molecule at the reducing end by two gene products of region B. Phospholipid substitution is a prerequisite for export of the polysaccharide through the inner and outer membrane, which occurs in an ATP-dependent manner by a transporter complex of the ABC-transporter family. These transporter proteins are encoded by genes of region C of the *cps* gene complex (for review see ref. 2). An additional DNA fragment of *cps,* termed region D, constitutes a cassette of genes from the LOS biosynthesis pathway, including *galE,* which synthesis the galactose as a part of the outer core moiety of the LOS molecule. Thus, deletion of *galE* results in expression of a truncated LOS molecule, which cannot be sialylated by the LOS-sialyltransferase (3).

The identification of genes required for capsule and LOS synthesis enabled us to construct isogenic mutants with isolated defects in capsule and/or LOS expression. These mutants were used to analyse the role of cell surface located sialic acids for mediating serum resistance and for their influence on invasion of epithelial cells. Summarising the results of these experiments, we could demonstrate, that the capsule does not mediate resistance to killing by alternative complement pathway activation. Serum resistance is exclusively mediated by LOS sialylation. Furthermore, it became evident, that invasion into epithelial cells is almost completely inhibited by the capsular polysaccharide, but not by the sialylated LOS. Surprisingly, the few cells of the encapsulated wildtype strains, which were able to invade into epithelial cells, were

proven to be capsule deficient derivatives. However, in high frequencies some of the capsule negative clones reverted to a capsule positive phenotype. This is the first evidence for a phase variation in capsule expression. There is preliminary evidence for a posttranscriptional control of capsule expression.

References

1. **Edwards, U., Müller, A., Hammerschmidt, S., and Frosch, M.** 1994. Molecular analysis of the biosynthesis pathway of the "2,8 polysialic acid capsule by *Neisseria meningitidis*. Mol. Microbiol., in press.
2. **Frosch, M., and Edwards, U.** 1993. Molecular mechanisms of capsule expression in *Neisseria meningitidis* serogroup B. In: Polysialic Acid (J. Roth, U. Rutishauser, F.A. Troy II eds.) Basel/Switzerland: Birkhäuser Verlag, pp. 49-57.
3. **Hammerschmidt, S., Birkholz, C., Zähringer, U., Robertson, B.D., van Putten, J., Ebeling, O., and Frosch, M.** 1994. Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol. Microbiol. **11**: 885-896.

Genetic and functional analysis of the lipopolysaccharide of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae synthesizes a rough LPS that does not contain any of the repetitive O-antigen units characteristic of the smooth LPS of the *Enterobacteriaceae*. We have identified genes involved in the biosynthesis of gonococcal LPS and have used these to construct isogenic strains carrying genetically defined null mutations. These mutants have been characterized and have allowed us to examine the role of components of the LPS structure in its biological function.

We have cloned a homologue for the gonococcal *galE* gene (1). Sequence alignments indicate extensive homologies with the homologous *E. coli* and *Salmonella* enzymes. Mutants were constructed which displayed deep rough phenotypes, and these were analyzed immunochemically and biologically. These analyses have allowed us to localize the target epitopes for killing by normal human serum, the acceptor site for sialic acid, and have provided some evidence about the role that LPS structure plays in the ability to invade human epithelial cells *in vitro*.

We have also identified gonococcal homologues for three *rfb* genes from *Salmonella* involved in the synthesis of the dTDP-rhamnose (2). Construction and analysis of gonococcal strains carrying null mutation in these genes showed no observable phenotypic changes with respect to LPS migration, sugar content, sialylation, monoclonal antibody binding, serum sensitivity or the interaction with eukaryotic cells *in vitro*.

References

1. **Robertson, B. D., M. Frosch and J. P. van Putten.** 1993. The role of *galE* in the biosynthesis and function of gonococcal lipopolysaccharide. Mol Microbiol. **8**: 891-901.
2. **Robertson, B. D., M. Frosch and J. P. van Putten**. 1994. The identification and characterization of cryptic rhamnose biosynthesis genes in *Neisseria gonorrhoeae*. submitted.

Isolation of mutants of *Neisseria meningitidis* producing altered LPS

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Mutants of *Neisseria meningitidis* with altered LPS were isolated in the following ways:

1. A novel method for random insertion/deletion mutagenesis in meningococci was devised. This consists of ligating a digest of total chromosomal DNA to a 1.1kb restriction fragment containing an erythromycin-resistance marker (*ermC*), and subsequent transformation of the ligation mixture to the meningococcal strain H44/76. The resulting erythromycin-resistant transformants all carried the *ermC* gene inserted at random positions in the chromosome, as was shown by Southern blotting. A pool of mutants was screened for binding of the mAb 4A8B2, which is specific for immunotype L3. Two independent L3-negative mutants were isolated, which could be distinguished from spontaneous LPS phase variants by their inability to revert to an $L3^+$ phenotype. In transformation experiments with chromosomal DNA from these mutants, erythromycin resistance and lack of 4A8B2 reactivity were always linked, showing that the insertion/deletion was in a locus or loci involved in LPS biosynthesis. Pulsed-field electrophoresis of chromosomal DNA showed that the two mutants mapped to the same *Nhe*I fragment. When the mutant LPS was analysed by SDS-PAGE, an electrophoretic mobility intermediate between previously isolated *galE* and *lsi-1* mutants was observed, suggesting an oligosaccharide structure truncated between the proximal heptose and galactose residues. Comparison of the binding pattern of mAbs specific for LPS confirmed the different nature of the new mutants. The chromosomal DNA flanking the *ermC* gene was cloned from both mutants; characterization of the region in both mutants and the wildtype strain is in progress.

2. Meningococcal strains synthesizing an LPS with reduced toxicity would be highly useful for production of outer membrane vesicle vaccines. To this end, spontaneous mutants of strain H44/76 resistant to the lipid A-binding antibiotic polymyxin B were isolated. Approximately 50% of the resistant mutants were found to have altered LPS as demonstrated by a strongly increased electrophoretic mobility on SDS-PAGE. The nature of the structural change in the LPS is currently under investigation. Preliminary experiments on the induction of IL-6 in human peripheral blood leukocytes demonstrated that this ability was reduced in whole cells or outer membrane complexes of the polymyxin-resistant mutants, but not in purified LPS. When mice were immunized with outer membrane complexes from the polymyxin-resistant mutants, the resulting antisera gave bactericidal titers against the parent strain H44/76

that were significantly lower as compared to the wildtype. This demonstrates that LPS has an effect on the immune response against the class 1 and 3 outer membrane proteins.

The biology of the lipooligosaccharides of the pathogenic *Neisseria*

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Lipooligosaccharides (LOS) are important surface components of *Neisseria*. They are a family of amphipathic molecules that consist of a hydrophilic carbohydrate portion and a hydrophobic lipid A portion. It has been shown that the oligosaccharide portion of the LOS is a target for potentially protective, bactericidal human antibody (1). The carbohydrate portion also contains determinants that mimic human glycosphingolipid antigens and may participate in molecular mimicry (3).

Recent studies in our laboratory and those of our collaborators has focused on studies related to the role of the oligosaccharide portion of the LOS in human disease pathogenesis. As part of these studies we have begun a systematic study of the genes involved in LOS biosynthesis. These studies have led to the identification of galactose epimerase gene and structural studies of the LOS produced in Neisseria with mutations in this gene. These analyses have shown that the oligosaccharide in these galactose deficient mutants can produce LOS oligosaccharides containing repetitive glucose moieties indicative of biosynthetic pathways different from that necessary to form the lacto-*N*-neotetraose moiety. Analysis of the parent strain confirms that similar structures are present among the LOS species of the wild type LOS. This suggests a wider heterogeneity of LOS structure than was previously considered. Studies of the LOS structure of organisms with mutations in the phosphoglucomutase gene (*pgm*) indicate that the LOS terminates at the second heptose and no additional sugars are added to the core region (5). Analysis of mutations in the heptosyltransferase I gene (*hepT1*) have indicated that the LOS is truncated at KDO. Sequence data revealed a thirty-two bp A+T-rich region between the putative -35 and -10 elements and the HepTI coding sequences. Previous studies suggested that such A+T-rich nucleotide elements that induce curved DNA structure may influence the promoter strength in *cis* (4). Since the average GC content in *N. gonorrhoeae* is about 50% (2), we believe that the continuous A+T-rich sequence may be involved in the regulation of expression of the *hepTI* gene in *N. gonorrhoeae*. In addition, a sixteen bp palindrome sequence encompassing the putative ribosomal binding site of *hepTI* is observed.

Functional studies of the adhesive interactions between gonococci indicate that terminal galactose of the LOS binds to the opa protein of neighbouring organisms. This is responsible for the clumping seen with opa+ organisms. Analysis of the interaction of gonococci with Hep2G cells has shown that terminal galactose moieties of the LOS bind to two calcium dependent carbohydrate receptors; one of these is the well characterized major hepatic asialoglycoprotein receptor and the other is a yet to be characterized 70kDa galactose binding protein. Expression of these receptors is increased in the presence of gonococci as compared to the insulin receptor which remains unchanged. Analysis of human sperm has shown that a 46 kDa galactose binding receptor is present and can bind the gonococcus through the galactose residues of LOS. This interaction can be competitively inhibited by asialoorosomucoid. Confocal and immunoelectron microscopy confirm that gonococci are binding to the sperm head.

Another cell known to express a galactose-binding protein on its surface is the macrophage. Serial immunoelectron micrographs of human urethral exudates infected with *N. gonorrhoeae* have demonstrated the presence of macrophage-like cells containing ingested gonococci. These organisms are present in the cytoplasm and have not been found in phagolysomal vacuoles. Of interest, gonococci in the process of ingestion do not bind anti-LOS MAbs on the bacterial surface fused to the macrophage membrane while gonococci which are in the cytoplasm stain circumferentially with anti-LOS MAbs. It is tantalizing to suggest that the LOS on cells during envelopment is occupied by receptors which are recycled when the organism is internalized. Studies are now in process to further define interactions of the LOS with macrophages and the role of macrophages in gonococcal infection.

References

1. **Apicella, M. A., Westerink, M. A. J., Morse, S. A., Schneider, H., Rice, P. A. and Griffiss, J. M.** 1986. The normal human serum bactericidal antibody response to the lipooligosaccharide of *Neisseria gonorrhoeae*. J. Infect. Dis. **153**: 520-526.
2. **Hoke, C. and Vedros, N. A.** 1982. Taxonomy of the *Neisseriae*: deoxyribonucleic acid base composition, interspecific transformation, and deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. **32**: 57-66.
3. **Mandrell, R. E., Lesse, A. S., Sugai, J. V., Shero, M., Griffiss, J. M., Cole, J., Parsons, N., Smith, H., Morse, S. A. and Apicella, M. A.** 1990. *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by N-acetyl neuraminic acid. J. Exp. Med. **171**: 1649-1664.
4. **Pérez-Martín, J., Rojo, F. and Víctor de Lorenzo.** 1994. Promoter responsive to DNA Bending: a common theme in prokaryotic gene expression. Microbiol. Rev. **58**: 268-290.
5. **Zhou, D., Stephens, D. S., Gibson, B. W., Engstron, J. J., McAllister, C. F., Lee, F. K. and Apicella, M. A.** 1994. Lipooligosaccharide biosynthesis in pathogenic *Neisseria*: cloning, identification, and characterization of the phosphoglucomutase gene. J. Biol. Chem. **269**: 11162-11169.

Structural relationships and sialylation among meningococcal lipooligosaccharide (LOS) serotypes

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Meningococci (mgc) are seldom of a single LOS serotype. 19/33 endemic B and C mgc in one US city were L8; 16 of these were also L3, L7 or both and 9 were L1. We compared the L1, L8 and L3,7 LOS structures. L1 strains made a common LOS; we estimated its M_r, and those of the L8 and L3,7 LOSs by co-SDS-PAGE with gonococcal LOS of known molecular mass. We used serotype-specific mAbs to mark each LOS. We used their M_rs to identify the L1 and L8 molecular ions in electrospray mass spectra and got mass and compositional information from this and other MS techniques.

As previously shown, the L8 " chain is lactose (Lac; Galß1-4Glc); the L1 LOS arises by terminal Gal" 1-4 substitution of the L8 Lac structure. This creates the human P^k blood group, and L1 LOS binds a P^k mAb. The L1/P^k glycose is partially sialylated, endogenously. Enzymatic removal of the terminal "-Gal from unsialylated L1 LOS creates the L8 LOS; removal of the terminal ß-Gal from L8 LOS causes loss of the L8 determinant. The L3,7 LOS arises by terminal substitution of the L8 LOS with lactosamine (Galß1-4GlcNAc); it is the paragloboside glycose and also is partially sialylated, endogenously.

L8 **Galß1-6-4Glc**ß1-6-4Hep"1-6-(KDO)$_2$
8
GlcNAc(OAc)"1-6-2Hep-3-7-**PEA**

L1 (NeuNAc"2-6-3)Gal"1-6-3**Galß1-6-4Glc**ß1-6-4Hep"1-6-(KDO)$_2$
8
GlcNAc(OAc)"1-6-2Hep

L3,7 (NeuNAc"2-6-3)Galß1-6-4GlcNAcß1-6-3**Galß1-6-4Glc**ß1-6-4Hep"1-6-(KDO)$_2$

The L1 mAb did not bind phosphoethanolamine (PEA) substituted LOS that had the L1 glycose. Dephosphorylation did not affect the mobility of the L1 LOS or its ability to bind its mAb, but caused the L8 LOS to lose the L8 epitope.

These data show that the L1 and L3,7 structures arise by alternative substitutions of the L8 " chain. Basal PEA substitutions variously effect L1 and L8 expression. Their structural relationships explain co-expression of L1, L3,7 and L8 serotypes. Both the L1 and L3,7 LOS may be sialylated, endogenously.

The structure of lipooligosaccharide produced by *Neisseria gonorrhoeae*, strain 15253 isolated from a patient with disseminated infection: Evidence for a new glycosylation pathway of the gonococcal lipooligosaccharide

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The lipooligosaccharides (LOSs) of *Neisseria gonorrhoeae* are important pathogenic and antigenic outer membrane components. Gonococcal LOS expression varies among strains, and some strains produce several structurally different LOS components (1, 2). In addition, gonococcal LOS expression can shift with the stage of infection (3), and a strain that produces a single LOS component has an ability to synthesize a variety of LOSs, depending on its environment (3). Further, changes in LOS structure can affect the interaction of gonococci with human cell surfaces and defences.

We have studied the structures and epitope expression of some prevalent LOS components produced by clinical gonococcal isolates (3-7). Our previous studies showed the following: (a) the structural differences among LOSs are primarily due to differences in the oligosaccharide moiety of the LOS; (b) gonococci produce higher molecular-weight (MW) LOSs such as 4.5 and 4.8 Kda by elongating the carbohydrate sequence of a small MW LOS; (c) the antigenic similarity between the 4.5 Kda LOS and human glycosphingolipid (4), paragloboside, is due the presence of identical OS, and this LOS component is suggested to be associated with the disease process (3).

Gonococci produce several other LOS components whose structures are unknown, and these unidentified LOSs could be important for understanding the role and functions of the LOS in gonococcal disease. In the present study, we investigated the structure of a LOS produced by strain 15253 isolated from a patient with disseminated gonococcal infection. MAb 3G9 binds to this LOS (2, 8) and its binding patterns to gonococcal LOS indicate that the 15253 LOS is structurally different from the LOSs studied previously (6, 7). This MAb 3G9-defined epitope appears to be specific for *Neisseria gonorrhoeae*.

We analyzed the structure of the oligosaccharide derived from the 15253 LOS by chemical and two-dimensional NMR methods and determined that the 15253 LOS

has two lactosyl residues in the molecule. This present finding reveals a newly recognized glycosylation pathway for gonococcal LOS. As described above, higher MW LOSs elongate from a LOS precursor. For example, gonococci synthesize a lactoneotetraose structure by elongating a lactosyl moiety that is attached to a heptose (Hep-1) in the LOS molecule (MS11mk LOS). In contrast to elongation of the Hep-1 linked lactosyl residue described above, strain 15253 modifies another heptosyl residue (Hep-2), producing a second lactosyl residue.

The novel structure represented by the presence of two lactosyl residues in the same LOS may be related to the antigenic specificity of MAb 3G9 for the gonococci. Strain 15253 may interact with human glycosyltransferase or cell lectins via either lactosyl residue, and these possible in vivo interactions may play some role in the infection and the disease. This new glycosylation pathway not only demonstrates additional complexity in gonococcal LOS biosynthesis but also provides further structural information on LOS expressed during different clinical states of infection.

References

1. **Schneider, H., T. L. Hale, W. Zollinger, R. Seid C, Jr., C. A. Hammack and J. M. Griffiss.** 1984. Heterogeneity of molecular size and antigenic expression within the lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. **45**:544-549.
2. **Mandrell, R. E., H. Schneider, M. Apicella, W. Zollinger, P. A. Rice and J. M. Griffiss.** 1986. Antigenic and physical diversity of *Neisseria gonorrhoeae* lipooligosaccharides. Infect. Immun. **54**:63-69.
3. **Schneider, H., J. M. Griffiss, J. W. Boslego, P. J. Hitchcock, K. M. Zahos and M. A. Apicella.** 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. Journal of Experimental Medicine. **174:**1601-1605.
4. **Mandrell, R. E., J. M. Griffiss and B. A. Macher.** 1988. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. Journal of Experimental Medicine. **168**:107-126.
5. **Yamasaki, R., W. Nasholds, H. Schneider and M. A. Apicella** 1991. Epitope expression and partial characterization of gonococcal F62 lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M) recognize non-reducing termini of the LOS components. Molecular Immunology. **28**:1232-1242.
6. **Yamasaki, R., B. E. Bacon, H. Schneider and J. M. Griffiss.** 1991. Structural determination of oligosaccharides derived from lipooligosaccharide F62 of *Neisseria gonorrhoeae* by chemical, enzymatic and two-dimensional NMR methods. Biochemistry. **30**:10566-10575.
7. **Kerwood, D. E., H. Schneider and R. Yamasaki.** 1992. Structural analysis of lipooligosaccharide produced by *Neisseria gonorrhoeae* strain

MS11mk (variant A): A precursor for a gonococcal lipooligosaccharide associated with virulence. Biochemistry. **31**:12760-12768.

8. **Mandrell, R. E., J. Sugai, J. Boslego, R. Chung, P. A. Rice and J. M. Griffiss.** 1987. Human immune response to monoclonal antibody-defined epitopes on lipooligosaccharides of *Neisseria gonorrhoeae*. p. 569-574. In J. T. Poolman, H. Zanen, T. Mayer, J. Heckels, P. H. Makela, H. Smith and C. Beuvery (ed.). Gonococci and meningococci. Kluwer Academic Publishers, Dordrecht.

Surface charge and hydrophobicity correlate with Gc behaviour

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Although surface charge and hydrophobicity dictate many interactions of inanimate colloidal particles with one another and with heterologous surfaces, whether these physicochemical attributes similarly influence the interactive behaviours of colloid-sized bacteria is incompletely defined. Nor are the molecular origins of surface charge of gram-negative bacteria defined comprehensively. Capsulation increases the negative charge of *E. coli*, with magnitude of the enhanced negativity related to capsule composition (1). Salmonella with "smooth" LPS are reported to be less negatively charged than "rough" LPS mutants by phase partition assessments, but charges of various rough mutants could not be differentiated by (5). A recent report finds no systematic differences in the electrophoretic mobility (EPM) of *E. coli* when they express different LPS molecules (1).

I have been exploring general surface properties of gonococci (Gc) to define their molecular origins and to assess the composite effects that accompany co-expression of multiple surface-exposed molecules. An added reason is to look for correlations between surface properties and biological behaviour of Gc, especially those that might relate to pathogenicity.

Lipooligosaccharide (LOS) molecules, as major surface-exposed constituents of the Gc outer membrane, are likely contributors to surface charge. Relationships between LOS structure and EPM of Gc were examined in the well-characterized set of pyocin-resistant mutants of strain 1291 (2). Single sugar truncations distinguish the LOS molecules expressed by 1291 and its variants 1291a-d (3); two of the variants have identical truncated side chains but differing numbers of KDO core moieties (1291d = 3 KDO, 1291e = 2 (2)). P- Opa- cells of each LOS variant were examined. The salient results follow:

LOS variants exhibit EPMs range from -0.15 to -0.77 ：m-cm/V-s; their EPM negativities are: 1291 < 1291a < 1291c < 1291c <1291e < 1291d.

Truncation of oligosaccharide side chains correlates with increased negative charge (correlation coefficient = 0.872 with all variants considered; = 0.976 when 1291b excluded); the EPM differences seen with single sugar truncations ranged from -0.12 to -0.36 ：m-cm/V-s.

Absence of a third KDO correlates with reduced negative EPM for variant 1291e (-0.71) compared to 1291d (-0.77).

These data suggest that phosphates of LOS core KDO and heptose moieties are the main ionogenic moieties of P- Opa- Gc outer membranes. The Gc LOS variants exhibit differing EPMs because of the spatial separation imposed by the oligosaccharide side chains of increased length between ionogenic core phosphates and the electrophoretic shear plane at the outermost surface of the bacteria. This is consistent with recent studies on the shape, orientation, and exposure for LPS molecules in the outer membranes of salmonella and *E. coli* (4); each side chain sugar adds approximately 2.3 - 3.5 nm to LPS molecule length, and all core phosphates orient toward the cell membrane exterior.

It could be argued that outer membrane proteins (such as Por and Rmp) might contribute to EPM and that the observed dissimilarities reflect different degrees of "shielding" of commonly expressed outer membrane proteins by the variant oligosaccharide side chains of differing length. This is inconsistent with a finding that proteinase K treatment of whole P- Opa- Gc, under conditions that appears to totally hydrolyse these proteins *in situ*, did not alter EPM. Nor does the surface lipoprotein (Lip, H.8) appear to be a major contributor to EPM, as deduced from examining Lip- mutants (from J. Cannon).

The LOS variants of strain 1291 exhibited differing in their comparative susceptibility/resistance to killing by the cationic "-helix forming peptide magainin-2 (GIGKFLHSAKKFGKAFVGEIMNS) as follows:

Resistance to magainin-2: 1291a > 1291 > b > c > d > e.

The inexact correlation between magainin-2 sensitivity of Gc and LOS side chain length resembles results with salmonella LPS mutants (6). Enhanced resistance of variant 1291a to magainin-2 might relate to this LOS variant being considerably more hydrophobic than all the others, (1291a >> 1291 > 1291b = c = d = e), as assessed by partitioning whole Gc between dextran and polyethylene glcol. Its heightened hydrophobicity likely relates to its having N-acetyl substituents on its terminal side chain saccharide.

Two variants (1291, 1291b) have galactose-terminating side chains and are sialylated when CMP-NANA is supplied. Another variant (1291c) also has a terminal galactose residue, but it was not sialylated. Sialylation produced enhanced negativity for both 1291 and 1291b, but not equally, and their hydrophobicities were virtually unchanged. It was somewhat surprising that sialylated 1291 and 1291b, because EPMs became decidedly more negative, were not altered in their sensitivities to magainin-2. Magainin-2 molecules, having little or no secondary structure in solution, are thought to be converted to "-helical oligomers upon interacting with negatively-charged cell surfaces; these oligomers subsequently can insert into the membrane, function as pores, and cause the cells to lyse.

Summary: Variation in LOS side chains is accompanied by distinctive changes in Gc surface charge as deduced from EPM measurements. Phosphate groups of the LOS core are probably the ionogenic groups responsible for the majority of negative charge on Gc. EPM differences among the LOS variants correlate inexactly with their sensitivities to magainin-2.

References

1. **Bayer, M. E. and J. L. Sloyer Jr.** 1990. The electrophoretic mobility of gram-negative and gram-positive bacteria: an electrokinetic analysis. J. Gen. Microbiol. **136**: 867-874.
2. **Dudas, K. C. and M. A. Apicella.** 1988. Selection and immunochemical analysis of lipooligosaccharide mutants of *Neisseria gonorrhoeae*. Infect. Immun. **56**: 499-504.
3. **John, C. M., J. M. Griffiss, M. A. Apicella, R. E. Mandrell and B. W. Gibson.** 1991. The structural basis for pyocin resistance in *Neisseria gonorrhoeae* lipooligosaccharides. J. Biol. Chem. **266**: 19303-19311.
4. **Kastowsky, M., T. Gutberlet and H. Bradaczek.** 1992. Molecular modelling of the three-dimensional structure and conformational flexibility of bacterial lipopolysaccharide. J. Bacteriol. **174**: 4798-4806.
5. **Magnusson, K. E. and G. Johansson.** 1977. Probing the surface of *Salmonella typhimurium* and *Salmonella minnesota* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. FEMS Microbiol. Lett. **2**: 225-228.
6. **Rana, F. R., E. A. Macias, C. M. Sultany, M. C. Modzrakowski and J. Blazyk.** 1991. Interactions between magainin 2 and *Salmonella typhimurium* outer membranes: effect of lipopolysaccharide structure. Biochem. **30**: 5858-5866.

Molecular analysis of mAb 2C7 and mAb CA1, an anti-idiotope surrogate for a conserved gonococcal oligosaccharide epitope

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We have identified a gonococcal lipooligosaccharide (LOS) epitope, recognized by mAb 2C7 (IgG3κ), that is widely expressed on gonococcal strains (2). We have previously shown that mAb 2C7 binds to oligosaccharide (OS) chains on LOS by the following methods. First, mAb 2C7 binds to OS chains derivatized to tyramine after acid hydrolysis of whole LOS and chromatographic separation of OS from lipid containing fractions. Second, proteinase K treatment did not alter binding of mAb 2C7 to immunoblots of whole gonococcal lysates and purified LOS. However, preincubation with $NaIO_4$ abolished mAb 2C7 binding to immunoblots of whole cell lysates or LOS. Third, mAb 2C7 bound to whole cell immunodot blots of the wild type gonococcal strain FA19, but not to two transformants possessing truncated LOS variants, FA628 (sac-1$^+$) and FA899 (sac-3$^+$).

We believe that this OS epitope may serve as an appropriate vaccine candidate for human immunization against *Neisseria gonorrhoeae*. However, purification of OS from LOS may modify its antigenicity and would result in a T-cell independent carbohydrate antigen, which may be poorly immunogenic. As an alternative strategy, we have generated an IgMκ anti-idiotope mAb in mice, named CA1, as a surrogate for the 2C7 OS epitope. mAb CA1 elicits a bactericidal and opsonophagocytic immune response in animals (1). Compared to gonococcal OS, use of a mAb surrogate would facilitate vaccine antigen purification and provide a protein antigen that should elicit a T-cell dependent immune response.

To simplify fragment production and allow more complete analysis of the interactions of these antibodies with each other and with LOS we decided to: a) clone and sequence the variable regions of both mAbs and b) produce them in both bacterial and mammalian expression systems. The heavy and light chain variable (V) region pair of each antibody (mAbs 2C7 and CA1) were separately amplified by polymerase chain reaction (PCR) from cDNA, using primer pools, and cloned into a phage display expression vector. This vector (Den, Sarantopoulos, and Sharon, submitted for publication) can be modified to produce soluble Fab. The nucleotide sequences of the 2C7 V_H and V_L region genes were determined. The 2C7 V_L region belongs to murine Vκ subgroup I and is encoded by the Vκ1 gene with no somatic mutations.

The 2C7 VH region belongs to murine subgroup II A and is partially encoded by the JH2 gene. These sequences are highly homologous with those of anti-dextran antibodies, as would be expected for an antibody that binds to a carbohydrate antigen. We are in the process of determining the nucleotide sequences of mAb CA1 (the anti-idiotope) V_H and V_L regions.

Fab fragments constructed from human constant (C) region and the V region sequences of mAbs 2C7 and CA1 will be expressed in bacterial and mammalian systems. Use of human C region sequences limits the non-human portions of the Fab to the V regions and V regions can be further humanized to eliminate unnecessary murine sequences. These strategies should decrease the chance of adverse reactions upon administration in humans. Bacterial and mammalian derived phage display and soluble CA1 Fab will be used to immunize animals. The cross-reactivity of the resultant Ab3 antibodies with a panel of gonococcal strains will be compared with the range of reactivity of mAb 2C7 (Ab1).

References

1. **Gulati, S., D.P. McQuillen and P.A. Rice**. Anti-idiotope vaccine against lipooligosaccharide of *N. gonorrhoeae* elicits bactericidal antibodies that mediate opsonophagocytosis. 1993. Program and Abstracts of the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy. p. 264.
2. **McQuillen, D.P., S. Gulati, Y.K. Tsung, J. Sharon and P.A. Rice**. Experimental immunization with gonococcal anti-idiotope vaccine against gonococcal lipooligosaccharide. 1992. Abstracts of the Eighth International Pathogenic Neisseria Conference. p. 33.

Functional characteristics of the immune response to the gonococcal lipooligosaccharide epitope defined by mAb 2C7 in natural infection and after immunization

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Monoclonal antibody (mAb) 2C7 (Ab1) recognizes a widely conserved, *in vivo* expressed lipooligosaccharide (LOS) epitope expressed by *Neisseria gonorrhoeae*. We have previously shown that mAb 2C7 is bactericidal against and promotes ingestion by human polymorphonuclear leukocytes (PMNs) of both serum-sensitive (SS) and -resistant (SR) gonococci (2, 3). The inherent toxicity of LOS and difficulties in purification of large quantities of non-toxic and immunogenic subcomponents of LOS antigen preclude its use as a convenient vaccine antigen. We therefore elected to generate an anti-idiotypic mAb (Ab2) surrogate that would also evoke a T-cell dependent immune response. Immunization of mice with mAb 2C7 elicited an anti-idiotope (Ab2), named mAb CA1, that was used in turn to immunize syngeneic mice and xenogeneic rabbits. Anti-anti-idiotypic (Ab3) antibodies elicited in both species recognized LOS, indicating that mAb CA1 represents an Ab2$ anti-idiotope (i.e., it carries the internal image of the nominal LOS antigen). These Ab3 were able to effect equivalent or greater complement-mediated killing of both SS and SR strains of gonococci expressing the 2C7 LOS epitope than LOS immunization. The Ab3 response in both species was predominantly IgG with essentially no IgM consistent with a T-cell dependent immune response.

We next assessed the capacity of rabbit Ab3 antibodies to promote gonococcal opsonophagocytosis by flow cytometry, based on a modification of previously published methods (5). Gonococci were labelled with Lucifer Yellow before opsonization with rabbit serum, then were allowed to adhere to human PMNs at 0°C followed by phagocytosis at 37/C. Surface adherent (but not internalized) gonococci were counter-stained with a biotinylated mAb directed against a conserved gonococcal lipoprotein antigen (H.8) coupled to streptavidin-phycoerythrin-Texas Red. PMN adherence of a SS and a SR strain bearing the 2C7 LOS epitope was moderately increased by immune (Ab3) rabbit serum compared to pre-immune rabbit serum. Immune (Ab3) rabbit serum mediated nearly complete ingestion of both the SS and SR strains bearing the 2C7 LOS epitope, while pre-immune rabbit serum mediated minimal ingestion of only the SS strain. Immune (Ab3) rabbit serum did not alter PMN adherence or ingestion of a control SR strain lacking the 2C7 LOS epitope, indicating specificity of the
response.

We also sought further evidence that the 2C7 epitope is immunogenic in the setting of both natural gonococcal infection and vaccination in humans. To evaluate the response elicited by natural infection to the 2C7 epitope, we assayed acute and convalescent sera from 6 patients with disseminated (DGI) and 7 with local (endometritis) infection. We used mAb 2C7 in an inhibition ELISA assay (1) to show that patients with DGI developed 8-fold IgG and 12-fold IgM increases against the 2C7 epitope. Women with endometritis had 3-fold IgG and 4-fold IgM antibody responses against the 2C7 epitope. Additionally, sera obtained from 62 adult male volunteers immunized with either a Por vaccine or placebo, as part of a 1985 enriched Por vaccine challenge trial (4), were assayed for antibody against the 2C7 epitope. The vaccine preparation consisted of approximately 85% Por (Protein I), but also contained 15% Rmp (Protein III) and small contaminating amounts of LOS (which contained the 2C7 epitope). Twenty-eight of 35 vaccinated individuals developed a greater than 2-fold rise in antibody levels against LOS and 21 of the 28 demonstrated a rise in antibody ($\geq$ 0.1 : g/ml) directed against the 2C7 epitope. Eleven individuals developed an excess level of antibody against the 2C7 epitope compared to their overall response to LOS. These results confirm that the 2C7 epitope is a target of the human immune response in the settings of both natural infection and LOS vaccination. In animal systems the anti-idiotope mAb CA1 (Ab2) elicits a T-cell dependent immune response that mediates gonococcal killing and PMN phagocytosis. Such an anti-idiotope, used as a surrogate immunogen for LOS, may form the basis for a potentially protective gonococcal vaccine.

References

1. **Dale, P.A., D.P. McQuillen, S. Gulati and P.A. Rice**. 1992. Human vaccination with *Escherichia coli* J5 mutant induces cross-reactive bactericidal antibody against *Neisseria gonorrhoeae* lipooligosaccharide. J. Infect. Dis. **166**: 316-25.
2. **Gulati, S., D.P. McQuillen and P.A. Rice**. Anti-idiotope vaccine against lipooligosaccharide of *N. gonorrhoeae* elicits bactericidal antibodies that mediate opsonophagocytosis. 1993. Program and Abstracts of the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy. p. 264.
3. **McQuillen, D.P., S. Gulati, Y.K. Tsung, J. Sharon and P.A. Rice**. Experimental immunization with gonococcal anti-idiotope vaccine against gonococcal lipooligosaccharide. 1992. Abstracts of the Eighth International Pathogenic Neisseria Conference. p. 33.
4. **Rice, P.A., E.W. Hook, M.S. Blake, R.S. Kaslow, S. Gulati, P.K. Kohl, M. VanRadden and T.M. Buchanan**. A possible influence of vaccine induced Por, LOS and Rmp antibodies on the outcome of intraurethral challenge with *Neisseria gonorrhoeae*.1994. Abstracts of the Ninth International Pathogenic Neisseria Conference (this volume).
5. **Sveum, R.J., T.M. Chused, M.M. Frank and E.J. Brown**. 1986. A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. J Immunol Methods. **90**: 257-64.

Cloning and sequencing of lipooligosaccharide monoclonal antibody regions for molecular level study of epitope-antibody interactions

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Various human invasive diseases are caused by mucosal pathogens, the virulence of which is mediated by an array of surface antigens, including their principal glycolipid, known as lipooligosaccharide (LOS) (3). The meningococcus is one of the two major pathogenic species of the genus *Neisseria*. Much of the pathogenicity of meningococcal strains is attributable to their surface LOS, which composes approximately 50% of the outer membrane. The hydrophobic portion of the molecule is lipid A, which is the active moiety of endotoxin. The more hydrophilic oligosaccharide portion contains the variability which provides a basis for epidemiological typing (immunotyping) (4, 8). 12 distinct LOS immunotypes have been defined and monoclonal antibodies (MAb's) against some of these epitopes have been produced (7).

Whereas work on the specific interactions of *Neisseria* OMP's and Mab's is quite advanced (5), this probably being due to the interest in these proteins as vaccine candidates and also the availability of techniques such as epitope mapping for protein antigens, the more complex area of anti-carbohydrate antibodies is only just beginning to be understood . Proteins that recognise carbohydrate moieties have to discriminate between a vast number of sugar structures, arising from the stereochemistry of hydroxyl groups and great variety of possible sugar linkages.

Antibody binding sites are primarily composed of six segments known as CDR's (complementarity determining regions), with three in each variable region of the light and heavy chains. Recombinant antibody and gene amplification technology have made it possible to clone antibody genes into bacteria, allowing them to be readily produced, sequenced and rearranged to alter specificities and affinities. The availability of Fab's (antibody fragments containing constant and variable regions) and ScFv's (single-chain constructs of both variable regions) now allows well-refined crystal structures of protein-carbohydrate complexes to be solved (2).

With this in mind, the genes for two anti-LOS L3,7,9 MAb's, 4A8-B2-L379 (J. Poolman) and 9-2-L379 (W. Zollinger), have been rescued from mice hybridomas and cloned either directly as Fab's into a phagemid expression vector (pGEM5Zf+/-), or used to construct ScFv's and cloned into a phage display system (pCANTAB5) (6). The relative binding affinities of these two Mab's have been

compared in ELISA assays using purified LOS, and have been found to show marked differences in their reactivities with LOS isolated from an *N. meningitidis* case isolate identified as immunotype L3,7,9 (strain K454, PHL Manchester). In order to determine the molecular basis for this variation, sequences of V region genes for MAb 4A8-B2-L379 have already been obtained from its ScFv, and are being aligned and compared to other antibody sequences. The Fab of MAb 9-2-L379 is presently being sequenced, and the comparison of sequence data from both anti-L3,7,9 MAb's will provide information on which areas of the V regions contribute to their differential binding. Using Fab DNA as a template, constant as well as variable region sequence can be obtained, which may be necessary in order to have a complete picture of all the nucleotides that may influence antibody binding capabilities.

Once all the genes encoding the antibody DNA's have been sequenced, studies of the interaction of these antibodies with LOS by co-crystallisation of *Nm*-LOS and ScFv/Fab regions will be possible, as will structural studies by X-ray crystallography and solution studies by NMR. Sequence information may also be used to generate models of the antibody combining sites with a commercial antibody modelling package, to help predict likely contact residues in the antibody combining sites. It is then possible to mutate these and analyse resulting alterations in affinities via an automated biosensor system, such as Biacore, which measure molecular interactions in real time (1).

References

1. **Borrebaeck, C. A. K., A.-C. Malmborg, C. Furebring, A. Michaellson, S. Ward, L. Danielsson and M. Ohlin**. 1992. Kinetic analysis of recombinant antibody-antigen interactions: relation between structural domains and antigen binding. Bio/Technology **10**: 697-698.
2. **Brummel, D.A., V.P. Sharma *et. al.*** 1993. Probing the combining site of an anti-carbohydrate antibody by saturation mutagenesis: Role of the heavy chain CDR 3 residues. Biochemistry **32**: 1180-1187.
3. **Griffiss, J. M., H. Schneider, R. E. Mandrell, R. Yamasaki, G. A. Jarvis, J. J. Kim, B. W. Gibson, R. Hamadeh and M. A. Apicella**. 1988. Lipooligosaccharides: the principal glycolipids of the neisserial outer membrane. Rev. Infect. Dis. **10(2)**: S287-S295.
4. **Kim, J. J., R. E. Mandrell, H. Zhen, M. A. J. Westerink, J. T. Poolman and J. M. Griffiss**. 1988. Electromorphic characterization and description of conserved epitopes of the lipopolysaccharides of group A *N. meningitidis*. Infect. Immun. **56**: 2631-2638.
5. **Larrick, J.W., M.J. Coloma *et. al.*** 1990. Immunoglobulin V regions of a bactericidal anti-*Neisseria meningitidis* outer membrane protein monoclonal antibody. Scand. J. Immunol. **32**: 121-128.
6. **McCafferty, J., A. D. Griffiths, G. Winter and D. J. Chiswell**. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature **348**: 552-554.

7. **Sugasawara, R. J., C. Prato and J. E. Sippel**. 1983. Monoclonal antibodies against *N. meningitidis* lipopolysaccharides. Infect. Immun. **42**: 863-868.
8. **Zollinger, W. D. and R. E. Mandrell**. 1977. Outer membrane protein and Lipopolysaccharide serotyping of *N. Meningitidis* by inhibition of a solid phase radio-immunoassay. Infect. Immun. **18**: 424-433.

Application of FPLC to purification of lipooligosaccharide from meningococcal strains

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Aqueous phenol has been widely used in the extraction of LPS from gram-negative bacteria because of its applicability to many groups of bacteria and its relative simplicity. It is also the only procedure which gives significant yields when extracting LOS-containing bacteria. Extraction of LOS by the standard phenol-water method (4) gives lower yields than for LPS, because the repeated water solubilisation and ultracentrifugation steps required for LPS purification results in considerable loss of the lower molecular weight LOS. Whilst traditional gel filtration has been used in LOS purification for some time (1, 2, 5), we chose to replace the traditional purification cycles by a single gel filtration step using FPLC (fast protein liquid chromatography), which has much greater separation power and vastly reduces elution times.

The group B meningococcal strains used, K454 and L352, were obtained from the reference laboratory in Manchester. Both were typed as B15:P1.7,16R. Crude LOS (prepared as above) from whole cells was applied to a Superose 6 HR10/30 column, and elution of LOS was followed by refractive index. Contaminant peaks were monitored at 260/280nm. The LOS eluted as a single peak in mostly one fraction (500µl) when a sample volume of 50µl was loaded. This was confirmed by analysis of all fractions collected by SDS-polyacrylamide gel electrophoresis (PAGE), followed by periodic acid-silver staining (3). The level of purity of this present FPLC method already compares very well with that for meningococcal LOS purified by traditional gel filtration; there is an average 50% reduction in nucleic acids, (~0.5% c.f. 1%) and % protein is an order of magnitude lower (<0.05% c.f. 0.5%) (1). By modifying the elution buffer and using a column with a fractionation range for dextrans closer to the molecular weight of LOS, lower levels of contamination, particularly with regard to RNA, should be possible.

References

1. **Gu, X.-X. and C.-M. Tsai**. 1991. Purification of rough-type lipopolysaccharides of *N. meningitidis* from cells and outer membrane vesicles in spent medium. Anal. Biochem. **196**: 311-318.
2. **Gu, X.-X. and C.-M. Tsai**. 1993. Preparation, characterization and immunogenicity of meningococcal lipooligosaccharide-derived oligosaccharide-protein conjugates. Infect. Immun. **61(5)**: 1873-1880.

3. **Tsai, C.-M. and C.E. Frasch**. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**: 115-119.
4. **Westphal, O. and K. Jann**. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**: 83-91.
5. **Wu, L. *et. al*.** 1987. A method for purification of bacterial R-type lipopolysaccharides (lipooligosaccharides). Anal. Biochem. **160**: 281-289.

Isolation of an *S. typhimurium rfaE* homolog in *Neisseria gonorrhoeae*

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Gram-negative bacteria have lipopolysaccharide or lipooligosaccharide (LPS or LOS) molecules attached to their outer membrane through a lipid A anchor. In pathogenic gram-negative bacteria, this molecule plays an important role in pathogenesis, conferring the bacteria ability to evade the immune system through molecular mimicry and anti-phagocytic properties. The structure and composition of the LPS or LOS have been elucidated for a number of gram-negative species and shows that the core oligosaccharides of different species share common sugars and/or linkages. The genetics of LPS biosynthesis in enteric gram-negative bacteria has been extensively studied and today, much is known about the genes involved in this complex pathway. However, in non-enteric pathogens, investigations into the genetics of LPS (or LOS-lipooligosaccharide) biosynthesis has been hampered due to difficulties encountered in manipulating the genomes of these non-enteric pathogens. Among the difficulties are the lack of an efficient and simple protocol for construction of mutant libraries and screening of a mutant library for LOS mutant phenotypes.

In this study, we report on the use of defined LPS biosynthesis mutants in enteric bacteria *S. typhimurium* LT-2 to identify and clone a LOS biosynthesis gene in *Neisseria gonorrhoeae*. A plasmid library of genomic DNA from *N. gonorrhoeae* strain 1291 was electroporated into an ADP-heptose synthase deficient mutant of *S. typhimurium* (*rfaE*827) and grown on ampicillin-novobiocin supplemented selection media. SDS-PAGE analysis of LPS isolated from the transformant showed an upward shift in mobility relative to the parent suggesting that the gonococcal DNA was able to complement the lesion in *rfaE*827. Phage sensitivity pattern of the transformant was similar to the pattern exhibited by the wild type. Southern blot hybridizations with the 6.8 kb insert suggested that the *rfaE* gene exists as a single-copy gene. This region appears to share high homology with the homologous region in *N. meningitidis*. Defined *S. typhimurium* LPS mutants appear to be a useful tool in identifying LOS biosynthesis genes of pathogenic gram-negative bacteria.

Lipooligosaccharide biosynthesis in *Neisseria gonorrhoeae*: Cloning, identification and characterization of the "1,5 heptosyltransferase I gene (*hepTI*)

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Neisseria gonorrhoeae is the causative agent of gonorrhoea. Lipooligosaccharide (LOS) is an important surface component of gonococcus. It is an amphipathic molecule that consists of a hydrophilic carbohydrate portion and a hydrophobic lipid A portion. Unlike most enteric bacteria, which make lipopolysaccharide (LPS), *N. gonorrhoeae* make LOS, which lack the O-antigen. Comparison of the LPS and LOS structures of *S. typhimurium* LT2 and *N. gonorrhoeae* strain 1291 revealed the identical partial deep core structure of Hep"1-3Hep"1-5KDO. This led us to assume that the two "1,3 and "1,5 heptosyltransferases in *S. typhimurium* and *N. gonorrhoeae* may be functionally exchangeable. By complementing the *S. typhimurium rfaC*630 mutant strain, we were able to clone the "1,5 heptosyltransferase genes in *N. gonorrhoeae* strain 1291.

SDS-PAGE analysis confirmed the production of wild type LPS in the transformant. Subcloning revealed that a 1.2 kb fragment was able to confer the complementation. Sequence analysis demonstrated a complete open reading frame corresponding to a 36~37 kDa peptide. *In vitro* transcription-translation analysis of the 1.2 kb clone confirmed that a 37 kDa protein was encoded by this DNA fragment. The DNA sequence-deduced protein had 36% identity and 58% isofunctional similarity to *S. typhimurium* heptosyltransferase I (*rfaC*). Primer extension analysis indicated that transcription of the cloned gene in *N. gonorrhoeae* strain 1291 begins 144 bp upstream of the start codon at a G nucleotide, indicating the cloned gene is transcribed from its own promoter. An isogenic mutant of *N. gonorrhoeae* strain 1291 with a m-Tn*3* insertion inside the coding sequences expressed a single truncated LOS with a similar molecular weight as *S. typhimurium rfaC* LPS. We conclude that the 1.2 kb fragment encodes for the "1,5 LOS heptosyltransferase I (*hepTI*) in *N. gonorrhoeae*. Our studies also provide further evidence to the observation that the third KDO in *S. typhimurium* LPS is added after the core synthesis is completed.

Genetic analysis of a locus for gonococcal LOS biosynthesis

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A locus involved in the biosynthesis of gonococcal LOS has been cloned from gonococcal strain F62. The locus contains five open reading frames. The first and the second reading frames are homologous, but not identical to the fourth and the fifth reading frames respectively. Interposed is an additional reading frame which has distant homology to the *E. coli rfaI* and *rfaJ* genes, both glucosyl transferases involved in LPS core biosynthesis (5). The second and the fifth reading frames show strong homology to the *lic2A* gene of *Haemophilus influenzae*, but do not contain the CAAT repeats seen in the *lic2A* gene (1,2).
Deletions of each of these five genes, of combinations of genes, and of the entire locus were constructed and introduced into parental gonococcal strain F62 by transformation. The LOS phenotypes were then analyzed by SDS-PAGE and reactivity with monoclonal antibodies. Analysis of the gonococcal mutants indicates that four of these genes are probably the transferases that add GalNAcβ1$\rightarrow$3Galβ1$\rightarrow$4GlcNAcβ1$\rightarrow$3Galβ1$\rightarrow$4 to the substrate Glcβ1$\rightarrow$4Hep$\rightarrow$R of the inner core region (8). The orf with homology to *E. coli rfaI/rfaJ* is involved with the addition of the α-linked galactose residue in the biosynthesis of the alternative LOS structure Galα1$\rightarrow$4Galβ1$\rightarrow$4Glcβ1$\rightarrow$4Hep$\rightarrow$R (3). The DNA sequence analysis revealed that the first, third and fourth reading frames contained poly-G tracts which in strain F62 were respectively 17, 10 and 11 nt. Thus, three of the LOS biosynthetic enzymes are potentially susceptible to premature termination by reading-frame changes, as has been reported for the gonococcal *pilC* genes (4,6). It is likely that these structural features are responsible for the high frequency genetic variation of gonococcal LOS (7).

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References

1. **Cope, L.D., R. Yogev, J. Mertsola, J.L. Latimer, M.S. Hanson and G.H. McCracken, Jr.** 1991. Molecular cloning of a gene involved in lipooligosaccharide biosynthesis and virulence expression by *Haemophilus influenzae* type B. Molec. Microbiol. **5**: 1113-1124.

2. **High, N.J., M.E. Deadman and E.R. Moxon.** 1993. The role of a repetitive DNA motif (5'-CAAT-3') in the variable expression of the *Haemophilus influenzae* lipopolysaccharide epitope "Gal(1-4)$Gal. Molec. Microbiol. **9**: 1275-1282.
3. **John, C.M., J. McLeod Griffiss, M.A. Apicella, R.E. Mandrell and B.W. Gibson.** 1991. The structural basis for pyocin resistance in *Neisseria gonorrhoeae* lipooligosaccharides. J. Biol. Chem. **266**: 19303-19311.
4. **Jonsson, A.-B., G. Nyberg and S. Normark.** 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. **10**: 477-488.
5. **Pradel, E., C.T. Parker and C.A. Schnaitman.** 1992. Structures of the *rfaB, rfaI, rfaJ,* and *rfaS* genes of *Escherichia coli* K-12 and their roles in assembly of the lipopolysaccharide core. J. Bacteriol. **174**: 4736-4745.
6. **Rudel, T., J.P. van Putten, C.P. Gibbs, R. Haas and T.F. Meyer.** 1992. Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. Molec. Microbiol. **6**: 3439-3450.
7. **Schneider, H., C.A. Hammack, M.A. Apicella and J.M. Griffiss.** 1988. Instability of expression of lipooligosaccharides and their epitopes in *Neisseria gonorrhoeae*. Infect. Immun. **56**: 942-946.
8. **Yamasaki, R., B.E. Bacon, W. Nasholds, H. Schneider and J.M. Griffiss.** 1991. Structural determination of oligosaccharides derived from lipooligosaccharide of *Neisseria gonorrhoeae* F62 by chemical, enzymatic, and two-dimensional NMR methods. Biochemistry **30**: 10566-10575.

Regulation of phase variation of gonococcal lipooligosaccharide expression

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Lipooligosaccharide (LOS) expression in *Neisseria gonorrhoeae* undergoes phase variation at high frequencies (4). In human challenge studies involving the inoculation of volunteers with gonococci expressing a 3.6 Kdal LOS (reacts with Mab 2-1-L8), isolates examined after infection expressed LOS of 4.8 Kdal (reacts with Mab 1B2) and larger, indicating a phase shift had occurred in vivo (5). Since we (6) had previously identified a genetic locus, *lsi*-2, that was responsible for this type of shift, we were interested in cloning this gene in order to better define the genetic basis for this variation.

We used a set of strains with defined LOS structures as a starting point for our studies. 1291_d is a spontaneous derivative of 1291 that produces a greatly truncated LOS (1). We have determined that the genetic basis of the altered phenotype is an in-frame deletion of 12 nucleotides in the gene encoding phosphoglucomutase. Although this strain fails to produce the appropriate levels of the glucose precursor required for its incorporation to produce wildtype LOS, it is capable of reverting to Mab 2-1-L8 reactivity at a frequency of about 10^{-3}/cell/generation (3). An LOS revertant of this strain, RS132L, was identified based on its ability to bind to Mab 2-1-L8.

In order to clone the gene responsible for the ability of 1291_d to revert to Mab 2-1-L8 reactivity, various restriction enzymes were used to digest RS132L chromosomal DNA and these digested DNAs were screened for the ability to transform 1291_d and 1291 to Mab 2-1-L8 reactivity. All digested DNAs capable of transforming 1291_d to Mab 2-1-L8 reactivity also transformed 1291 to Mab 2-1-L8 reactivity indicating that the reversion of 1291_d to Mab 2-1-L8 reactivity is not the result of a second site suppressor mutation of 1291_d. Digested DNA was fractionated on a sucrose gradient and a fraction was identified that could transform 1291 to Mab 2-1-L8 reactivity. The DNA in this fraction was cloned into the *E. coli* cloning vector pUC19. Many clones were identified but only one orientation of the insert was obtained. One of these clones, pREV1, was selected for further analysis. The insert of pREV1 was subsequently cloned into pKUP, a derivative of pK18 that contains a gonococcal uptake sequence, resulting in clone pREV2. Again, only a single orientation was obtained suggesting that the insert contains a gene that is lethal when expressed from the *lac* promoter of pUC19 and pKUP.

The ability of this cloned gene to modulate LOS expression in strains other than 1291 was also examined. The cloned region was used in a transformation assay to transform several other strains that are able to revert to Mab 2-1-L8 reactivity to Mab reactivity. Transformants of strains F62 and DOV were identified that bound Mab 2-1-L8. LOS was isolated from each of these strains and analyzed by SDS-PAGE. The data indicate that each transformant had an altered LOS profile, where it had lost the ability to express the 1B2 reactive LOS and now expressed the smaller Mab 2-1-L8 reactive LOS.

To map the location of the gene responsible for LOS phase variation, unidirectional deletion clones were generated and used to transform 1291 to Mab 2-1-L8 reactivity. The DNA sequence of the region responsible for the transformation event was determined and revealed an open-reading-frame that contained a stretch of guanine residues that corresponds to the region required for transformation of 1291 to Mab 2-1-L8 reactivity. This data suggests that the mechanism of LOS phase variation involves a DNA slip-strand mechanism identical to that previously reported for gonococcal *pilC* phase variation (2).

References

1. **John, C.M., J.M. Griffiss, M.A. Apicella, R.E. Mandrell and B.W. Gibson**. 1991. The structural basis for pyocin resistance in *Neisseria gonorrhoeae* lipooligosaccharides. J. Biol. Chem. **266**: 19303-19311.
2. **Jonsson, A., G. Nyberg and S. Normark**. 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. **10**: 477-488.
3. **Sandlin, R.C. and D.C. Stein**. 1994. Role of phosphoglucomutase in lipooligosaccharide biosynthesis in *Neisseria gonorrhoeae*. J. Bacteriol. **176**: 2930-2937.
4. **Schneider, H., C.A. Hammack, M.A. Apicella, and J.M. Griffiss**. 1988. Instability of expression of lipooligosaccharides and their epitopes in *Neisseria gonorrhoeae*. Infect.Immun. **56**: 942-946.
5. **Schneider, H., J.M. Griffiss, J.W. Boslego, P.J. Hitchcock, K.M. Zahos and M.A. Apicella**. 1991. Expression of paragloboside-like lipooligosaccharides may be anecessary component of gonococcal pathogenesis in men. J. Exp. Med. **174**: 1601-1605.
6. **Stein, D.C., E.F. Petricoin, J.M. Griffiss and H. Schneider**. Use of transformation to construct *Neisseria gonorrhoeae* strains with altered lipooligosaccharides. Infect. Immun. **56**: 762-765.

Influence of lipooligosaccharide structure on the sensitivity of serogroup B *Neisseria meningitidis* to normal human serum

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To further define the role of lipooligosaccharide (LOS) in resistance to killing by normal human serum, a mutant library in a serogroup B *N. meningitidis* strain was constructed by transformation using the *tetM* containing conjugative transposon, Tn*916*. Over 1500 transformants, accumulated from multiple transformations, were screened for mutations that affected LOS structure. The LOS of the parent strain was 4.5 kDa, contained a lacto-neotetraose moiety (Gal$1-4GlcNAc$1-4Glcl-4Hep1-KDO-Lipid A), and was ~50% sialyated under in vitro aerobic growth conditions. The library was probed in immunoblots with the monoclonal antibody (MAB), 3F11, which recognizes the terminal galactose of the lacto-neotetraose. If the terminal galactose was substituted with a different sugar, completely sialyated or was not present, the transformant would not react with 3F11. The 3F11 negative transformants were further characterized by immunoreactivity with the capsule specific MAB, SC1-3 and the LOS biochemical profile was determined by tricine-SDS PAGE analysis. Transformants that did not react with 3F11 and that had an altered LOS profile by SDS PAGE, but expressed normal amounts of capsule (SC1-3 positive), were selected for further study. Linkage between *tetM* and the phenotype expressed by these strains was demonstrated by transformation of the wild type parent with chromosomal DNA from the transformants. Using these methods, two new LOS mutants designated 559 and 469, which express altered LOS of 3.1 kDa and 2.9 kDa respectively, were identified.

The ability of 559, 469 and two previously identified Tn*916* LOS mutants, R6 and SS3, to resist killing by normal human serum (NHS) was compared to the parent strain. R6 (Hep1-KDO-LipidA), in which the phosphoglucomutase gene (*pgm*) has been interrupted by Tn*916*, has a LOS of 3.1-3.2 kDa (1). SS3 (Glc1-4Hep1-KDO-LipidA) has an insertion in the UDP glucose 4-epimerase gene (*galE*) and expresses a truncated LOS of 3.4 kDa (2). The bactericidal assays were performed with 10% and 25% normal human serum with colony counts determined at 0, 5, 15 and 30 minute intervals. Mutants SS3, 469 and the wild type parent strain, demonstrated >90% survival in 10% and 25% NHS. The R6 mutant showed a one log decrease in survival in 25% serum after 30 minutes, whereas no significant decrease was seen in 10% serum. The LOS mutant, 559, showed a one log decrease in survival after 30 minutes in 10% serum and was completely killed after 30 minutes in 25% serum. In conclusion, truncations in the core oligosaccharide of

meningococcal LOS influence the sensitivity of serogroup B encapsulated *N. meningitidis* to killing by NHS.

References

1. **Zhou, D., D.S. Stephens, B.W. Gibson, J.J. Engstrom, C.F. McAllister, F.K. Lee, and M.A. Apicella.** 1994. Lipooligosaccharide biosynthesis in pathogenic *Neisseria*. Cloning, identification, and characterization of the phosphoglucomutase gene. J. Biol. Chem. **269**: 11162-11169.
2. **Stephens, D.S., C.F. McAllister, D. Zhou, F.K. Lee, and M.A. Apicella.** 1994. Tn*916*-generated, lipooligosaccharide mutants of *Neisseria meningitidis* and *Neisseria gonorrhoae*. Infect. Immun. **62**: 2947-2952.

Influence of capsular polysaccharide and LOS sialylation on serum resistance and invasive properties in meningococci serogroups A, B and C

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Sialic acids are components of the capsular polysaccharide and most of the lipooligosaccharide (LOS) serotypes in meningococci with the serogroups B and C. In contrast, group A strains express a capsule composed of "-2,6-N-acetylmannosamine-1-phosphate, and sialic acids are only LOS-associated with some LOS serotypes. Since sialic acids on bacterial cell surfaces mediate resistance to complement mediated bacteriolysis by inhibition of alternative complement pathway activation, we analysed the role of LOS associated sialic acids on serum resistance in correlation to the capsules of serogroup A, B and C strains. Furthermore, since there was evidence that invasion is inhibited by capsule expression (3), we analysed the role of the capsular polysaccharide and of LOS sialylation with isogenic mutants of meningococcal serogroups A, B and C with defects in capsule and/or LOS sialylation.

A first group of mutants (I) was constructed by transformation of the wild type strains with plasmid pMF32.35:Tn*1725* (1), which contains the capsule gene complex of *Neisseria meningitidis* group B with an inactivated poly-"-2,8 sialyltransferase gene (*siaD*), resulting in a capsule negative phenotype. Mutants of group II were defective in the *galE* gene (2). Since the terminal galactose of the LOS molecule is the acceptor for sialic acid, the *galE* mutants are characterized by an inability to sialylate the LOS. Additionally, mutants of group III exhibited a combined defect in the *siaD* and *galE* genes. The mutants and the parental strains were incubated with 90% C4-deficient guinea pig serum for and the CFU were determined. Mutants I and III of each serogroup were completely killed by the alternative complement pathway, but the *galE* mutants (II) and the wild type strains survived and multiplied about threefold. This indicates, that the LOS associated sialic acid, but not the capsular polysaccharides play a pivotal role in mediating serum resistance to meningococci.

The three serogroups and their mutants were also tested for their ability to adhere to and to invade epithelial cells. Adhesion and invasion were strongly increased in mutants III and I compared to the *galE* mutants (II) and to the wild type strains. These results indicated, that the LOS associated sialic acid has no influence on invasion, but adherence and invasion are strongly inhibited by expression of the capsular polysaccharide in all investigated serogroups. Therefore, since invasion and,

thus, outbreak of meningococcal disease seems to be limited by expression of the capsule, we hypothesize a mechanism, which allows phase variation of encapsulation in meningococci.

References

1. **Frosch, M., Weisgerber, C. and Meyer, T.F.** 1989. Molecular characterization and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria meningitidis* group B. Proc. Natl. Acad. Sci. USA. **86**: 1669-73.
2. **Hammerschmidt, S., Birkholz, C., Zähringer, U., Robertson, B.D., van Putten, J., Ebeling, O., and Frosch, M.** 1994. Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol. Microbiol. **11**: 885-896.
3. **Virji, M., Makepeace, K., Ferguson, D.J.P., Achtman, M., and Moxon, E.R.** 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol. Microbiol. **10**: 499-510.

Effects of capsule and lipooligosaccharide sialylation on the serum resistance of *Neisseria meningitidis*

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During an epidemiological study of an outbreak of meningococcal disease in Gloucestershire, UK (Stonehouse meningococcal survey), the L3,7,9 LOS immunotype of *N. meningitidis* (serotype B15 P1.7,16) was found to be associated with invasive disease, whereas the L1,8,10 immunotype was more commonly expressed by strains isolated from carriers (2). When strains expressing the various LOS immunotypes (L1,8,10 only, L3,7,9 only or both simultaneously) were tested for virulence in an intranasal (i.n.) infant mouse model, case isolates and groupable carrier isolates possessing the L3,7,9 immunotype produced higher levels of nasal colonisation and were more invasive than isolates expressing the L1,8,10 LOS immunotype (3). It was thought that the enhanced virulence in the mouse model was linked to serum resistance.

Capsule is an important factor for survival in the blood, since it was previously shown that mutants lacking in capsule were effectively avirulent in intraperitoneal infection in mice (4). However, recent findings by Hammerschmidt *et al.* (1), using isogenic mutants lacking in either capsule production or in the ability to sialylate LOS, suggested that LOS sialylation might also contribute to resistance of meningococci to complement-mediated lysis. In the present study, both encapsulation and LOS sialylation of the meningococcal strains previously tested for virulence in the i.n. mouse infection model, were assessed for their contribution to serum resistance in an assay using fresh human serum.

LOS sialylation of meningococcal strains: Endogenous sialylation was determined by measuring binding of mab 3F11 to the sialylation site on LOS (by whole cell ELISA). Exogenous sialylation was determined by measuring mab 3F11 binding after growth in the presence of CMP-NANA (5 : g/ml). Only strains expressing the L3,7,9 LOS immunotype were found to possess the sialic acid acceptor site defined by mab 3F11. In most case strains expressing the L3,7,9 LOS immunotype, 60% of the total sialylation was found to be endogenous. Also, groupable carrier isolates that expressed the L3,7,9 LOS immunotype were 60% endogenously sialylated. In contrast, two other carrier isolates (S/H1114 and S/H1497) which expressed high levels of the target epitope for sialic acid were unable to endogenously sialylate their LOS component and only became sialylated after growth in the presence of exogenous CMP-NANA.

Contribution of capsule and LOS sialylation to resistance to complement-mediated killing by human serum: The presence of the polysialyl capsule was found to be an important factor in serum resistance of meningococci, since all isolates expressing high levels of capsule had some resistance in serum and all isolates expressing low amounts of capsule were sensitive to serum, regardless of levels of LOS sialylation.

LOS sialylation however, also appeared to contribute to serum resistance, since after growth in exogenous CMP-NANA, the serum resistance of all strains that were endogenously sialylated, increased. In contrast, exogenous sialylation afforded no protection against serum killing of carrier strains (S/H1114 and S/H1497) despite these strains expressing high levels of the sialylatable LOS component. Two case strains expressing the L1,8,10 LOS immunotype also experienced an increase in serum resistance after growth in exogenous CMP-NANA, despite lacking the terminal galactose acceptor for sialic acid (defined by mab 3F11), indicating that other acceptor sites for sialic acid may exist on LOS that have not yet been defined.

Levels of endogenous LOS sialylation were shown to affect serum survival rates. A carrier strain possessing only 24% capsule but being 90% endogenously sialylated had a higher serum survival rate (24.5%) than other fully encapsulated strains.

Serum resistance as a factor in murine virulence: A direct correlation was observed between resistance to human serum and virulence of meningococci in the i.n. infant mouse model. Most serum resistant strains produced high levels of nasal colonisation (above $5.0x10^4$ CFU/mouse) and disseminated to cause blood infection. In contrast, serum sensitive strains produced low levels of nasal colonisation (below $5.5x10^3$ CFU/mouse) and failed to cause blood infection (3).

Thus, serum resistance was found to be directly related to virulence of meningococcal strains in infant mice following i.n. infection. However, the relative importance of capsule expression and LOS sialylation on serum resistance, varied between strains and neither alone resulted in high levels of serum resistance, suggesting an interactive mechanism between the two factors. Capsule appeared to be a primary determinant conferring serum resistance, but the resistance of capsulate strains was enhanced by LOS sialylation.

References

1. **Hammerschmidt, S., C. Birkholz, U. Zahringer, B.D. Robertson, J. Vanputten, O. Ebeling and M. Frosch**. 1994. Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria Meningitidis*. Mol. Microbiol. **11**: 885-896.
2. **Jones, D.M., R. Borrow, A.J. Fox, S. Gray, K.A. Cartwright and J. T. Poolman**. 1992. The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb. Pathog. **13**: 219-224.
3. **Mackinnon, F.G., R. Borrow, A.R. Gorringe, A.J. Fox, D.M. Jones and A. Robinson**. 1993. Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. Microb. Pathog. **15**: 359-366.
4. **Masson, L. and B.E. Holbein**. 1982. Virulence linked to polysaccharide capsule production in serogroup B *Neisseria meningitidis*. FEMS Microbiol Letts. **13**: 187-190.

Phenotypic switching of LOS immunotype expression during murine infection with *Neisseria meningitidis*

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Lipooligosaccharide (LOS) is a surface exposed component of the bacterial cell subject to attack by bactericidal antibody. A number of pathogens such as *Neisseria gonorrhoeae* (4) and *Haemophilus influenzae* (3) have demonstrated a phenotypic change of LOS during infection. Similarly, meningococcal LOS has been shown to undergo change during human infection (2) from the non-sialylatable form in local infection isolates to the sialylatable form in disseminated infection isolates.

During previous virulence studies in mice of serotype B:15:P1.7,16 meningococcal case and carrier isolates, the L3,7,9 LOS immunotype was found to be associated with increased virulence (1). Two case isolates (L91 1134 and L352) which were atypical in that they expressed only the L1,8,10 LOS immunotype, were found to switch LOS expression and also expressed the L3,7,9 immunotype, following intranasal infection in infant mice.

Characterisation of the phenotypic change in LOS immunotype expression: After further investigation, populations of strains L91 1134 and L352 isolated during murine infection were found to consist of mixed populations of individual cells expressing either the L1,8,10 immunotype, the L3,7,9 immunotype or both simultaneously. Proportions of each immunotype varied depending on the time and site of sample. All Isolates sampled from mice during infection were found to be fully encapsulated.

The presence of single colony murine isolates expressing both the L3,7,9 and L1,8,10 immunotypes indicates that the immunotype switch observed *in vivo* represents a true phenotypic switch rather than selection of a small number of L3,7,9 bacteria present in the inocula. To support this data, a hundred colonies of strain L352 inoculum isolates were analysed for immunotype expression and all were found to express the L1,8,10 LOS immunotype only.

Since LOS switching was shown to occur at high frequency *in vivo*, the stability of immunotype expression of LOS variants *in vitro* was assessed. After 10 subcultures on blood agar (containing vancomycin supplement), the immunotype expression was found to be stable, suggesting that environmental conditions are crucial to the control of LOS expression. In a previous *in vitro* study on *N. meningitidis*, levels of aeration were shown to effect LOS expression, with low aeration giving rise to an increase in

the high MW LOS which was sialylatable; these growth conditions may have mimicked the *in vivo* situation (5).

Sialylation and serum resistance of LOS variants: Only the strain L91 1134 LOS variants (isolated from murine infection) expressing the L3,7,9 LOS immunotype possessed the acceptor site for sialic acid (defined by mab 3F11). Sialylation of this epitope has previously been shown to induce serum resistance in *N. gonorrhoeae*. Therefore the levels of endogenous and exogenous sialylation and their effects on resistance to killing by fresh human serum was assessed in the LOS variants. Endogenous sialylation was determined by measuring binding of mab 3F11 to the sialylation site on LOS by whole cell ELISA. Exogenous sialylation was determined by measuring mab 3F11 binding after growth in the presence of CMP-NANA (5 ∶g/ml). There was an increase in serum resistance of all variants after growth in CMP-NANA, despite the fact that the L1,8,10 variant possessed no detectable sialylatable epitope (defined by mab 3F11). This was also observed with the parent strains (L91 1134 and L352) and suggests that other target epitopes for sialylation may exist on meningococcal LOS. There was a direct correlation between 3F11 epitope expression and survival rates of meningococci in human serum, since the L3,7,9 LOS variant expressed the highest amount of the sialylatable epitope and was the most the most serum resistant.

In this study LOS expression was shown to be subject to high frequency switching under *in vivo* conditions, but remained stable after *in vitro* subculture. The expression of the sialylatable LOS component following i.n. infection in mice was found to confer an increased level of serum resistance on LOS variants, possible explaining the association of its expression to invasive meningococcal infection.

References

1. **Mackinnon, F.G., R. Borrow, A.R. Gorringe, A.J. Fox, D.M. Jones and A. Robinson.** 1993. Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. Microb. Pathog. **15**: 359-366.
2. **Patrick, C.C., G.T. Furuta, M. Edwards, M. Estabrook, M.S. Blake and C.J. Baker.** 1993. Variation in phenotypic expression of the opa outer membrane protein and lipooligosaccharide of *Neisseria meningitidis* serogroup C causing periorbital cellulitis and bacteremia. Clin. Inf. Dis. **16**: 523-527.
3. **Rubin, L.G. and J.W. St. Geme.** 1993. Role of lipooligosaccharide in virulence of the Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup *aegyptius* for infant rats. Infect. Immun. **61**: 650-655.
4. **Schneider, H., J.M. Griffiss, J.W. Boslego, P.J. Hitchcock, K.M. Zahos and M.A. Apicella.** 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. J. Exp. Med. **174**: 1601-1605.
5. **Tsai, C., R Boykins and C.E. Frasch.** 1983. Heterogeneity and variation among *Neisseria meningitidis* lipopolysaccharides. J. Bact. **155**: 498-504.

The relationship between encapsulation and sialylation of meningococcal lipooligosaccharide

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The importance of polysialic acid capsules as a major virulence determinant for invasive meningococcal disease is well established (1). Meningococci undergo phase variation in capsular polysaccharide expression whereby organisms exist in either the capsulate form, associated with invasive disease, or the noncapsulate form which is associated with isolates from the nasopharynx and the establishment of the carrier state. Recent work has suggested that the importance of sialic acid in meningococcal pathogenesis extends to the sialylation of another major surface component, the lipooligosaccharide. Since 1982 there has been a prolonged outbreak of meningococcal disease due to B:15:P1.7,16 sulphonamide resistant strains in the Gloucester Health district, England. This outbreak prompted an epidemiological investigation (Stonehouse Meningococcal Survey, SMS) into meningococcal disease resulting in the recovery of meningococcal isolates from asymptomatic carriers including strains with the same phenotype as the outbreak strain with the exception that a number of strains were noncapsulate (3). Selected strains from this collection of epidemiologically related isolates have since been demonstrated to be genetically similar and related to members of the ET-5 complex, a group of related organisms which have been responsible for numerous epidemics of meningococcal disease in Europe and elsewhere (4).

We have examined a selection of case and carrier strains from this collection for their LOS immunotype and there appears to be an association between expression of the L3,7,9 immunotype and invasive disease, whereas the L1,8,10 immunotype was associated with carriage (5). The relationship between the L3,7,9 and L1,8,10 immunotypes and meningococcal virulence of this collection of case and carrier strains was examined using an infant mouse intranasal infection model. The results indicate that in addition to the capsule, the L3,7,9 immunotype confers increased virulence due to greater colonisation of the nasopharynx and ability to invade infant mice (6). We concluded that the L3,7,9 LOS immunotype acts as a secondary virulence factor and others have shown, using capsule$^-$ and LOS$^-$ isogenic, mutants that both meningococcal capsule and LOS contribute to resistance to serum-mediated killing (7). These phenomena are of fundamental importance to understanding the pathogenesis of meningococcal infection and the regulation of sialic acid biosynthetic processes may be central to both these phenomena.

We have examined related case and carrier isolates, representing the observed capsule and LOS phenotypes, using reactivity with the monoclonal antibody (mab) 3F11 as a surrogate for sialylation (8). Briefly, capsulate case and capsulate and noncapsulate carrier strains, expressing the LOS immunotype L3,7,9 were examined for 3F11 binding using a whole cell ELISA. 3F11 is directed against the terminal lacto-N-neotetraose residue and antibody binding is blocked by sialylation. Capsule expression was quantified using a group B polysaccharide specific mab and whole cell ELISA. Several of the case strains were found to express only trace amounts of the 3F11 epitope which was not detectable on other strains. This lack of reactivity was not due to sialylation since no further increase in 3F11 binding was demonstrated following neuraminidase treatment. Those case strains which were expressing low levels of 3F11 were endogenously sialylated. Similar results were obtained for the groupable carrier strains (capsule expression for all of these strains was determined to be between 50 and 100% that of a collection of groupable case isolates), whereas the non-groupable carrier strains for which the level of capsule expression was less than 5% or not detectable were found to have greatly increased expression of the 3F11 epitope and all were found to exogenously sialylate their LOS in the presence of CMP-NANA.

These results demonstrate a relationship between group B capsule biosynthesis, 3F11 expression and LOS sialylation. It is possible that the down-regulation of the capsular polysaccharide results in the loss of endogenous substrate (CMP-NANA) for LOS sialylation. Mandrell *et al* (9) have presented evidence for the existence of a LOS-specific silayltransferase which may be regulated independently of the *cps* complex thus enabling the use of exogenous substrate for LOS sialylation in the absence of endogenous CMP-NANA from capsule biosynthesis. The phenomena described above are central to the control of virulence by group B meningococci and knowledge in this area has major implications for the design of effective vaccines for group B meningococci.

References

1. **Cross, AS.** In: Current topics in microbiology and immnuology, (Jann, K. and Jann, B., Eds). 1990. **150**: 87-96, Springer, Berlin.
2. **Cartwright, K.A.V., Stuart J.M., Jones D.M. and Noah N.D.** The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidem. Infect. 1987. **99**: 591-601.
3. **Caugant, D.A., Froholm L.O., Bovre K., Holten E., Frasch C.E., Mocca L.F., Zollinger W.D., Selander R.K.** Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc. Natl. Acad.Sci. USA. 1986. **83**: 4927-4931.
4. **Jones, D.M., Borrow R., Fox A.J., Gray S., Cartwright K.A.V. and Poolman J.T.** The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb. Pathogen. 1992. **13**: 219-224.

5. **MacKinnon, F.G., Borrow R., Gorringe A.R., Fox A.J., Jones D.M., Allison A. and Robinson A.** Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. Microb. Pathogen. 1993. **15**:359-366.
6. **Hammerschmidt, S., Birkholz C., Zahringer U., Robertson B.D., van Putten J., Ebeling O. and Frosch M.** Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol. Microbiol. 1994. **11**: 885-896.
7. **Yamasaki, R., Nasholds W., Schneider H. and Apicella M.A.** Epitope expression and partial structural characterisation of F62 lipooligosaccharide (LOS) of *Neisseria gonorhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M) recognise non-reducing termini of the LOS components. Mol. Immunol. 1991. **28**: 1233-1242.
8. **Mandrell R.E., Griffiss J.M., Smith H. and Cole J.A.** Distribution of a lipooligosaccharide-specific sialyltransferase in pathogenic and non-pathogenic *Neisseria*. Microb. Pathogen. 1993. **14**: 315-327.