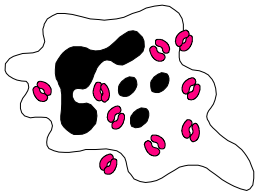


Lipooligosaccharides

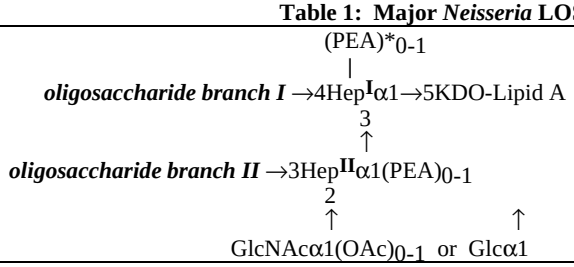
The lipooligosaccharides of the pathogenic *Neisseria.*

MA Apicella[1], P Densen[1], A Preston[1], RE Mandrell[2], DS Stephens[3], DC Stein[4], BW Gibson[5]

[1]Department of Medicine and Microbiology, The University of Iowa College of Medicine, [2]USDA, Albany, CA, [3]Department of Medicine, Emory University School of Medicine, [4]Department of Microbiology, The University of Maryland and [5]Department of Pharmaceutical Chemistry, University of California at San Franscisco, San Francisco, CA

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

Table 1: Major *Neisseria* LOS Structures



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

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

Physicochemical analysis: A structural model has been proposed for the LOS of gram-negative pathogenic bacteria that colonize human mucosa, e.g. pathogenic *Neisseria* and *Haemophilus* (Table 1). This model shows that the deep core of *Neisseria* LOSs contain two KDO and two heptoses moieties. The proposed LOS model has several unique features that distinguish it from those developed for the lipopolysaccharides of enteric bacteria. To simplify the nomenclature, the heptoses are numbered with the first heptose being the one directly linked to KDO. Oligosaccharide chains extending from the heptoses are designated "oligosaccharide branch I" if it extends from the first heptose and "oligosaccharide branch II" if the chain extends from the second heptose. The development of this model has involved analysis of a series of pyocin-resistant gonococcal mutants with altered LOS and other recent immunochemical and structural

data. Analysis of this series of pyocin mutants revealed that the oligosaccharide attached to the first heptose, branch I, of the LOS structure was sequentially assembled to the structure Galβ1→4Glc (Table 1). After this point in assembly, divergence in assembly occurs. The Galβ1→4GlcNAcβ1→3Galβ1→4Glc structure is the predominant structure formed. The Galβ1→3Galβ1→4Glc structure can also be formed from the Galb1→4Glc.

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

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

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

It had been observed that gonococci in urethral exudates possess a virulence factor that is lost when the bacteria are subcultured onto an artificial medium. Attempts to purify the inducing factor resulted in both a high and a low Mr factor being detected in extracts from human red blood cells. The low Mr activity was discovered to be the nucleotide sugar for sialic acid, cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA). Purified CMP-NANA in nanogram amounts duplicated all of the effects of the low Mr factor with gonococci including a major alteration in the strain's LOS. Immunoelectronmicroscopy of gonococcal infected male urethral exudates indicated <u>in vivo</u> sialylation of the 4.5 kDa paragloboside-like LOS component.

Previous studies had indicated that the 4.5 kDa lactoneoseries LOS is present in some strains of meningococcal serogroup B and C strains. However, the pattern of binding of MAb 3F11 to

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

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

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

An operon responsible for the inner core biosynthesis of the LOS of *N. meningitidis* has been identified. Using *Tn916* mutagenesis, the a1,2 *N*-acetylglucosamine transferase gene (*rfaK*) was

identified which, when inactivated, prevents the addition of GlcNAc to hepII of the meningococcal LOS inner core. This mutant was also deficient in the chain I oligosaccharide extension suggesting that the lack of extension of this chain was due to structural constraints imposed by the incomplete biosynthesis of the LOS inner core.

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

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

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

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

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

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

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

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

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

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

species suggests that sialylation may interfere with the cross linking of the bactericidal epitopes that is required for IgM conformational change prior to complement activation (Abstract this meeting).

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

Studies of the human immune response *N. meningitidis* has demonstrated a striking inverse correlation between the presence of complement dependent bactericidal activity in serum and susceptibility to meningococcal disease. This activity was antibody (IgG) dependent and was acquired with increasing age beginning around the age of 6 months to a year. Anti-capsular antibodies were shown to be enormously effective in initiating complement dependent killing as well as opsonophagocytic killing of appropriate isolates. However, persons convalescing from meningococcal disease rarely develop a second infection despite the existence of multiple meningococcal serogroups. Those individuals who do experience recurrent disease commonly have some immunologically identifiable defect - typically a defect in one of the terminal complement components that mediate complement dependent killing. In addition, quantitation of anti-capsular antibodies in the sera possessing bactericidal activity for a given strain demonstrates only weak correlation with the extent of that activity. In total, these observations suggest that antibodies, in addition to those directed at capsular polysaccharides, are important in mediating natural protection against meningococcal disease. This conclusion is of enormous impact for the prevention of serogroup B meningococcal disease, since the bulk of evidence suggests that immune exclusion in humans prevents them from making antibodies to the homopolymeric sialic acid which forms the capsule of this meningococcus.

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

former and that these cross reactive antibodies form the basis for a large part of the natural immunity against meningococcal disease. The epitopes on subcapsular antigens that give rise to these cross protective antibodies remain poorly defined.

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

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

References

Because of space limitations, the authors of this review have limited their refernce list to one current extensive review article which cites the work discussed in this abstract.

1 Preston A, Mandrell RE, Gibson BW, Apicella MA. The lipooligosaccharides of pathogenic Gram-negative bacteria. CRC Critical Reviews in Microbiology, in Press.

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

CM Kahler, RW Carlson, MM Rahman, LE Martin and DS Stephens.

Departments of Medicine and Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia; Research Service, VA Medical Center, Atlanta, Georgia; and Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia.

Lipooligosaccharide (LOS) is a critical virulence factor involved in many aspects of meningococcal pathogenesis. Gotschlich et al. (1) identified the *lgtA-E* operon required for the biosynthesis of the Galb1-4GlcNAcb1-3Galb1-4 epitope of the a-chain of LOS which is added to the substrate Glcb1-4Hep_2-R. We have used *Tn*916 mutagenesis to identify a second operon, termed *ice* (inner core extension), which is required for LOS inner core assembly and the addition of the a chain to this structure. We recently characterized a LOS mutant, 559, in which the a 1,2 *N*-acetylglucosamine transferase (*rfaK*) was inactivated by *Tn*916 (2). Inactivation of *rfaK* in the L2 serogroup B strain NMB results in a LOS structure without GlcNAc or glucose attached to HepII, as well as preventing the extension of a chain from HepI (ie. Hep_2-R). We concluded from these results that the addition of GlcNAc to HepII was a prerequisite for further LOS synthesis. During the study of *rfaK*, a second ORF (*lgtF*) of 720 bp was found upstream of *rfaK*. The amino terminus of LgtF had significant homology with a family of b-glucosyltransferases involved in the biosynthesis of polysaccharides and O-antigens of LPS (3). *LgtF* was inactivated by insertion of a non-polar *aphA-3* cassette, thereby minimizing potential polar effects on *rfaK*. Tricine SDS-PAGE and composition analysis of the LOS from the non-polar *lgtF* mutant showed that this strain produced a truncated LOS structure containing a complete LOS inner core, $GlcNAc_1Hep_2$-R, but without the a-chain attached to HepI or glucose to HepII. These results and the amino acid homology with b-glycosyltransferases indicate that *lgtF* is involved in LOS biosynthesis and suggest that it encodes the previously undefined UDP glucose:lipooligosaccharide b1,4 glucosyltransferase which attaches the first residue of the a chain to HepI to form Glcb1-4Hep_2-R which is the substrate for the *lgt* operon. RT-PCR and primer extension analysis indicate that both *lgtF* and *rfaK* are co-transcribed as a polycistronic message from a promoter upstream of *lgtF*. This arrangement suggests that the completion of the LOS inner core and the extension of the a-chain are coordinated in *N. meningitidis* through the *ice* operon.

References.

1. Gotschlich EC. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. J Exp Med **1994**; 180:2181-90
2. Kahler CM, Carlson RW, Rahman, MM, Martin, LE, and Stephens, DS. Inner core biosynthesis of lipooligosaccharide in *Neisseria meningitidis* serogroup B: identification and role in LOS assembly of the a 1,2 *N*-acetylglucosamine transferase (RfaK). J Bacteriol **1996**; 178:1265-73

3. Saxena IM, Brown Jr. RM, Fevre M, Geremia RO, Henrissat B. Multidomain architecture of b-glycosyl transferases: implications for mechanism of action. J Bacteriol **1995**; 177:1419-24

Regulation of gonococcal sialyltransferase expression

DJ McGee and RF Rest

MCP-Hahnemann School of Medicine, Allegheny University of the Health Sciences, Philadelphia, PA

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

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

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

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

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

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

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

References

1. Smith H, Parsons NJ and Cole JA. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. Microb Pathog **1995**; 19:365-377.
2. Rest RF and Mandrell RE. *Neisseria* sialyltransferases and their role in pathogenesis. Microb Pathog **1995**; 19:379-390.
3. Britigan BE, Klapper D, Svendsen T and Cohen MS. Phagocyte-derived lactate stimulates oxygen consumption by *Neisseria gonorrhoeae*. An unrecognized aspect of the oxygen metabolism of phagocytosis. J Clin Invest **1988**; 81:318-324.
4. Parsons NJ, Boons GJ, Ashton PR, Redfern PD, Quirk P, Gao Y, Constantinidou C, Patel J, Bramley J, Cole JA and Smith H. Lactic acid is the factor in blood cell extracts which enhances the ability of CMP-NANA to sialylate gonococcal lipopolysaccharide and induce serum resistance. Microb Pathog **1996**; 20:87-100.
5. Norrod EP, Burnham JS, Williams RP and Ding MJ. Induced surface changes in the surface of *Neisseria gonorrhoeae*. Can J Microbiol **1983**; 29:584-592.
6. Norrod EP. A role for sulfite in inducing surface changes in *Neisseria gonorrhoeae*. Can J Microbiol **1984**; 30:1297-1301.

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

NJ Parsons[1], MJ Gill[2], DP McQuillen[3], JPM van Putten[4], LM Wetzler[3], JP Émond[5], M Goldner[6], J Bramley[1], H Crooke[1], JA Cole[1], and H Smith[7]

[1]School of Biochemistry, [2] Department of Infection and the [7] Medical School, [1-3]University of Birmingham, Birmingham, B15 2TT, UK; [3]Maxwell Finland Laboratory for Infectious Diseases, Boston City Hospital, Boston, Mass. 02118, USA: [4]Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, NIAD, NIH, Hamilton, MT 59840-2999, USA: [5]Laboratoire de Bactériologie, Centre Hospitalier Intercommunal de Villeneuve-Saint-Georges, France and [6]Department of Microbiologie, Faculté de Medécine, Université Laval, Quebec, Canada G1K 7P4

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

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

The assay involved sialylation of LPS by CMP-^{14}CNANA and scintillation counting of the labelled LPS after fixing it on filter paper strips by trichloracetic acid and washing it free from unincorporated CMP-^{14}CNANA. It was rapid, reproducible and, although the enzyme preparations contained endogenous LPS, dependent on added LPS for maximum activity. Using these methods it was shown that a wide range of concentrations of lithium-L-lactate did not enhance the activity of the extracted sialyltransferase (4).

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

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

References

1. Bramley J, Demarco de Hormaeche R, Constantinidou C, Nassif Y, Parsons NJ, Jones P, Smith H, Cole JA. A serum sensitive sialyltransferase-deficient mutant of *Neisseria gonorrhoeae* defective in conversion to serum resistance by CMP-NANA or blood cell extracts. Microb Pathog **1995**; 18:187-95.
2. Gill MJ. McQuillen DP, van Putten JPM, Wetzler LM, Bramley J, Crooke H, Parsons NJ, Cole JA, Smith H. Functional characterization of a sialyltransferase-deficient mutant of *Neisseria gonorrhoeae.* Infect Immun **1996;** 64: In Press.
3. Parsons NJ, Boons GJ, Ashton PR, Redfern PD, Quirk P, Gao Y, Constantinidou C, Patel J, Bramley J, Cole JA, Smith H. Lactic acid is the factor in blood cell extracts which enhances the ability of CMP-NANA to sialylate gonococcal lipopolysaccharide and induce serum resistance. Microb Pathog **1996**; 20; 87-100.
4. Parsons NJ, Emond J-P, Goldner M, Bramley J, Crooke H, Cole JA, Smith H. Lactate enhancement of sialylation of gonococcal lipopolysaccharide and of induction of serum resistance by CMP-NANA is not due to direct activation of the sialyltransferase: metabolic events are involved. Microb Pathog **1996;** 21: In Press.

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

DC Stein, CL Burch and RJ Danaher

Department of Microbiology, University of Maryland , College Park, Maryland 20742.

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

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

N. gonorrhoeae 1291 contains a functional *lsi-2 (lgtA)* gene and it expresses a single LOS component that reacts with MAb 1B2; when this gene is nonfunctional due to a +1 frame shift caused by the insertion of a guanine into a polyguanine tract within the gene, the gonococcus expresses a single LOS component that reacts with the MAb 2-1-L8 (3). We will present data that shows that *N. gonorrhoeae* strain FA19 simultaneously expresses in the same cell both of these LOS components. The genetic locus responsible for this phenotype in FA19 was identified by isolating a clone that is able to impart on strain 1291 the ability to simultaneously express both LOS molecules. This clone, pCLB1, was characterized by DNA sequence analysis. The data indicate that the gene responsible for the expression of both LOS components on the same cell is also *lsi-2*. DNA sequence analysis of $lsi\text{-}2_{FA19}$ indicated that there were several differences in the DNA sequence, relative to $lsi\text{-}2_{1291}$. The region responsible for the LOS-specific phenotype change in $lsi\text{-}2_{FA19}$ was identified by deletion and transformation analysis. The region responsible for the phenotypic change mapped to the polyguanine tract within *lsi-2* where $lsi\text{-}2_{FA19}$ possessed a +2 frameshift within this polyguanine repeat, relative to $lsi\text{-}2_{1291}$. The polyguanine tract in $lsi\text{-}2_{FA19}$ was modified by site directed mutagenesis to change the sequence GGGGGGGGGGGGG to GGGAGGTGGCGGA. This change in DNA sequence does not alter the protein sequence of the in- frame gene. This change in the DNA sequence will

prevent the DNA sequence from changing during DNA replication. Transformants of 1291 generated with this clone only expressed a single LOS component that reacted with MAb 2-1-L8. From this data, we concluded that FA19, even though *lsi-2*$_{FA19}$ is out of frame with respect to *lsi-2*$_{1291}$, is able to generate a small amount of a functional LSI2 protein via transcriptional frame shifting, and this limited amount of protein allows for the expression of some "wild type" LOS molecules. This results in the production of cells that can express two different LOSs on its cell surface at the same time. These data indicate that a cell only needs to express a small amount of LSI2 protein to produce MAb 1B2 reactivity.

References

1. Apicella MA, Shero M, Jarvis GA, Griffiss JM, Mandrell RE, Schneider H. Phenotypic variation in epitope expression of the *Neisseria gonorrhoeae* lipooligosaccharide. Infect Immun **1987**; 55: 1755-1761.
2. Gibson BW, Webb JW, Yamasaki R, Fisher SJ, Burlingame AL, Mandrell RE, Schneider H, Griffiss JM. Structure and heterogeneity of the oligosaccharides from the lipopolysaccharides of pyocin-resistant *Neisseria gonorrhoeae*. Proc Natl Acad Sci. USA **1989**; 86:17-21.
3. Schneider H, Hale TL, Zollinger WD, Seid RC, Hammack CA, Griffiss JM. Heterogeneity of molecular size and antigenic expression of lipooligosaccharides of individual strains of *Neisseria gonorrhoeae.* Infect Immun **1984**; 45:544-549.
4. Yamasaki R, Bacon BE, Nasholds W, Schneider H, Griffiss JM. Structural determination of oligosaccharides derived from lipooligosaccharides of *Neisseria gonorrhoeae* F62 by chemical, enzymatic and two-dimensional NMR methods. Biochemistry **1991**; 30:10566-10575.
5. Schneider H, Griffiss JM, Boslego JW,Hitchcock PJ, Zahos KM, Apicella MA. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. J Exp Med **1991**; 174:1601-1605.
6. Gotschlich E. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. J Exp Med **1994**; 180:2181-2190.
7. Danaher RJ, Levin JC, Arking D, Burch CL, Sandlin R, Stein DC. Genetic basis of *Neisseria gonorrhoeae* lipooligosaccharide antigenic variation. J Bacteriol **1995**; 177:7275-7279.
8. Yang QL, Gotschlich EC. Variation of gonococcal lipooligosaccharide structures is due to alterations in poly-G tracts in *lgt* genes encoding glycosyl transferases. J Exp Med **1996**; 183:323-327.

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

P van der Ley, M Kramer, L Steeghs and JT Poolman

Laboratory of Vaccine Development and Immune Mechanisms, National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands

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

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

Up to date, no genes involved in Neisserial lipid A biosynthesis have been reported. By complementation of a temperature-sensitive *E.coli lpx*D mutant, we have cloned a meningococcal chromosomal fragment that carries the *lpx*D homologue. At the restrictive temperature of 420C, LPS biosynthesis was restored in this E. coli mutant when a plasmid carrying the meningococcal *lpx*D was present. Cloning and sequence analysis of chromosomal DNA downstream of *lpx*D revealed the presence of the *fab*Z and *lpx*A genes, followed by an inverted repeat that might function as transcriptional terminator. In contrast to *E. coli* and several other bacterial species, no *lpx*B gene was found directly downstream of *lpx*A. The LpxA and LpxD proteins catalyze early steps in the lipid A biosynthesis pathway, adding the O- and N-linked 3-OH fatty acyl chains (3). In E. coli and N. meningitidis, the LpxD proteins have the

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

References

1. van der Ley P, Kramer M, Steeghs L, Kuipers B, Andersen SR, Jennings MP, Moxon ER, Poolman JT. Identification of a locus involved in meningococcal lipopolysaccharide biosynthesis by deletion mutagenesis. Mol Microbiol **1996**; 19:1117-1125.
2. Jennings MP, Hood DW, Peak IRA, Virji M, Moxon ER. Molecular analysis of a locus for the biosynthesis and phase-variable expression of the lacto-N-neotetraose terminal lipopolysaccharide structure in *Neisseria meningitidis*. Mol Microbiol **1995**; 18:729-740.
3. Raetz CRH. Bacterial endotoxins: extraordinary lipids that activate eukaryotic signal transduction. J Bacteriol **1993**; 175:5745-5753.
4. Raetz CRH, Roderick SL. A left-handed parallel ? helix in the structure of UDP-N-acetylglucosamine acyltransferase. Science **1995**; 270:997-1000.

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

J Bramley[1], H Crooke[1], NJ Parsons[1], H Smith[2] and JA Cole[1]

[1]School of Biochemistry and [2]The Medical School, University of Birmingham, Birmingham B15 2TT, UK

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

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

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

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

Acknowledgment. The authors are grateful to David McGee and Rick Rest for communicating their data in advance of publication and to John Heckels and Cathy Ison for gifts of strains.

References.

1. Bramley J, Demarco de Hormaeche R, Constantinidou C, Nassif X, Parsons N, Jones P, Smith H, Cole J. A serum-sensitive, sialyltransferase-deficient mutant of *Neisseria gonorrhoeae* defective in conversion to serum resistance by CMP-NANA or blood cell extracts. Microb Pathogen **1995**; 18:187-195.
2. Mandrell RE, Smith H, Jarvis JA, Griffiss JM, Cole JA. Detection and some properties of the sialyltransferase implicated in the sialylation of lipopolysaccharide of *Neisseria gonorrhoeae*. Microb. Pathogen **1993**; 14:307-313.

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

B Chan, O Daramola, U Grunwald, and M Levin

Paediatric Infectious Disease Unit, Imperial College School of Medicine at St. Mary's, South Wharf Road, London W2 1NY, UK.

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

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

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

LPS induced TF expression in HUVECs was blocked by MAbs CD14 when either added before or 5 min after the LPS. However, MAbs CD14 were ineffective at blocking meningococcal induced TF expression. Similarly, $rBPI_{23}$ blocked LPS induced TF expression if added before the LPS but had no effect on meningococcal induced TF expression.

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

References

1. Brandtzaeg P, Kierulf P, Gaustad P, Skulberg A, Bruun JN , Halvorsen S, Sorensen E. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J Infect Dis **1989**; 159 (2)**:** 195-204.
2. Dentener MA, Bazil V, Von AE, Ceska M, Buurman WA. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J Immunol **1993**; 150: 2885-91.
3. Haziot, A., G. W. Rong, J. Silver, and S. M. Goyert. Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. J Immunol **1993**; 151: 1500-7.
4. Chan B, Kalabalikis P, Klein N, Heyderman R, Levin M. Assessment of candidate anti-inflammatory treatments in an *in vitro* whole blood model of meningococcal sepsis [abstract]. In: Neisseria 94: Proceedings of the Ninth International Pathogenic Neisseria Conference (Winchester, England). **1994**: 297-298.
5. Heyderman RS, Klein NJ, Daramola OA, Levin M. Modulation of the endothelial procoagulant response to lipopolysaccharide and tumour necrosis factor-a in-vitro: The effects of dexamethasone, pentoxifylline and a polyclonal anti-human IL-1a antibody. Inflamm Res **1995**; 44: 275-280.

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

MM Estabrook

Case Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

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

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

All 9 strains made LNnT that was variously endogenously sialylated, as judged by the binding of MAb 1B2 before and after removal of sialic acid by neuraminidase. For endogenously sialylated meningococcal strains, (grown without CMP-NANA), susceptibility to SBA of PHS correlated with the amount of unsialylated LNnT ($r^2 = 0.83$) above a threshold of LNnT expression; strains that expressed less than the threshold survived in 25% PHS. Non-disseminated isolates were not more sensitive to SBA than case isolates that expressed an equivalent amount of LNnT.

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

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

References

1. Mandrell RE, Kim JJ, John CM, et al. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J Bacteriol **1991**;173:2823-2832.
2. Mandrell RE, Griffiss JM, Macher BA. 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. J Exp Med **1988**;168:107-126.
3. Estabrook MM, Christopher NC, Griffiss JM, Baker CJ, Mandrell RE. Sialylation and human neutrophil killing of group C *Neisseria meningitidis*. J Infect Dis **1992**;166:1079-1088.
4. Apicella MA, Mandrell RE, Shero M, et al. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: An immunoelectron microscopic analysis. J Infect Dis **1990**;162:506-512.
5. Mandrell RE, Lesse AJ, Sugai JV, et al. In vitro and in vivo modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. J Exp Med **1990**;171:1649-1664.
6. Parsons NJ, Andrade JRC, Patel P, A CJ, Smith H. Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5'-monophospho-N-acetyl neuraminic acid. Microb Path **1989**;7:63-72.

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

MM Estabrook and M Berger

Case Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

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

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

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

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

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

References

1. Estabrook MM, Christopher NC, Griffiss JM, Baker CJ, Mandrell RE. Sialylation and human neutrophil killing of group C *Neisseria meningitidis.* J Infect Dis **1992**;166:1079-1088.
2. Ross SC, Rosenthal PJ, Berberich HM, Densen P. Killing of *Neisseria meningitidis* by human neutrophils: implications for normal and complement-deficient individuals. J Infect Dis **1987**;155:1266-1275.
3. Schlesinger M, Greenberg R, Levy J, Kayhty H, Levy R. Killing of meningococci by neutrophils: Effect of vaccination on patients with complement deficiency. J Infect Dis **1994**;170:449-453.
4. Stephens DS, McAllister CF, Zhou D, Lee FK, Apicella MA. Tn*916*-generated lipooligosaccharide mutants of *Neisseria meningitidis* and *neisseria gonorrhoeae.* Infect Immun **1994**;62:2947-2952.
5. Zhou D, Stephens DS, Gibson BW, et al. Lipooligosaccharide biosynthesis in pathogenic *Neisseria.* Cloning, identification, and characterization of the phosphoglucomutase gene. J Biol Chem **1994**;269:11162-11169.

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

S Gulati, DP McQuillen, RE Mandrell, and PA Rice

The Maxwell Finland Laboratory for Infectious Diseases, Boston Medical Center, Boston Univ. School of Medicine, USDA-Agricultural Research Service, Albany CA.

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

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

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

We examined mAb 2C7 binding to Western blots of LOS prepared from gonococcal strains. MAb 2C7 binds to strain 15253 LOS, which possesses lactosyl (Galβ1$\rightarrow$4Glc) substitutions at both heptose 1 and 2 (8), but not to strain 1291c LOS, which differs from 15253 by the lack of

this lactosyl substitution on heptose 2 (4). Collectively these data suggest that the 2C7 epitope may involve the lactosyl (Galβ1→4Glc) substitution of heptose 2.

Acknowledgments. This work was supported by grants R01 AI32725 and P01 AI33087 (PAR) and K11 AI01061 (DPM) from the National Institutes of Health, USA.

References

1. Gulati S, McQuillen DP, Jani DB, Rice PA. Immunogenicity of *Neisseria gonorrhoeae* lipooligosaccharide epitope, 2C7, widely expressed *in vivo* with no immunochemical similarity to human glycosphingolipids. J Infect Dis **1996** (In press).
2. McQuillen DP, Gulati S, Hook III EW, Rice PA. Functional characteristics of the immune response to the gonococcal lipooligosaccharide epitope defined by mAb 2C7 in natural infection and after immunization. In: Evans JS, Yost SE, Maiden MCJ, Feavers IM, eds. Neisseria 94. Winchester, England: S.C.C., **1994**:21-2.
3. Gotschlich EC. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. J Exp Med **1994**; 180:2181-90.
4. John CM, Griffiss JM, Apicella MA, Mandrell RE, Gibson BW. The structural basis for pyocin resistance in *Neisseria gonorrhoeae* lipooligosaccharides. J Biol Chem **1991**; 266:19303-11.
5. Virji M, Weiser JN, Lindberg AA, Moxon ER. Antigenic similarities in lipopolysaccharides of *Haemophilus* and *Neisseria* and expression of a digalactoside structure also present on human cells. Microb Pathog **1990**; 9:441-50.
6. Mandrell RE. Further antigenic similarities of *Neisseria gonorrhoeae* lipooligosaccharides and human glycosphingolipids. Infect Immun **1992**; 60:3017-20.
7. Mandrell RE, Griffiss JM, Macher BA. 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. J Exp Med **1988**; 168:107-26.
8. Yamasaki R, Kerwood DE, Schneider H, Quinn KP, Griffiss JM, Mandrell RE. The structure of lipooligosaccharide produced by *Neisseria gonorrhoeae*, strain 15253, isolated from a patient with disseminated infection: evidence for a new glycosylation pathway of gonococcal lipooligosaccharide. J Biol Chem **1994**; 269:30345-51.

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

MP Jennings[1], P van der Ley[2], M Kramer[2], D Hood[1], JT Poolman[2], ER Moxon[1]

[1]School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Nathan, Queensland, Australia; [2]National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands; [3]Molecular Infectious Diseases Group, Department of Paediatrics, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, England.

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

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

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

Using a set of hybridization probes, based on the *lgt*ABE genes from Nm MC58 and the *lgt*C and *lgt*D genes from Nm 126E, we had surveyed the IT strains for the presence of these genes. This survey revealed that the Nm IT strains were not like the Ng F62 example (8) in that none of them

contain all 5 lgt genes - consistent with a more restricted repertoire of terminal structures which can be made by an individual Nm strain. For example, the *lgt*C gene is present only in the L1 and L8 IT strains, *lgt*D is only present in the L1 IT strain, and *lgt*A is present in all strains except L1 and L10. This survey has allowed the prediction of the phase variation repertoire of individual strains based on the lgt genes present. The predicted phase variation repertoire of a selection of these strains has been confirmed using colony immunoblot with IT specific monoclonal antibodies. Refinement of this system with new probes to other key LPS biosynthetic genes may lead to an improvement of the IT system such that individual strains may be classified by the potential repertoire of LPS structures that may be expressed.

References

1. Verheul AFM, Snippe H, Poolman JT. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol. Rev. **1993**; 57:34-49.
2. Scholten RJPM, Kuipers B, Valkenberg HA, et al. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA using monoclonal antibodies and association of immunotype with serogroup, serotype and subtype. J Med Microbiol **1994**; 41:236-243.
3. Jones DM, Borrow R, Fox AJ, et al. The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb Pathogen **1992**; 13: 219-224.
4. Moran EE, Brandt BL, Zollinger WD. Expression of the L8 lipopolysaccharide determinant increases the sensitivity of *Neisseria meningitidis* to serum bactericidal activity. Infect Immun **1994**; 62:5290-5295.
5. Virji M, Makepeace K, Peak IRA, et al. Opc- and Pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. Mol Microbiol **1995**; 18:741-54.
6. Jennings MP, Hood D, Peak IRA, et al. Molecular analysis of a locus which controls the biosynthesis and phase variable expression of the lacto-N-neotetraose terminal LPS structure in *Neisseria meningitidis*. Mol Microbiol **1995**; 18:724-740.
7. Warkarchuk W, Martin A, Jennings MP, et al. Functional relationships of the gene locus encoding the glycosyltransferase enzymes involved in the expression of the lacto-N-neotetraose terminal lipopolysaccharide structure in *Neisseria meningitidis*. J Biol Chem **1996**; In press.
8. Gotschlich EC. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. J Expt Med **1994**; 180:2181-2190.

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

AS Johnson[1], DM Jones[1], M Potter[2], A Moran[2], M Heaney[2], AJ Fox[1]

[1]Meningococcal Reference Unit, Manchester Public Health Laboratory, Withington Hospital, Manchester, M20 2LR, UK; [2]Department of Immunology, Hope Hospital, Stott Lane, Salford, M6 8HD, UK

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

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

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

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

References

1. Goldschneider IE, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibody. J Exp Med **1969**; 129:1307-26.
2. Fredlund H, Sjoholm AG, Selander B, et al. Serum bactericidal activity and induction of chemiluminescence of polymorphonuclear leukocytes: Complement activation pathway requirements in defense against *Neisseria meningitidis*. Int Arch Allergy Immunol **1993**; 100:135-43.
3. Jones DM, Borrow R, Fox AJ, et al. The lipooligosaccharide immunotype as a virulence determinant in *Neisseria meningitidis*. Microb Pathogen **1992**; 13:219-24.
4. Kim JJ, Zhou D, Mandrell RE, et al. Effect of exogenous sialylation of the lipooligosaccharide of *Neisseria gonorrhoeae* on opsonophagocytosis. Infect Immun **1992**; 60:4439-42.

Molecular characterization of antibodies specific for meningococcal lipooligosaccharide

JS Evans, IM Feavers and MCJ Maiden

Division of Bacteriology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, EN6 3QG, UK.

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

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

The nucleotide sequence of cDNA encoding the variable domains of the antibody heavy and light chains from two of the monoclonal antibodies against the L3,7,9 epitopes has been determined. The framework region sequences show that the heavy chains of antibodies 4A8-B2-L379 (J. Poolman) and 9-2-L379 (W. Zollinger) both belong to the V_H family J558, although their low homology (86%) to each other suggests that they are probably from different subfamilies. The heavy chains showed similar levels of homology to the germline genes VMU3.2 and 186-2, and use J_H2 and J_H3, respectively. The light chains are from different families: Vκ8 and Vκ-ARS, respectively. Comparison of the nucleotide sequences with those of other murine antibody genes revealed that the 4A8-B2-L379 κ chain is 82% homologous to the

D23 germline gene and uses the Jκ1 segment, whereas the 9-2-L379 κ chain is 84% identical to the germline gene 28.4.10A(κ) and uses Jκ2.

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

Acknowledgements. The authors are grateful to Dr Jan Poolman and Dr Wendell Zollinger for providing the hybridomas used in this study.

References

1. Griffiss JM, Schneider H, Mandrell RE, et al. Lipooligosaccharides: The principal glycolipids of the neisserial outer membrane. Rev Infect Dis **1988**; 10:S287-95.
2. Verheul AF, Boons GJ, van der Marel GA, et al. Minimal oligosaccharide structures required for induction of immune responses against meningococcal immunotype L1, L2, and L3, 7,9 lipopolysaccharides determined by using synthetic oligosaccharide-protein conjugates. Infect Immun **1991**; 59:3566-73.
3. Hammerschmidt S, Birkholz C, Zahringer U, et al. Contribution of genes from the capsule gene complex (cps) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. Mol Microbiol **1994**; 11:885-96.
4. Sugasawara RJ, Prato C, Sippel JE. Monoclonal antibodies against *Neisseria meningitidis* lipopolysaccharide. Infect Immun **1983**; 42:863-8.
5. Mackinnon FG, Borrow R, Gorringe AR, Fox AJ, Jones DM, Robinson A. Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for *Neisseria meningitidis* using an infant mouse intranasal infection model. Microb Pathog **1993**; 15:359-66.
6. Malmborg A-C, Michaelsson A, Ohlin M, Jansson B, Borrebaeck CAK. Real time analysis of antibody-antigen reaction kinetics. Scand J Immunol **1992**; 35:643-50.
7. Borrebaeck CAK, Malmborg A-C, Furebring C, et al. Kinetic analysis of recombinant antibody-antigen interactions: relation between structural domains and antigen binding. Biotechnology **1992**; 10:697-8.

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

DJ McGee, GC Chen and RF Rest

MCP-Hahnemann School of Medicine, Allegheny University of the Health Sciences, Philadelphia, PA

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

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

The mutants deficient in Stase activity described here have numerous properties in common with the previously described *Tn 1545D3* derived Stase-deficient mutant, JB1 (6): all of these mutants lack Stase activity, and in the presence of CMP-NANA, cannot sialylate their LOS, are serum sensitive, and react with anti-gal-glcNac mabs. The only notable difference is that JB1 expresses altered amounts of several LOS species, whereas our Stase null mutants express similar amounts

of individual LOS species compared to WT F62. The specific defect(s) in these mutants is unknown.

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

References

1. Smith H, Parsons NJ and Cole JA. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. Microb Pathog **1995**; 19:365-377.
2. Rest RF and Mandrell RE. *Neisseria* sialyltransferases and their role in pathogenesis. Microb Pathog **1995**; 19:379-390.
3. van Putten JVM and Robertson BD. Molecular mechanisms and implications for infection of lipopolysaccharide variation in *Neisseria*. Mol Microbiol **1995**; 16:847-853.
4. Bramley J, DeMarco de Hormaeche R, Constantinidou C, Nassif X, Parsons N, Jones P, Smith H and Cole J. A serum-sensitive, sialyltransferase-deficient mutant of *Neisseria gonorrhoeae* defective in conversion to serum resistance by CMP-NANA or blood cell extracts. Microb Pathog **1995**; 18:187-195.

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

D Post, W Nichols, M Sunshine, A Zaleski, A Esch, and MA Apicella

Department of Microbiology, The University of Iowa College of Medicine, Iowa City, IA.

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

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

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

The MLK217 strains containing plasmid expressing *N. meningitis htrB*+ candidates were grown a second time at 37°C and were patched onto 5% and 10% deoxycholate plates. After an overnight incubation at 37°C, all of the colonies were able to grow on the 5% deoxycholate plates. All of the colonies except one were able to grow on the 10% deoxycholate plates. The colony that did not grow on the 10% deoxycholate plate, but did grow on the 5% deoxycholate

plate exhibited a phenotype similar to MLK2 (htrB+). The colony, A3, was tested again on 5% and 10% deoxycholate plates, and similar results were obtained.

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

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

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

References:

1. Karow M, Fayet O, Cegielska A, Ziegelhoffer T, Georgopoulus C. Isolation and characterization of the *Escherichia coli htrB* gene, whose product is essential for bacterial viability above 33°C in rich media. J Bacteriol **1991**; 173:743-750.
2. Karow M, Georgopoulos C. Isolation and characterization of the *Escherichia coli msbB* gene, a multicopy suppressor of null mutations in the high-temperature requirement gene htrB. J Bacteriol **1992**; 174:702-710.
3. Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol Rev **1993**;57:655-682.
4. Lee N, Sunshine M, Engstrom J, Gibson B, Apicella M. Mutation of the *htrB* locus of *Haemophilus influenzae* nontypable strain 2019 is associated with modifications of lipid A and phoshporylation of the Lipo-oligosaccharide. J Biological Chemistry. **1995**; 270:27151-27159.

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

MM Rahman[1], RW Carlson[1], CM Kahler[2] and DS Stephens[2]

[1]Complex Carbohydrate Research Center, The University of Georgia, 220 Riverbend Road, Athens, GA 30602, and [2] Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30303, and Department of Veterans Affairs Medical Center, Atlanta, GA.

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

References

1. Gamian A, Beurret M, Michon F, Brisson J-R, and Jennings HJ. Structure of the L2 lipopolysaccharide core oligosaccharides of *Neisseria meningitidis*. J. Biol. Chem. **1992**; 267:922-925.
2. Kulshin VA, Zahringer U, Linder B, Frasch CE, Tsai C-M, Dmitriev BA, and Rietschel ET. Structural characterization of the lipid A component of pathogenic *Neisseria meningitidis*. J. Bacteriol. **1992**; 174:1793-1800

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

C-M Tsai, WH Chen and PA Balakonis

Division of Bacterial Products, Center for Biologics Evaluation and Research, FDA, Bethesda, Maryland, USA

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

References

1. Mandrell RE, Kim JJ, John CM, Gibon BW, Sugai JV, Apicella MA, Griffiss JM, Yamasaki R. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J. Bacteriol. **1991**; 173:2823-2832.
2. Tsai CM, Civin CV. Eight lipooligosaccharides of *Neisseria meningitidis* react with monoclonal antibody which binds lacto-*N*-neotetraose (Galß1-4GlcNAcß1-3Galß1-4Glc). Infect. Immun. **1991**; 59:3604-3609.
3. Pavliak V, Brisson JR, Michon F, Uhrin D, and Jennings HJ. Structure of the sialylated L3 lipopolysaccharide of *Neisseria meningitidis*. J. Biol. Chem. **1993**; 268:14146-14152.
4. Yamasaki R, Griffiss JM, Quinn KP, Mandrell RE. Neuraminic acid is α2-3 linked in the lipooligosaccharide of *Neissria meningitidis* serogroup B strain 6275. J Bacteriol. 1993; 175:4565-4568.

5. Jennings HJ, Johnson KG, Kenne L. The structure of an R-type oligosaccharide core obtained from some lipopolysaccharides of *Neisseria meningitidis*. Carbohydr. Res. 1983; 121:233- 241.
6. Mandrell RE, Griffiss JM, Macher BA. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immuno-chemically similar to precursors of human blood group antigens. J. Exp. Med. 1988; 168:107-126.
7. Mandrell RE, Apicella MA. Lipo-oligosaccharides (LOS) of mucosal pathogens: Molecular mimicry and host-modification of LOS. Immunobiol. 1993; 187:382-402.

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

U Vogel, S Hammerschmidt and M Frosch

Institute for Medical Microbiology, Medical School Hannover, Germany

The capability of *Neisseriae* to survive the host defense mechanisms has been shown *in vitro* to depend largely on the incorporation of the sialic acid (NeuNAc) into the capsule as well as its use as a terminal modification of the neisserial lipooligosaccharide (LOS) (1,2). In the present study we investigated the *in vivo* contribution of the polysialic-acid capsule and the terminal LOS-modification by sialic acid to the pathogenicity of *Neisseria meningitidis* serogroup B using a set of defined isogenic mutants of the wild type strain B1940. The mutants were deficient in either the capsule synthesis (B1940*sia*D$^-$) or LOS-sialylation (B1940*gal*E$^-$). A spontaneous capsule deficient variant (3) (B1940*sia*D$_{SSM}$) was also used which is capable of switching on the capsule synthesis *in vitro* in a frequency of 3x10^{-3}. Infection of infant rats with the wild type strain revealed a high potential of to cause bacteremia. An infective dose of 100 CFU applied intraperitoneally resulted in a bacteremia of 10$^{3-5}$ CFU/ml after 9 h of infection. This potential was attenuated 100-1000 fold in the spontaneous capsule deficient variant (LOS sialylation$^-$) which was capable of switching on the capsule synthesis in a frequency of 3x10^{-3} *in vitro*. All reisolates of B1940*sia*D$_{SSM}$ from the blood or the peritoneal fluid were encapsulated. Using a mutant irreversibly deficient in capsule synthesis, but nevertheless sialylating its LOS, bacteremia could only be achieved using 10^6 times higher numbers of bacteria when compared to the wild type strain. All reisolates of this mutant from the blood and the peritoneal fluid were proven to be unencapsulated suggesting that defense mechanisms directed against unencapsulated meningococci were exhausted using very high doses. Interestingly, bacteremia could never be achieved when the encapsulated, LOS-sialylation deficient mutant B1940*gal*E$^-$ was inoculated into the newborn rats, probably because LOS sialic acid more potently than the polysialic-acid capsule protects against the action of the rat complement system which cannot be exhausted. Microscopic analysis of peritoneal cells revealed that the mutants B1940*gal*E$^-$ and B1940*sia*D$^-$ were phagocytosed in contrast to the wild type strain suggesting that both the capsule and the LOS sialic acid are prerequisites for the resistance of meningococci against phagocytosis by rat intra peritoneal phagocytes. In conclusion our study demonstrates that in the infant rat model of meningococcal infection both forms of sialic acid on the bacterial cell surface are indispensable for systemic survival.

References

1. Jarvis GA. Recognition and control of neisserial infection by antibody and complement. Trends in Microbiology **1995**; 3:198-201.
2. Klein NJ, Ison CA, Peakman M, Levin M, Hammerschmidt S, Frosch M, Heyderman RS. The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by *Neisseria meningitidis*. J Infect Dis **1996**; 173:172-179.
3. Hammerschmidt S, Müller A, Sillmann H, Mühlenhoff M, Barrow R, Fox A, van Putten J, Zollinger WD, Gerardy-Schahn R, Frosch M. Correlation of capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*sia*D) with bacterial invasion and the outbreak of meningococcal disease. Mol Micobiol (in press).

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

U Vogel[1], A Weinberger[1], A Müller[1], JP Atkinson[2] and M Frosch[1]

[1]Institute for Medical Microbiology, Medical School Hannover, Germany, [2]Department of Medicine, Washington University School of Medicine, St. Louis, USA

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

We conclude that C3b deposition and bacterial lysis via the CP of complement activation is exclusively inhibited by the expression of LOS sialic acid irrespective of the capsular phenotype.

References:

1. Pangburn M, Müller-Eberhard HJ. Complement C3 convertase: Cell surface restriction of β 1H control and generation of restriction on neuraminidase-treated cells. Proc Natl Acad Sci USA **1978**; 5:2416-2420.
2. Jarvis GA. Recognition and control of neisserial infection by antibody and complement. Trends Microbiol. **1995**; 3:198-201.
3. Klos A, Ihrig V, Messner M, Grabbe J, Bitter-Suermann D. Detection of native human complement components C3 and C5 and their primary activation peptides C3a and C5a (anaphylatoxic peptides) by ELISAs with monoclonal antibodies. J Immunol Methods **1988**; 111:241-252.
4. Densen P, McRill CM, Ross SC. Assembly of the membrane attack complex promotes decay of the alternative pathway C3 convertase on *Neisseria gonorrhoeae.* J Immunol **1988**; 141:3902-3909.

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

M Gilbert[1], DC Watson[1], A Cunningham[1], MP Jennings[2], NM Young[1], and WW Wakarchuk[1]

[1]Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada, K1A 0R6; [2]School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Kessels Road, Nathan 4111, Queensland Australia

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

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

J Wang, H Schneider, and JMcL Griffiss

111W1, VA Medical Center, Department of Laboratory Medicine University of California, San Francisco. CA 94121 and Walter Reed Army Institute of Research, Washington, DC.

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

Materials and Methods. We used MS11mkA, a gonococcal variant that makes only lactosyl LOS, MS11mkC, a variant that makes paraglobosyl and gangliosyl-like LOS, F62 and FA1090. The latter two strains makes paraglobosyl and gangliosyl-like LOS. We subcultured organisms onto GC agar base supplemented with IsoVitaleX® (BBL, Cockeysville, MD) (GC agar) and used standard visual criteria to select piliated and opaque.

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

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

All the adherence and invasion experiments were performed in 24 well cell culture plates, $2x10^5$ epithelial cells were seeded into each well and incubated for 16 hours before the addition of $2x10^7$ bacteria to each well. The inoculated cells were incubated for 2-6 hours, and then rinsed with PBS 3 times. For assays of invasion, gentamicin (50μG/mL) was added to each cell and the plates incubated for two hours in order to kill extra cellular bacteria. The wells were again rinsed with PBS 3 times. Cells then were dislodged from the wells with trypsin and lysed with 1% saponin in PBS. Lysates diluted 1:10 and 100μL were inoculated onto GC agar. Bacterial colonies were counted after overnight growth.

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

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

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

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

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

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

A Zaleski and P Densen

Department Of Internal Medicine, The University of Iowa, Iowa City, Iowa, USA

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