INFECTION AND IMMUNITY, Feb. 2006, p. 1360–1367 Vol. 74, No. 2
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.2.1360–1367.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Lipooligosaccharide Structure Contributes to Multiple Steps in the Virulence of *Neisseria meningitidis*

Laura Plant,[1]* Johanna Sundqvist,[1] Susu Zughaier,[2] Lena Lövkvist,[1] David S. Stephens,[2,3] and Ann-Beth Jonsson[1]

Department of Medical Biochemistry and Microbiology, Biomedical Centrum, Uppsala University, Uppsala, Sweden[1]; Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, Georgia[2]; and Laboratories of Microbial Pathogenesis, VA Medical Center, Atlanta, Georgia[3]

Received 19 September 2005/Returned for modification 7 November 2005/Accepted 22 November 2005

Lipooligosaccharide (LOS) of *Neisseria meningitidis* has been implicated in meningococcal interaction with host epithelial cells and is a major factor contributing to the human proinflammatory response to meningococci. LOS mutants of the encapsulated *N. meningitidis* serogroup B strain NMB were used to further determine the importance of the LOS structure in in vitro adherence and invasion of human pharyngeal epithelial cells by meningococci and to study pathogenicity in a mouse (CD46 transgenic) model of meningococcal disease. The wild-type strain [NeuNAc-Galβ-GlcNAc-Galβ-Glcβ-Hep$_2$ (GlcNAc, Glcα) 3-deoxy-D-manno-2-octulosonic acid (KDO$_2$)-lipid A; 1,4′ bisphosphorylated], although poorly adherent, rapidly invaded an epithelial cell layer in vitro, survived and multiplied early in blood, reached the cerebrospinal fluid, and caused lethal disease in the mouse model. In contrast, the Hep$_2$ (GlcNAc) KDO$_2$-lipid A (*pgm*) mutant, which was highly adherent to cultured epithelial cells, caused significantly less bacteremia and mortality in the mouse model. The Hep$_2$-KDO$_2$-lipid A (*rfaK*) mutant was shown to be moderately adherent and to cause levels of bacteremia and mortality similar to those caused by the wild-type strain in the mouse model. The KDO$_2$-lipid A (*gmhB*) mutant, which lacks the heptose disaccharide in the inner core of LOS, avidly attached to epithelial cells but was otherwise avirulent. Disease development correlated with expression of specific LOS structures and was associated with lower adherence but rapid meningococcal passage to and survival in the bloodstream, induction of proinflammatory cytokines, and the crossing of the blood-brain barrier. Taken together, the results of this study further define the importance of the LOS structure as a virulence component involved in multiple steps in the pathogenesis of *N. meningitidis*.

Neisseria meningitidis is a frequent inhabitant of the upper respiratory tract of humans and is a major cause of meningitis and fulminant septicemia in otherwise healthy individuals. Among the important virulence factors involved in meningococcal pathogenesis, endotoxin, or lipooligosaccharide (LOS), is a major component inducing proinflammatory responses in meningococcal sepsis and meningitis (15, 36). Furthermore, the morbidity and mortality of meningococcal sepsis are directly correlated with levels of circulating meningococcal LOS (3, 4).

Meningococcal LOS interacts with human cells, resulting in the production of proinflammatory cytokines and chemokines, including interleukin 1 (IL-1), IL-6, and tumor necrosis factor (TNF), that are important in the pathogenesis of meningococcal disease (3, 42). While pili and Opa and Opc outer membrane proteins are also critical, LOS is one of the structures important in mediating meningococcal attachment to (23, 24) and invasion into (30) epithelial cells. The role of LOS in these events is further substantiated by the findings that LOS-deficient meningococcal mutants show impaired adherence (2) and reduced induction of serum cytokines (35) compared to the wild-type strain.

The structure of *N. meningitidis* LOS has been characterized both immunologically (33) and biochemically (6, 8, 19, 20, 40). Meningococcal LOS lacks the repeating O antigens of enteric lipopolysaccharide but maintains a conserved inner core composed of heptose and 3-deoxy-D-manno-2-octulosonic acid bound to lipid A, to which variable α and β chain saccharides are attached (15). Lipid A is the active moiety through its ability to upregulate the inflammatory response. Changes in the configuration or the conformational structure of lipid A affect the biological response (27). Variation in the composition of meningococcal LOS is also postulated to mediate changes in host immune responses and bacterial virulence (28, 37).

In order to further define the role of LOS in meningococcal pathogenesis, each of a series of genetically and structurally defined LOS mutants of the serogroup B meningococcal strain NMB was examined for its capacity to adhere to and invade epithelial cells in comparison to the wild-type parent strain. Also, the pathogenicities of the parent and of each of the mutants were examined by use of a mouse model of meningococcal disease (9), and survival in the blood of these mice was assessed. Our results show that structural variations of LOS affect attachment and invasion of epithelial cells, access to and survival in the bloodstream, and the subsequent innate immune response and morbidity in vivo.

MATERIALS AND METHODS

Bacterial strains. *Neisseria meningitidis* serogroup B strain NMB (encapsulated; L2/4 immunotype) and the genetically defined mutants of this strain (*pgm*, *rfaK*, and *gmhB* mutants) are shown in Fig. 1 and described in references 12, 15, 25, 29, 34, and 41. The meningococcal strain NMB and the *pgm*, *rfaK*, and *gmhB*

* Corresponding author. Mailing address: Department of Medical Biochemistry and Microbiology, Biomedical Centrum, Uppsala University, PO Box 582, Uppsala, Sweden. Phone: 46 18 471 46 05. Fax: 46 18 50 98 76. E-mail: Laura.Plant@imbim.uu.se.

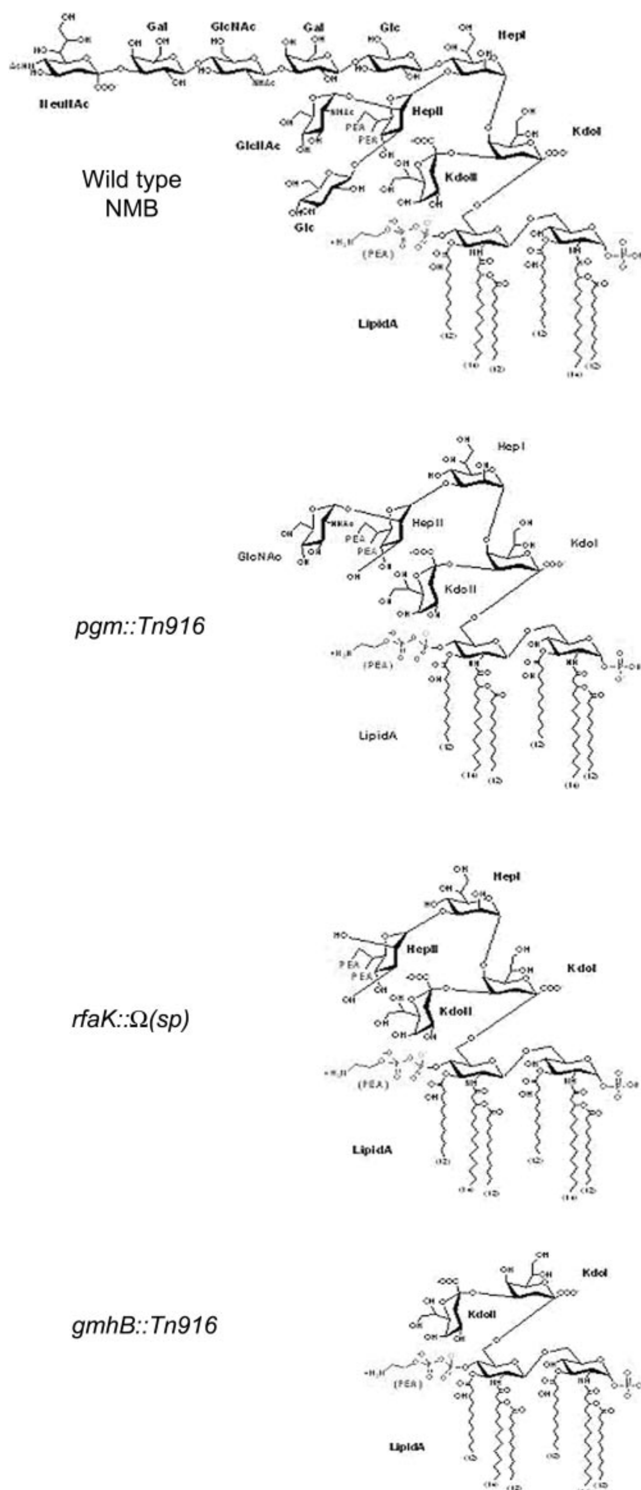

FIG. 1. Genotypes and structures of the serogroup B *N. meningitidis* LOS mutants used in this study. The wild-type NMB strain and the *pgm*::Tn*916* (enzyme deficiency: phosphoglucomutase), *rfaK*::Ω(*sp*) (enzyme deficiency: α1-2-*N*-acetyl-glucosamine transferase), and *gmhB*::Tn*916* (enzyme deficiency: 2-D-manno-heptose phosphatase) LOS mutants were assessed for virulence.

mutants were grown on GC agar with Kellogg's supplement (16) at 37°C in 5% CO_2. The antibiotics for LOS mutants were used at the following concentrations: for the *pgm* and *gmhB* mutants, tetracycline at 5 μg/ml; and for the *rfaK* mutant, spectinomycin at 60 μg/ml. The M7 strain used in the whole-cell enzyme-linked immunosorbent assay (ELISA) is an unencapsulated mutant (NMB *synA*::Tn*916* mutant) (32).

Cells. Detroit 562 human pharyngeal cells (ATCC CCL 138) were maintained in modified Eagle medium (Sigma) supplemented with 10% inactivated fetal bovine serum (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Gibco), and 1× nonessential amino acids (Gibco) at 37°C in 5% CO_2.

Electron microscopy. Bacteria were resuspended to an optical density at 600 nm (OD_{600}) of 0.3 in Tris-Mg buffer (10 mM Tris-HCl [pH 7.4] and 10 mM $MgCl_2$) and mounted on copper grids. The grids were stained with 1% ammonium molybdate and analyzed with a JEOL 1230 electron microscope.

Western blotting. Bacterial protein extracts (750 ng) were separated by 10% (PilC expression) and 15% (Opa expression) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by standard methods and transferred to polyvinylidene difluoride membranes. The membranes were blocked overnight with 5% nonfat dried milk in Tris-buffered saline (TBS; 0.05 M Tris and 2 M NaCl, pH 7.4) containing 0.05% Tween 20. PilC was identified by incubation with the K3 antibody that recognizes both PilC1 and PilC2 (polyclonal rabbit antibody diluted 1:5,000). The Opa proteins were detected with the 4B12/C11 antibody, which recognizes all Opa proteins (1) (monoclonal mouse antibody diluted 1:5,000). Incubation with primary antibodies was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad; diluted 1:10,000) or horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnologies; diluted 1:10,000). A chemiluminescence kit from Perkin Elmer Life Sciences was used for detection.

Examination of LOS expression. Tricine SDS-PAGE was used to examine the LOS expression of all bacterial mutants. Whole-cell lysates of bacterial strains were prepared by incubation of bacterial suspensions (8 mg) with proteinase K (2.5 μg/ml), Tris-HCl (50 mM), $CaCl_2$ (1 mM), and glycerol (2%) at 60°C for 18 h. Following digestion, loading buffer (1 M Tris-HCl, 10% glycerol, 2% SDS, 0.5% 2-β-mercaptoethanol, and 0.0125% bromophenol blue) was added, 200 ng of sample was separated on a 16.5% gel, and LOS was detected by silver staining (39).

Capsule quantification by whole-cell ELISA. The expression of capsule by various mutants used in this study was quantified by a whole-cell ELISA method as described previously (31). Briefly, cells were harvested from overnight growth on GC plates and suspended in phosphate-buffered saline. A 50-μl aliquot of a 1:3 dilution of cell suspensions at an OD_{650} of 0.1 was applied and dried at 37°C overnight. Antibodies specific for serogroup B (2-2-B) and serogroup A (14-1-A) were used at 1:2,000 and 1:30,000 dilutions, respectively. The M7 strain does not express capsule due to the *synA* mutation and was used as a negative control for the whole-cell ELISA.

Adhesion and invasion assay. Detroit 562 cells were grown in 24-well tissue culture plates to full confluence. Suspensions of meningococci were added to triplicate wells at a multiplicity of infection of 100. Infected cells were incubated at 37°C in 5% CO_2 for 2 h and washed five times with media to remove unbound bacteria. Adhered bacteria were quantified after 2 h by lysing the cells for 5 min with 1% saponin and serially diluting the samples and spreading them on GC agar plates. The number of invaded bacteria was determined after 3, 4, 5, and 6 h by incubating cells in medium containing 100 μg/ml gentamicin for 1 h to kill extracellular bacteria before the saponin treatment. Both assays were conducted in at least three independent experiments, and data were normalized to results for the original inocula. The sensitivity of all bacterial strains to gentamicin was determined in cell media by recovery of viable bacteria following a 1-h incubation in gentamicin at 0, 0.1, 1, 10, and 100 μg/ml.

Growth curve. To monitor the growth of bacteria in cell culture medium, 100-μl suspensions of bacteria (OD_{600} = 0.1) were inoculated into 2 ml of cell culture medium and incubated at 37°C in 5% CO_2. The optical density at 600 nm was measured at various time points after inoculation.

Animal infection assay. A mouse model of meningococcal infection (9, 10) was used to examine the pathogenicity of the LOS mutants. Briefly, 5- to 8-week-old CD46 transgenic mice (n = 17) were infected intraperitoneally (i.p.) with 3×10^8 bacteria in two independent experiments. Blood samples were taken from the tail 1, 4, 8, and 24 h after infection and spread on GC agar plates to determine the bacterial load in blood. At 4, 8, and 24 h postinfection, blood smears were made for analysis of neutrophil infiltration. Serum was collected 24 h postchallenge for analysis of proinflammatory cytokine levels in blood. The cisterna magna was punctured 24 h postinfection, and the cerebrospinal fluid (CSF) was checked for absence of red blood cells and spread on GC plates for a viable count.

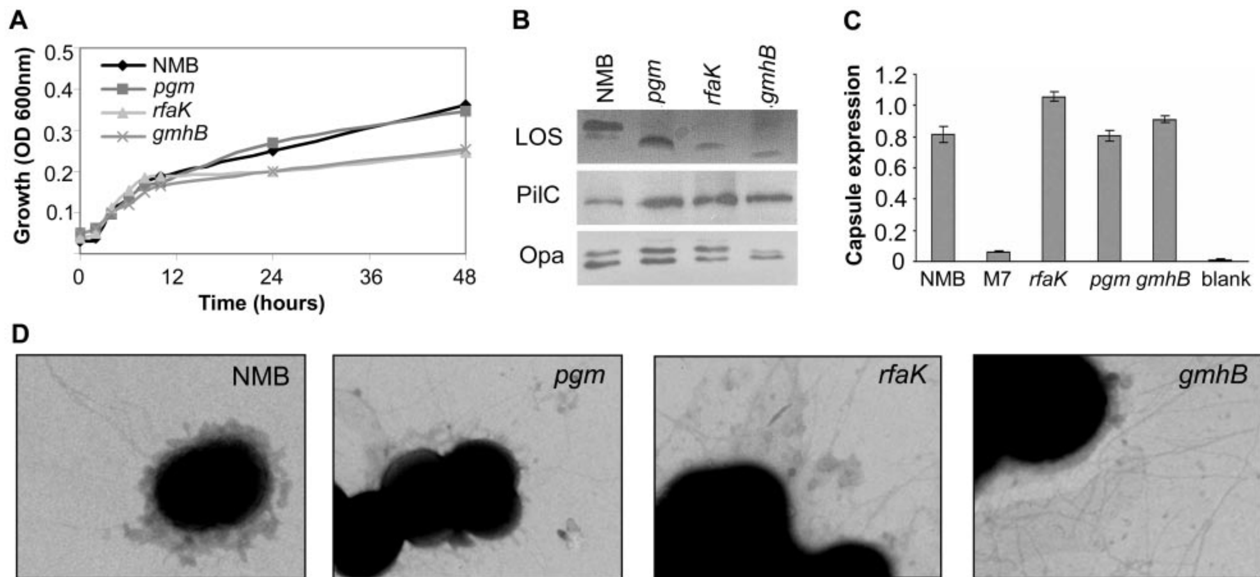

FIG. 2. Phenotypic analysis of the wild-type meningococcal strain NMB and the *pgm*, *rfaK*, and *gmhB* LOS mutants. (A) Bacterial strains showed similar growth rates, measured as optical density at 600 nm, in cell medium. (B) Lipooligosaccharide structure was assessed by Tricine SDS-PAGE, and PilC and opacity proteins were examined by Western blotting using PilC-specific and Opa-specific antisera, respectively. (C) Capsule expression was measured by ELISA, and the unencapsulated strain M7 was used as a negative control for capsule expression. (D) Transmission electron microscopy of whole bacterial cells showed expression of pili.

Survival assay. Blood from CD46 transgenic mice was collected by retro-orbital bleeding and heparin treated. Whole blood was diluted 1:1 with bacterial suspension in Detroit cell media (1×10^7 bacteria) in a final volume of 300 μl. Bacterial survival was determined by serial dilutions and plating of the blood-bacteria mixture at 3, 6, 9, and 12 h postinfection. Survival was monitored in freshly isolated blood in two independent experiments.

Immunoassays for cytokines in serum. Concentrations of murine IL-6 and TNF were measured in the sera of *N. meningitidis*-challenged mice at 24 h postinjection by using sandwich ELISAs according to the manufacturer's recommendations (Diaclone).

Blood smears. Samples (approximately 5 μl) of blood were obtained from the tail vein of infected mice, and smears were made on glass slides. The slides were fixed with methanol and left to dry. The smear samples were stained with Wright's stain according to the manufacturer's recommendations (Sigma). Samples were analyzed by light microscopy.

Statistical analysis. Experiments were evaluated with Microsoft Excel. A two-tailed Student's *t* test was used to assess significance in the adhesion and invasion assays and in the cytokine assays. Lethality experiments were assessed using Fisher's exact test, and bacteremia and in vitro blood survival was monitored with a nonparametric Mann-Whitney test. All experiments were performed in sets of at least two independent experiments.

RESULTS

Expression of virulence factors by LOS mutants. *N. meningitidis* strain NMB and the LOS mutants have been previously described and are shown in Fig. 1 (12, 15, 25, 29, 34, 41). The mutant strains showed growth rates similar to that of the wild-type strain, with the *rfaK* and *gmhB* mutants having lower final bacterial numbers in the stationary phase (Fig. 2A); however, growth rates were not significantly different ($P > 0.05$). *N. meningitidis* NMB and each mutant expressed the SDS-PAGE LOS profiles expected (Fig. 2B) on the basis of their nuclear magnetic resonance-defined structures (Fig. 1), and Western blotting of whole-cell preparations revealed that the parent and the mutants expressed PilC (Fig. 2B). The wild-type strain expressed a two-band Opa repertoire and the *pgm* and *rfaK* mutants expressed a third Opa of higher molecular weight, while the *gmhB* mutant expressed one Opa in common with the parent and a unique Opa of higher molecular weight (Fig. 2B). All strains showed levels of capsule expression that were not significantly different ($P < 0.05$) from that of the wild-type strain, NMB (Fig. 2C), as determined by a whole-cell ELISA (34). Electron microscopy analysis showed that all strains were piliated (Fig. 2D).

Mutations in LOS change the capacity of meningococci to adhere to and invade epithelial cells. An assay to assess adhesion of the wild-type strain and LOS mutants to the epithelial cell line Detroit 562 revealed that mutations resulting in a truncated structure of LOS modulated the capacity of the meningococci to associate with these cells (Fig. 3A). The wild-type strain NMB showed a level of adherence to Detroit 562 cells significantly lower than those of the mutants ($P < 0.05$), with approximately five associated bacteria per cell at 2 h postinfection. Although the NMB strain appeared to express less PilC (Fig. 2B), it is unlikely that this difference alone mediated the decrease in adhesion, since the mutant strains expressed similar levels of PilC but also had different capacities to adhere. The *pgm* [Hep_2(GlcNAc) 3-deoxy-D-manno-2-octulosonic acid (KDO_2)-lipid A] and *gmhB* (KDO_2-lipid A) mutants demonstrated that between 50 and 80 bacteria adhered to each cell, which was a level significantly enhanced ($P < 0.05$) compared to that seen for the *rfaK* mutant (Hep_2-KDO_2-lipid A), which adhered at levels of approximately 20 bacteria per cell. The percentage of associated meningococci that entered and survived within cells was also examined using gentamicin to kill extracellular meningococci. The wild-type strain showed a steady increase in bacterial counts with time, with significantly greater numbers of bacteria within cells at 3, 4, and 5 h ($P < 0.05$). The *pgm* and *rfaK* mutants showed decreased

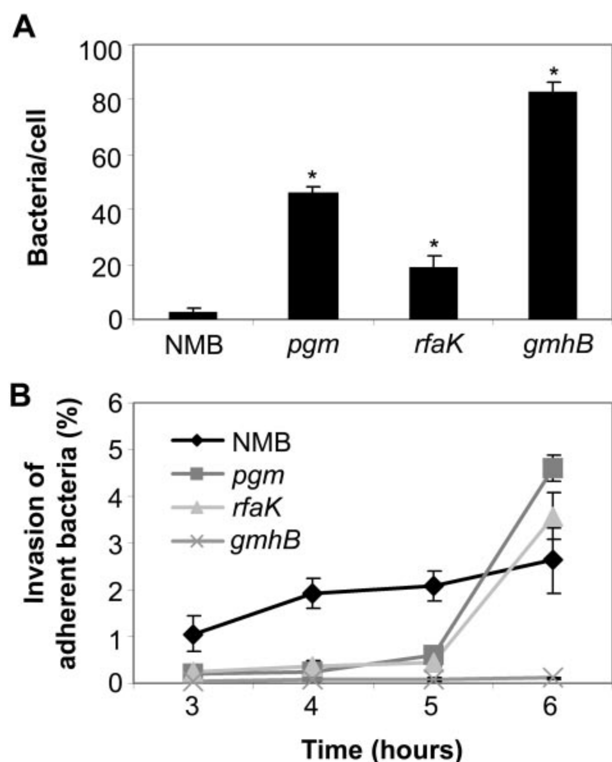

FIG. 3. Adhesion to (A) and invasion into (B) Detroit 562 epithelial cells by the wild-type strain NMB and the meningococcal LOS mutants. Viable counts of adherent meningococci were determined 2 h after infection, and the percentages of adhered bacteria that invaded were determined after 3, 4, 5, and 6 h. Significant increases in adhesion over that of the wild-type strain are indicated with asterisks ($P < 0.05$).

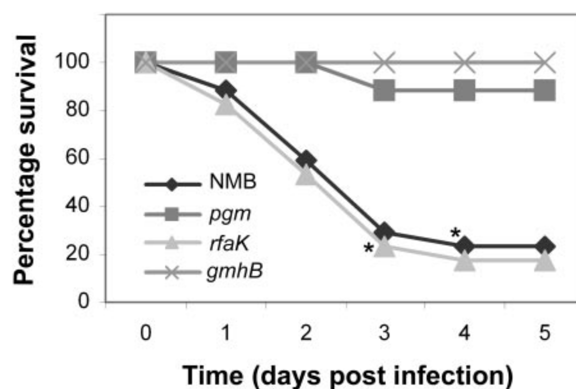

FIG. 4. Survival of mice following i.p. infection of CD46 transgenic mice ($n = 17$) with the wild-type NMB strain and the LOS mutants. The infection dose was 3×10^8 CFU/mouse. Survival of mice was monitored over a 6-day period. Significant mortality was determined using Fisher's exact test and is indicated with an asterisk ($P < 0.05$).

bacterial counts compared to the wild-type strain at the early time points (Fig. 3B), but at 6 h postinfection, the numbers of these mutants in epithelial cells were not different from that of the parent. The *gmhB* mutant was unable to enter and survive within epithelial cells. The differences in intracellular numbers of bacteria were not related to differences in sensitivity to gentamicin (data not shown).

LOS structure affects the pathogenicity of meningococci and mortality in a mouse model of infection. The pathogenicities of the wild-type strain and LOS mutants were further examined in a mouse model of meningococcal infection (9). Adult CD46 transgenic mice, which have increased susceptibility to meningococci in the absence of additional supplements, such as iron, were infected intraperitoneally with 3×10^8 meningococci/mouse. The wild-type strain and the *rfaK* mutant showed similar mortalities, with significant fatality noted for the wild-type strain at 4 days postinfection and for the *rfaK* mutant at 3 days postinfection (Fig. 4). The *pgm* mutant showed a mortality significantly lower than the that of the wild-type strain and that of the *rfaK* mutant. The *gmhB* mutant was avirulent in the mouse model.

LOS structure affects the levels of meningococci in the bloodstream. At 1 and 4 h postinfection, mice infected with the wild-type strain NMB and the *rfaK* mutant had bacterial blood counts ($P < 0.05$) higher than those for to the *pgm* and *gmhB* mutant-infected mice (Fig. 5). At 24 h postinfection, no significant difference in bacteremia levels in the *pgm* mutant- and *rfaK* mutant-infected mice was observed, but the mice infected with the wild-type strain still showed significantly enhanced bacteremia ($P < 0.05$). Hence, it appears that a correlation between the level of bacteremia at the early time points and mouse mortality exists. Meningococci were detected in the CSF of all mice infected with the wild-type strain NMB and in 12.5% of the mice infected with the *pgm* mutant and 25% of mice infected with the *rfaK* mutant (data not shown). No bacteria were detected in the CSF of mice infected with the *gmhB* mutant. These data suggest that LOS structure, including a requirement for inner core heptose, affects the level of bacteremia seen following infection and the ability of meningococci to enter into the CSF. It also suggests that the mortality associated with the *rfaK* mutant is caused by sepsis rather than meningitis, whereas the mortality associated with the wild-type strain is probably due to both sepsis and meningitis.

In vitro survival in whole mouse blood. In order to evaluate if the wild-type strain and the LOS mutants differed in their abilities to survive in blood, bacteria were incubated with heparin-treated mouse blood in vitro. As shown in Fig. 6, each of the mutations resulting in truncation of meningococcal LOS structure was associated with a decrease in the capacity of meningococci to survive in whole blood taken from CD46 mice. These data suggest that the wild-type LOS structure best promoted the survival of meningococci in blood and correlated with the persistent bacteremia of the wild-type strain in the mouse model. However, pathogenesis in the mouse model was not solely mediated by the capacity to survive in blood, since no significant differences were observed in the survival rates of the LOS mutants in the mouse blood in vitro ($P > 0.05$), in contrast to the differences observed in mouse mortality rates.

Mouse mortality is associated with high serum cytokine levels. Meningococcal sepsis-associated death is often due to hypotension and organ failure characteristic of septic shock, which occurs as a result of uncontrolled release of proinflammatory cytokines, such as IL-6 and TNF. Host cytokine responses were analyzed by measuring cytokine levels in mouse serum 24 h postinfection by ELISA. As shown in Fig. 7A, IL-6 increased significantly in serum after i.p. challenge with the wild-type strain NMB and the *rfaK* mutant. IL-6 was not elevated in sera of mice at 24 h after

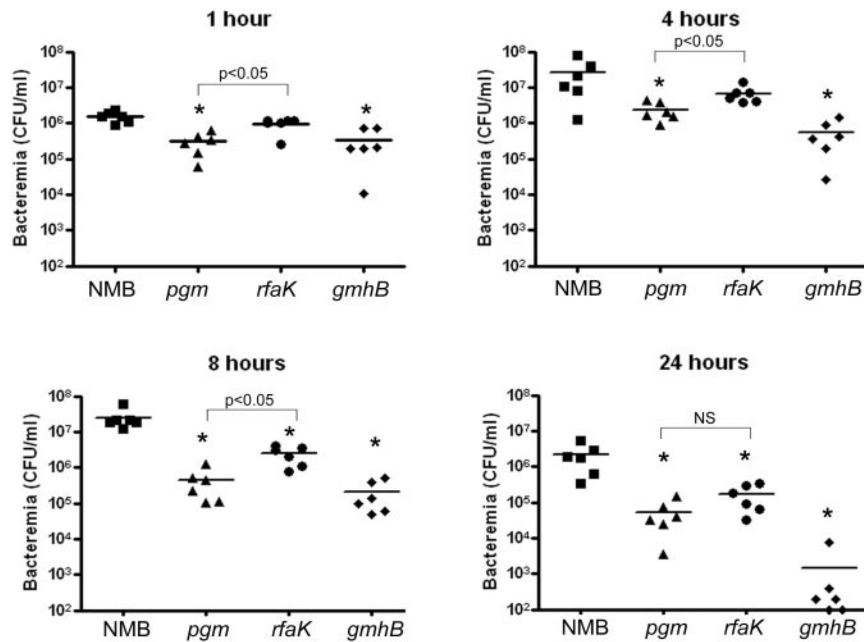

FIG. 5. Bacterial counts (CFU/ml) in blood of CD46 transgenic mice challenged i.p. with wild-type strain NMB and meningococcal LOS mutants. Blood samples were collected 1, 4, 8, and 24 h following infection and spread on GC plates for a viable count. Significant decreases in bacteremia compared to that seen with the wild-type strain have been calculated using a Mann-Whitney test and are indicated with asterisks ($P < 0.05$).

infection with the *pgm* mutant or the *gmhB* mutant. The wild-type strain, the *rfaK* mutant, and the *gmhB* mutant induced serum TNF (Fig. 7B). TNF was not induced in serum following i.p. infection of the *pgm* mutant. Cytokine levels did not correlate with a shift of neutrophil levels in blood samples following challenge (Fig. 7C), since all strains induced comparable influxes of neutrophils into the blood at 8 h postinfection. Interestingly, the *gmhB* mutant, which was avirulent in the mouse model, did induce TNF but did not induce IL-6 and also caused increased neutrophil infiltration after infection.

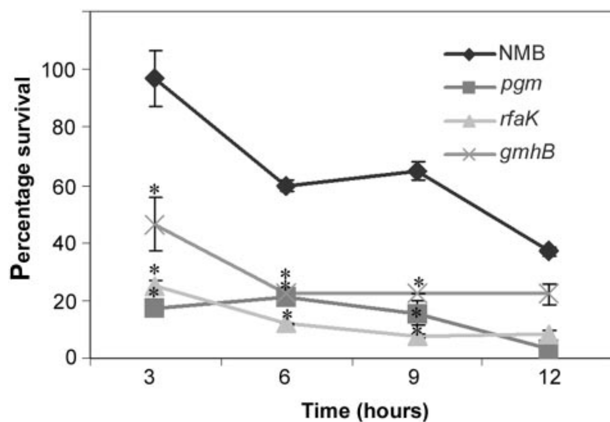

FIG. 6. Survival of the wild-type strain NMB and meningococcal LOS mutants in whole mouse blood. Bacterial survival in whole blood from CD46 transgenic mice was determined 3 to 12 h after infection by plating bacteria on GC agar and counting CFU. Significant decreases in survival from the level of the wild-type strain have been calculated using a Mann-Whitney test and are indicated with asterisks ($P < 0.05$).

DISCUSSION

LOS, or endotoxin, including the ability to express a full repertoire of LOS structures, is an important virulence factor involved in multiple steps in the pathogenesis of *N. meningitidis* and is a significant contributor to the pathologies of meningococcal disease (2–4, 15, 23, 36). In this study, wild-type meningococci of serogroup B strain NMB (LOS immunotype L2/4) and mutants of this strain with defined mutations in enzymes required for LOS assembly (12, 15, 25, 29, 34, 41) were used to further determine the role of the LOS structure in meningococcal virulence. Variations of meningococcal LOS α chain and inner core structures are known to occur at high frequencies (15) and to influence pathogenesis. Immunotypes with full-length, sialylated α chain L3/7/9 and L2/4 immunotypes are those most frequently associated with invasive disease, while LOS with a truncated α chain {L8 immunotype [Galβ-Glcβ-Hep$_2$ (GlcNAc, Glcα) KDO$_2$-lipid A]} are more often found on carrier isolates (11). However, recent evidence indicates the L2/4 immunotype (strain NMB) has the genetic capability to express all other immunotypes (13).

Piliation is known to be critically important in the initial steps of the adhesion cascade of pathogenic *Neisseria* spp. (21). The wild-type parent and all mutants showed similar levels of piliation; however, the wild-type strain had slightly decreased PilC expression compared to the mutant strains. Other important meningococcal surface components influencing adherence and invasion are capsule, Opa, and Opc (2, 5, 38). The parent and mutants expressed the same amounts of capsule, although the Opa repertoire expressed by all the mutants was different from that expressed by the parent strain. To analyze the interaction between bacteria and host cells, in vitro adhesion and

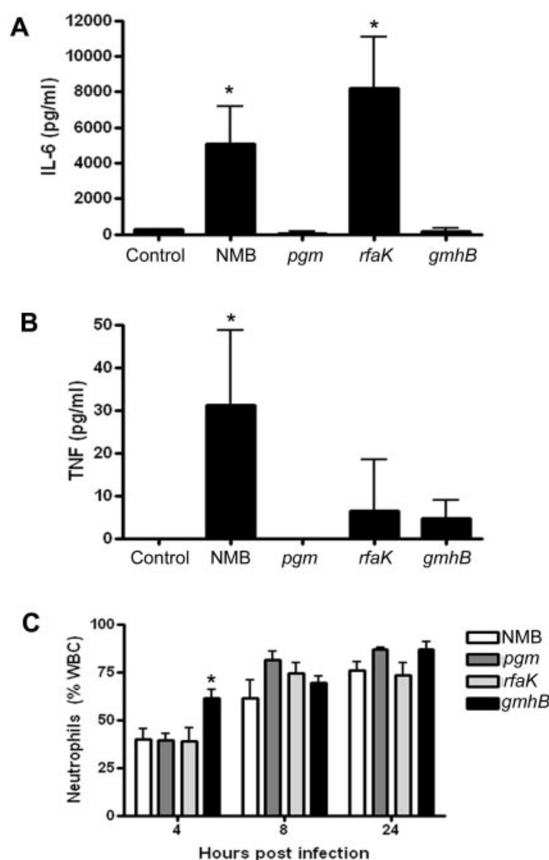

FIG. 7. Serum cytokine levels in CD46 transgenic mice following infections with the meningococcal wild-type strain NMB and with the LOS mutants. Mice were inoculated i.p. with 3×10^8 CFU/mouse, and serum levels of IL-6 (A) and TNF (B) were measured 24 h postinfection. Significant increases over the levels seen with the uninfected mouse serum have been calculated using Student's *t* test and are indicated with asterisks ($P < 0.05$). (C) Blood neutrophil counts were taken at different time points postchallenge. Blood samples were taken from the tail, and blood smear samples were stained and analyzed by light microscopy. Significant increases in neutrophil levels over that of the wild-type strain have been calculated using Student's *t* test and are indicated with an asterisk ($P < 0.05$). WBC, white blood cells.

invasion of human pharyngeal cells by meningococci with LOS truncation was studied. As anticipated on the basis of previous studies (5, 18, 35), the capacity to express a full-length LOS with a sialylated α chain and inner core structure, as in the wild-type strain, was associated with the lowest levels of adhesion to the epithelial cells. The very truncated *gmhB* mutant (KDO_2-lipid A) was, in contrast, quite adherent. This may be explained by the expression of different Opa proteins or better exposure of these or other adherence ligands due to the marked truncation in LOS structure. The *rfaK* mutant (Hep_2-KDO_2-lipid A) and the *pgm* mutant [Hep_2(GlcNAc)-KDO_2-lipid A] were also more adherent than the wild-type parent. Interestingly, meningococci expressing the Hep_2-KDO_2-lipid A LOS inner core structure were less adherent than meningococci expressing a Hep_2(GlcNAc)-KDO_2-lipid A structure, suggesting a possible ancillary role of the inner core GlcNAc in the adherence pathway. Changes in LOS structure may also enhance charge and other nonspecific interactions that facilitate adhesion; however, the mutants used in this study did not show visibly different levels of autoaggregation when observed microscopically.

In contrast to adhesion, invasion of meningococci into epithelial cells was best correlated with the capacity to produce α chain LOS structures and a requirement for the heptose disaccharide in the LOS inner core. As shown by the kinetics of invasion of epithelial cells, meningococci capable of expressing the LOS α chain appeared to invade epithelial cells rapidly. Although poorly adherent, the wild-type strain showed a steady increase in epithelial cell invasion over time. This may be due to the capacity of the wild-type strain to express the α chain, nonsialylated L3, L8 structure, which can facilitate invasion (5). Mutants unable to express or modify the α chain exhibited slower kinetics but were eventually as invasive as the wild-type strain. However, the strongly adherent *gmhB* mutant lacking the heptose disaccharide in the inner core did not invade epithelial cells. The data suggest that the minimal LOS structure required for the invasion of epithelial cells was Hep_2-KDO_2-lipid A. Similar observations have also been made in regard to the invasion of human nasopharyngeal epithelial cells in an organ culture model (D. S. Stephens, unpublished). The Opa protein expression differences may also have contributed to the low levels of meningococcal invasion of cells observed with the *gmhB* mutant in this study.

Other studies have indicated a role of LOS in bacterial cell invasion (24, 30). Harvey et al. (7) showed that the lacto-*N*-neotetraose-terminal LOS of *N. gonorrhoeae* facilitated gonococcal entry via the asialoglycoprotein receptors into primary human urethral epithelial cells. LOS α chain structures are known to bind asialoglycoprotein receptors on host cell surfaces (23, 24), and Opc-mediated cell invasion is best observed in meningococci expressing nonsialylated LOS structures (38). Furthermore, it was recently demonstrated that complementation of an *rfaE Salmonella* mutant (KDO_2-lipid A) to wild-type lipopolysaccharide permitted the invasion of different epithelial cells that were not seen with the mutant (17).

Infection of CD46 transgenic mice with the wild-type strain and LOS mutants revealed that the wild-type strain and the *rfaK* mutant caused mortality in 77 to 82% of mice and in 0 to 12% of mice infected with the *pgm* and *gmhB* mutants. Mortality in the mouse model (Fig. 4) was correlated with significantly higher levels of bacteremia at early time points after peritoneal infection (Fig. 5). The detection of meningococci in the CSF of infected mice also correlated with mortality in the model. All mice infected with the wild-type NMB strain showed bacteria in the CSF.

The link between high levels of bacteremia early in infection and mortality was also associated with the induction of cytokine responses in the mouse model. TNF and IL-6 were not induced by infection with the *pgm* mutant and were induced at low levels following infection with the *gmhB* strain. The wild-type NMB strain and the *rfaK* mutant both induced high levels of IL-6 and increased levels of TNF. The greater-than-twofold increase in the number of meningococci in blood at these early time points is reminiscent of the studies of Brandtzaeg et al. (3, 4) defining the association of human morbidity and mortality to meningococcemia with serum levels of circulating meningococcal LOS. Thus, the ability of meningococci to rapidly enter and survive in the bloodstream after invasion and cause early

high-level bacteremia is an important virulence factor, and this effect is related to LOS structure. The molecular basis for differences between the LOS structures is not clear. Equal molar quantities of endotoxins from the parent and each of these mutants are not different in terms of mouse or human macrophage cytokine activation (5). However, changes in LOS inner core structure do influence meningococcal survival in blood (26), and differences in interactions of the LOS with a variety of other host cells in vivo might also contribute to the differences observed. For example, the lack of cytokine response by the *pgm* mutant might be due to stimulation of a negative inhibitory pathway through C-lectin or other receptor recognition facilitated by the inner core structure. The difference in responses warrants further investigation into the mechanisms involved.

LOS structure is important in the resistance of meningococci to serum bactericidal activity (18, 22). The wild-type strain showed the greatest resistance to killing by whole blood taken from CD46 transgenic mice. The LOS mutants all showed decreased survival compared to the wild-type strain. Previous work by Kahler et al. (14) showed the *pgm* and *rfaK* mutants were more susceptible to normal human serum than the wild-type parent, supporting the findings of this study.

An encapsulated serogroup B wild-type strain capable of expressing a sialylated α chain or other structures containing α chain sugars was highly virulent in all the assays elucidated in this study. This strain had the best invasive capacity and had the highest serum resistance in whole CD46 transgenic mouse blood. A mutant with complete loss of inner core and α chain structures, although capable of avid adherence and bloodstream infection in the mouse model, was completely avirulent. Structural variations resulting in the loss of α chain structures were shown to exhibit increased adherence and decreased rates of cellular invasion in in vitro studies. However, meningococcal mutants deleted for LOS α chain structures but retaining inner core heptoses, although more sensitive to whole mouse blood, remained significantly virulent, providing evidence that virulence strongly associated with meningococcal LOS structure may be quite specific and not simply due to in vivo strain viability.

ACKNOWLEDGMENTS

We gratefully acknowledge Margareta Hagelin and Maj-Britt Alter for excellent assistance with animal experiments and Ann von Euler for the electron microscopy.

This work was supported by grants from the Swedish Medical Research Council (Dnr 10846), the Swedish Cancer Society, the Swedish Society for Medicine, Lars Hiertas Stiftelse, Magnus Bergvalls Stiftelse, Åke Wibergs Stiftelse, Wenner-Gren foundations, Stiftelsen Wenner-Grenska Samfundet, and Karolinska Institutet. This work was also supported by Public Health Service grant AI-033517 (to D.S.S.) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

REFERENCES

1. **Achtman, M., M. Neibert, B. A. Crowe, W. Strittmatter, B. Kusecek, E. Weyse, M. J. Walsh, B. Slawig, G. Morelli, A. Moll, et al.** 1988. Purification and characterization of eight class 5 outer membrane protein variants from a clone of Neisseria meningitidis serogroup A. J. Exp. Med. **168:**507–525.
2. **Albiger, B., L. Johansson, and A. B. Jonsson.** 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. Infect. Immun. **71:**155–162.
3. **Brandtzaeg, P., K. Bryn, P. Kierulf, R. Ovstebo, E. Namork, B. Aase, and E. Jantzen.** 1992. Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. J. Clin. Investig. **89:**816–823.
4. **Brandtzaeg, P., P. Kierulf, P. Gaustad, A. Skulberg, J. N. Bruun, S. Halvorsen, and E. Sorensen.** 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J. Infect. Dis. **159:**195–204.
5. **de Vries, F. P., A. van der Ende, J. P. van Putten, and J. Dankert.** 1996. Invasion of primary nasopharyngeal epithelial cells by *Neisseria meningitidis* is controlled by phase variation of multiple surface antigens. Infect. Immun. **64:**2998–3006.
6. **Gamian, A., M. Beurret, F. Michon, J. R. Brisson, and H. J. Jennings.** 1992. Structure of the L2 lipopolysaccharide core oligosaccharides of Neisseria meningitidis. J. Biol. Chem. **267:**922–925.
7. **Harvey, H. A., M. P. Jennings, C. A. Campbell, R. Williams, and M. A. Apicella.** 2001. Receptor-mediated endocytosis of Neisseria gonorrhoeae into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. Mol. Microbiol. **42:**659–672.
8. **Jennings, H. J., A. K. Bhattacharjee, L. Kenne, C. P. Kenny, and G. Calver.** 1980. The R-type lipopolysaccharides of Neisseria meningitidis. Can. J. Biochem. **58:**128–136.
9. **Johansson, L., A. Rytkonen, P. Bergman, B. Albiger, H. Kallstrom, T. Hokfelt, B. Agerberth, R. Cattaneo, and A. B. Jonsson.** 2003. CD46 in meningococcal disease. Science **301:**373–375.
10. **Johansson, L., A. Rytkonen, H. Wan, P. Bergman, L. Plant, B. Agerberth, T. Hokfelt, and A. B. Jonsson.** 2005. Human-like immune responses in CD46 transgenic mice. J. Immunol. **175:**433–440.
11. **Jones, D. M., R. Borrow, A. J. Fox, S. Gray, K. A. Cartwright, and J. T. Poolman.** 1992. The lipooligosaccharide immunotype as a virulence determinant in Neisseria meningitidis. Microb. Pathog. **13:**219–224.
12. **Kahler, C. M., R. W. Carlson, M. M. Rahman, L. E. Martin, and D. S. Stephens.** 1996. Inner core biosynthesis of lipooligosaccharide (LOS) in *Neisseria meningitidis* serogroup B: identification and role in LOS assembly of the α1,2 *N*-acetylglucosamine transferase (RfaK). J. Bacteriol. **178:**1265–1273.
13. **Kahler, C. M., A. Datta, Y. L. Tzeng, R. W. Carlson, and D. S. Stephens.** 2005. Inner core assembly and structure of the lipooligosaccharide of Neisseria meningitidis: capacity of strain NMB to express all known immunotype epitopes. Glycobiology **15:**409–419.
14. **Kahler, C. M., L. E. Martin, G. C. Shih, M. M. Rahman, R. W. Carlson, and D. S. Stephens.** 1998. The (α2→8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. Infect. Immun. **66:**5939–5947.
15. **Kahler, C. M., and D. S. Stephens.** 1998. Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). Crit. Rev. Microbiol. **24:**281–334.
16. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and D. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.
17. **Kim, C. H.** 2003. A Salmonella typhimurium rfaE mutant recovers invasiveness for human epithelial cells when complemented by wild type rfaE (controlling biosynthesis of ADP-L-glycero-D-mannoheptose-containing lipopolysaccharide). Mol. Cell **15:**226–232.
18. **Klein, N. J., C. A. Ison, M. Peakman, M. Levin, S. Hammerschmidt, M. Frosch, and R. S. Heyderman.** 1996. The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis. J. Infect. Dis. **173:**172–179.
19. **Kulshin, V. A., U. Zahringer, B. Lindner, C. E. Frasch, C. M. Tsai, B. A. Dmitriev, and E. T. Rietschel.** 1992. Structural characterization of the lipid A component of pathogenic *Neisseria meningitidis*. J. Bacteriol. **174:**1793–1800.
20. **Maisner, A., J. Alvarez, M. K. Liszewski, D. J. Atkinson, J. P. Atkinson, and G. Herrler.** 1996. The N-glycan of the SCR 2 region is essential for membrane cofactor protein (CD46) to function as a measles virus receptor. J. Virol. **70:**4973–4977.
21. **Merz, A. J., and M. So.** 2000. Interactions of pathogenic neisseriae with epithelial cell membranes. Annu. Rev. Cell Dev. Biol. **16:**423–457.
22. **Moran, E. E., B. L. Brandt, and W. D. Zollinger.** 1994. Expression of the L8 lipopolysaccharide determinant increases the sensitivity of *Neisseria meningitidis* to serum bactericidal activity. Infect. Immun. **62:**5290–5295.
23. **Porat, N., M. A. Apicella, and M. S. Blake.** 1995. A lipooligosaccharide-binding site on HepG2 cells similar to the gonococcal opacity-associated surface protein Opa. Infect. Immun. **63:**2164–2172.
24. **Porat, N., M. A. Apicella, and M. S. Blake.** 1995. *Neisseria gonorrhoeae* utilizes and enhances the biosynthesis of the asialoglycoprotein receptor expressed on the surface of the hepatic HepG2 cell line. Infect. Immun. **63:**1498–1506.
25. **Rahman, M. M., D. S. Stephens, C. M. Kahler, J. Glushka, and R. W. Carlson.** 1998. The lipooligosaccharide (LOS) of Neisseria meningitidis serogroup B strain NMB contains L2, L3, and novel oligosaccharides, and lacks the lipid-A 4′-phosphate substituent. Carbohydr. Res. **307:**311–324.

26. **Ram, S., A. D. Cox, J. C. Wright, U. Vogel, S. Getzlaff, R. Boden, J. Li, J. S. Plested, S. Meri, S. Gulati, D. C. Stein, J. C. Richards, E. R. Moxon, and P. A. Rice.** 2003. Neisserial lipooligosaccharide is a target for complement component C4b. Inner core phosphoethanolamine residues define C4b linkage specificity. J. Biol. Chem. **278:**50853–50862.
27. **Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, et al.** 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. **8:**217–225.
28. **Schneider, H., J. M. Griffiss, R. E. Mandrell, and G. A. Jarvis.** 1985. Elaboration of a 3.6-kilodalton lipooligosaccharide, antibody against which is absent from human sera, is associated with serum resistance of *Neisseria gonorrhoeae*. Infect. Immun. **50:**672–677.
29. **Shih, G. C., C. M. Kahler, R. W. Carlson, M. M. Rahman, and D. S. Stephens.** 2001. gmhX, a novel gene required for the incorporation of L-glycero-D-manno-heptose into lipooligosaccharide in Neisseria meningitidis. Microbiology **147:**2367–2377.
30. **Song, W., L. Ma, R. Chen, and D. C. Stein.** 2000. Role of lipooligosaccharide in Opa-independent invasion of Neisseria gonorrhoeae into human epithelial cells. J. Exp. Med. **191:**949–960.
31. **Swartley, J. S., L. J. Liu, Y. K. Miller, L. E. Martin, S. Edupuganti, and D. S. Stephens.** 1998. Characterization of the gene cassette required for biosynthesis of the (α→6)-linked *N*-acetyl-D-mannosamine-1-phosphate capsule of serogroup A *Neisseria meningitidis*. J. Bacteriol. **180:**1533–1539.
32. **Swartley, J. S., and D. S. Stephens.** 1994. Identification of a genetic locus involved in the biosynthesis of *N*-acetyl-D-mannosamine, a precursor of the (α2→8)-linked polysialic acid capsule of serogroup B *Neisseria meningitidis*. J. Bacteriol. **176:**1530–1534.
33. **Tsai, C. M., L. F. Mocca, and C. E. Frasch.** 1987. Immunotype epitopes of *Neisseria meningitidis* lipooligosaccharide types 1 through 8. Infect. Immun. **55:**1652–1656.
34. **Tzeng, Y. L., A. Datta, C. Strole, V. S. Kolli, M. R. Birck, W. P. Taylor, R. W. Carlson, R. W. Woodard, and D. S. Stephens.** 2002. KpsF is the arabinose-5-phosphate isomerase required for 3-deoxy-D-manno-octulosonic acid biosynthesis and for both lipooligosaccharide assembly and capsular polysaccharide expression in Neisseria meningitidis. J. Biol. Chem. **277:**24103–24113.
35. **van der Ley, P., and L. Steeghs.** 2003. Lessons from an LPS-deficient Neisseria meningitidis mutant. J. Endotoxin Res. **9:**124–128.
36. **van Deuren, M., P. Brandtzaeg, and J. W. van der Meer.** 2000. Update on meningococcal disease with emphasis on pathogenesis and clinical management. Clin. Microbiol. Rev. **13:**144–166.
37. **Verheul, A. F., H. Snippe, and J. T. Poolman.** 1993. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol. Rev. **57:**34–49.
38. **Virji, M., K. Makepeace, I. R. Peak, D. J. Ferguson, M. P. Jennings, and E. R. Moxon.** 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. Mol. Microbiol. **18:**741–754.
39. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide cells. Anal. Biochem. **118:**197–203.
40. **Yamasaki, R., J. M. Griffiss, K. P. Quinn, and R. E. Mandrell.** 1993. Neuraminic acid is alpha 2→3 linked in the lipooligosaccharide of *Neisseria meningitidis* serogroup B strain 6275. J. Bacteriol. **175:**4565–4568.
41. **Zhou, D., D. S. Stephens, B. W. Gibson, J. J. Engstrom, C. F. McAllister, F. K. Lee, and M. A. Apicella.** 1994. Lipooligosaccharide biosynthesis in pathogenic Neisseria. Cloning, identification, and characterization of the phosphoglucomutase gene. J. Biol. Chem. **269:**11162–11169.
42. **Zughaier, S. M., Y. L. Tzeng, S. M. Zimmer, A. Datta, R. W. Carlson, and D. S. Stephens.** 2004. *Neisseria meningitidis* lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. Infect. Immun. **72:**371–380.

Editor: J. N. Weiser