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Note

Phase-variation of the truncated lipo-oligosaccharide of *Neisseria meningitidis* NMB phosphoglucomutase isogenic mutant NMB-R6

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Abstract

The detection of antibodies specific to meningococcal lipo-oligosaccharides (LOSs; outer-core → inner-core → lipid A) in sera of patients convalescent from meningococcal infection suggests the potential use of LOS as a vaccine to combat pathogenic *Neisseria* spp. Removal of the outer-core region, which expresses glycans homologous to human blood-group antigens, is a required first-step in order to avoid undesirable immunological reactions following vaccination. To this end, we describe here the structural makeup of the LOS produced by serogroup B *N. meningitidis* NMB isogenic phosphoglucomutase (Pgm) mutant (NMB-R6). The dominant LOS types produced by NMB-R6 expressed a deep-truncated inner-core region, GlcNAc-(1 → 2)-LDHepII-(1 → 3)-LDHepI-(1 → 5)-[Kdo-2 → 4]-Kdo → lipid A, with one PEA unit attached at either O-6 or O-7 of LDHepII, or with two simultaneously PEA moieties attached at O-3 and O-6 or O-3 and O-7 of the same unit. Unexpectedly, this mutation did not completely deactivate the production of Glc, as some LOS molecules were observed to carry Glc at O-4 of LDHepI and at O-3 of LDHepII. A glycoconjugate vaccine comprised of NMB-R6 LOSs is currently being evaluated in our laboratory.

Keywords: *Neisseria meningitidis*; Lipo-oligosaccharide; 2-Aminoethyl phosphate; Vaccine

Neisseria meningitidis is an encapsulated Gram-negative bacterium that causes meningococcal disease exclusively in humans. Despite the availability of antibiotics, meningococcal disease remains a health problem, especially in the very young.[1] The invasive disease occurs only in patients devoid of specific bactericidal or opsonizing antibodies. Therefore, one way to prevent meningococcal disease is to induce antibodies by vaccination. New *N. meningitidis* glycoconjugate vaccines that trigger a T-cell dependent immune response, composed of capsular polysaccharides (CPSs) of serotypes A, C, Y and W-135 covalently linked to a protein carrier hold considerable promise.[2] Even though serogroup B accounts for at least 50% of meningococcal infections in Europe and North America,[3] the serogroup B CPS has been excluded from the aforementioned CPS-based vaccine family because of the poor immunogenicity in both its native[4] and conjugated forms.[5] This fact is in all likelihood due to the structural similarity of serogroup B CPS with α-(2 → 8)-linked polysialic acid structures present in human neuronal cell adhesion molecules.[6] Therefore, another type of vaccine component is needed to combat *N. meningitidis* group B infections.

Several studies have suggested the employment of meningococcal lipo-oligosaccharide (LOS) as a potential vaccine candidate.[7–9] The glycolipid LOS (outer-core → inner-core → lipid A) is a major component of the outer membrane of *N. meningitidis* and sera-derived bactericidal antibodies, from patients with meningococcal infection, were found to be directed against the LOS, among other cell-surface antigens.[10,11] Importantly, some of these bactericidal LOS antibodies appear to be directed at conserved low-molecular weight inner-core LOS epitopes.[9,12,13] The pioneering work by Jennings and co-workers led to several observations regarding the structures of meningococcal LOSs[14–18]

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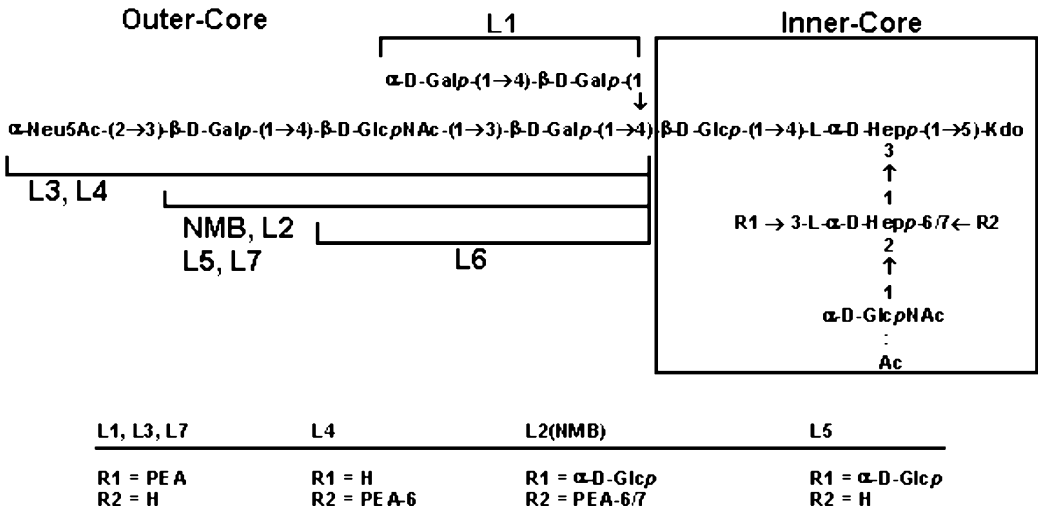

Fig. 1. The structures of the core oligosaccharides of *N. meningitidis* immunotypes. 12 (L1–L12) LOS immunotypes have been serologically characterized; they differ structurally in composition and extension of outer-core and in the presence and location of PEA on HepII. In some LOSs, sialic acid may be attached to O-3 or O-6 of the terminal Gal unit.

(Fig. 1). Serologically, there are 12 (L1–L12)[19–21] presently recognized LOS immunotypes among meningococci that differ in outer-core composition and in 2-aminoethyl phosphate (PEA) substitution at the distal L-*glycero*-α-D-*manno*-heptose (HepII), more precisely at positions O-3, O-6, and O-7 (Fig. 1). L2 and L5 immunotype LOSs may also carry a D-glucose (α-D-Glc) unit at O-3 of HepII (Fig. 1). Meningococci lack the biosynthetic machinery to produce an O-chain polysaccharide, typical of lipopolysaccharides (LPSs; O-chain → core → lipid A), and thus produce solely a LOS (core → lipid A) consisting of a structurally conserved lipid A moiety and a core OS (outer-core → inner-core) that displays some variability in glycosylation and phosphorylation, structural features that seem to be responsible for the observed LOS serotypic diversity. Previous structural studies have revealed that *N. meningitidis* NMB (serotype 2b:P1.2,5) LOSs (L2 immunotype) carry the PEA at O-6 or at O-7 of HepII and also a Glc unit at O-3 of HepII[22] (Fig. 1), similar to other examined L2 immunotype LOS from *N. meningitidis* strain 2241.[17] The outer-core region of many LOS immunotypes is composed of glycans that mimic human blood-group antigens (lacto-*N*-tetraose, paraglobosides, *N*-acetyl-lactosamine, and pk blood-group). Hence, in order to avoid the risk of inducing autoimmune responses, it is a precondition that the outer-core region be removed from any *N. meningitidis* LOS being contemplated as a vaccine candidate. Here, we describe the structure of the LOS from *N. meningitidis* phosphoglucomutase (Pgm) isogenic mutant NMB-R6, an enzyme involved in the production of D-glucose 1-phosphate, which tentatively should not carry Glc and thus no outer-core region. The genetic generation of NMB-R6 has been described elsewhere.[23]

That a "deep" truncation in the NMB-R6 LOS mutant had indeed taken place was observed at the outset by comparing the gel-electrophoresis profile of the wild-type NMB LOS (Fig. 2, lane B) with that of NMB-R6 LOS (Fig. 2, lane C). Monosaccharide analyses by the alditol acetate[24] and but-2-yl glycoside[25] procedures were carried out on the NMB-R6 lipid A-free core oligosaccharide (OS), generated by treatment

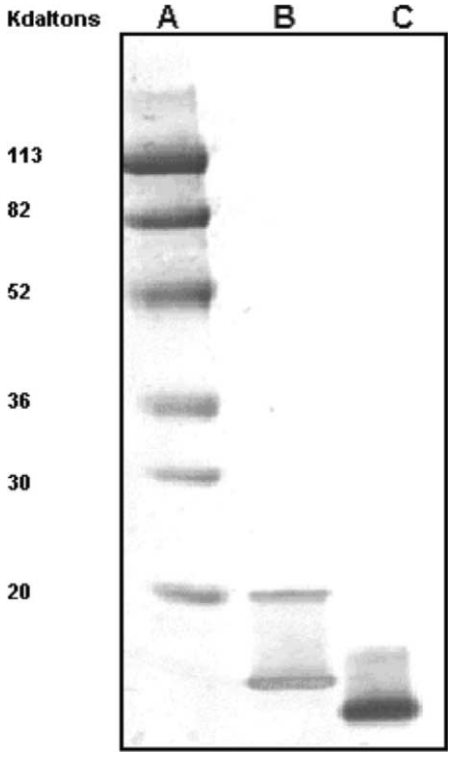

Fig. 2. The gel-electrophoresis profiles of NMB wild type LOS (lane B) and of Pgm mutant NMB-R6 LOS (lane C).

of NMB-R6 LOS with 1% acetic acid (100 °C, 2 h), which cleaved the core OS form the lipid A region through the ketosidic linkage. The gas–liquid chromatography (GLC) profile (Fig. 3) of the alditol acetate derivatives from NMB-R6 core OS, obtained from fractionation on a Bio-Gel P6 column with water as eluent, revealed that the NMB-R6 LOS was composed of D-Glc, *N*-acetyl-D-glucosamine (GlcNAc), and L-*glycero*-D-*manno*-heptose (LDHep). The derivative corresponding to 1,6-anhydro LDHep originates from LDHep due to the conditions of alditol acetate formation. These composition analyses indicated that galactose (Gal), a main component of NMB wild-type LOS outer-core, was present only in trace amount, and was no longer a major key constituent of NMB-R6 LOS. Although in lesser amounts than GlcNAc and Hep, Glc was still present in NMB-R6 (Fig. 1), and thus the expected removal of Glc through the Pgm knockout mutation was not absolute. The core OS obtained by Bio-Gel P6 fractionation was subjected to a sugar linkage analysis by the permethylated alditol acetate procedure.[26] The linkage types detected were terminal Glc, Hep, and GlcNAc, 3-substituted LDHepI, and 3,4-disubstituted LDHepI in the approximate respective ratios of 0.4:0.11:0.7:0.3. Trace amounts of terminal Gal and 4-substituted Glc were also observed. These results were in line with the known structure of the inner regions of *N. meningitidis* NMB wild-type LOS (Fig. 1), with the exception that, within our limits of detection, no derivatives of the distal HepII, which is typically substituted by PEA units, were observed. The presence of trace amounts of the Gal-(1→4)-Glc region, attached to HepI (Fig. 1), was suggested due to the presence of minor amounts of terminal Gal and 4-substituted Glc.

In order to gain an insight into the overall makeup of the glycan molecule(s) present in NMB-R6 LOS, mass spectroscopy (MS)-based experiments using electron

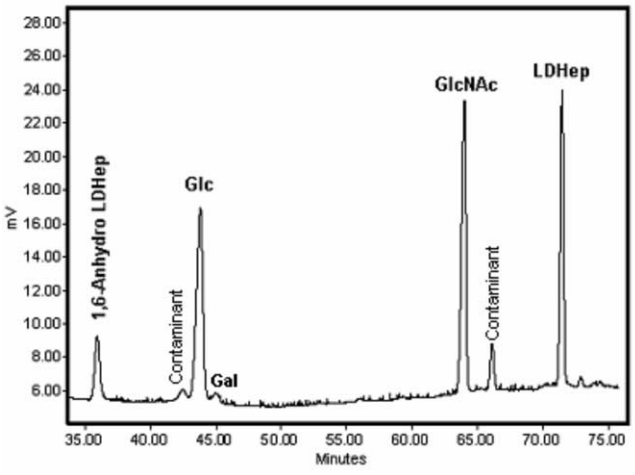

Fig. 3. The GLC profile of the alditol acetate derivatives of NMB-R6 core OS; *contaminant.

spray (ES) and matrix-assisted laser-desorption ionization–time of flight (MALDI-TOF) were carried out on the lipid A-free core OS and on de-*O*-acylated LOS. For these analyses, core OS material was further fractionated by Bio-Gel P4 with 50 mM pyridine acetate buffer as eluent. This fractionation yielded two carbohydrate-positive peaks (core OS1 and OS2) that were analyzed independently. The ES-MS analysis of core OS2 (second eluting peak) (Fig. 4A) revealed a dominant OS molecule (*m*/*z* 947.4) that was composed of 1 Kdo (220 amu), 1 GlcNAc (203 amu), 2 Hep (384 amu) units, and 1 PEA (123 amu) moiety. Additional substitutions by acetyl at GlcNAc (*m*/*z* 989.3) and by Glc at HepI and at HepII (*m*/*z* 1109.3 and 1271.3) were also detected. Fig. 4B displays the ES-MS spectrum of core OS1 (first eluting peak), which furnished a major ion at *m*/*z* 1070.4 that corresponded to 1 Kdo, 1 GlcNAc, 2 Hep residues, and 2 PEA substituents. As with the mono-PEA core OS2 (Fig. 4A), additional substitutions by acetyl at GlcNAc (*m*/*z* 1113) and by Glc at HepI (*m*/*z* 1233) were also observed. The previous detection of 3- and 3,4-substituted LDHepI derivatives in the methylation linkage analysis indicated that the PEA substitutions were at HepII, in line with previous assigned structures of *N. meningitidis* LOSs (Fig. 1). This ES-MS data showed that NMB-R6 core OS1 expressed mainly an inner-core carrying 2 PEA units and NMB-R6 core OS2 was composed of a similar inner-core, but with a single PEA substitution. Both core OSs, to some extent, could be further acetylated and glucosylated. The composition of the typical *N. meningitidis* lipid A was confirmed by MALDI-TOF/MS analysis of the LOS after de-*O*-acylation. The *N*-acylated diphosphorylated lipid A [PO_4→4)-GlcN-(1→6)-GlcN-1→PO_4] (*m*/*z* 952) region was defined by MALDI-TOF/MS (Fig. 5). As previously observed by ES-MS (Fig. 4), the presence of two PEA units (*m*/*z* 2225.95) in the inner-core was also established, as well as the two additional Glc residues (*m*/*z* 2265.2 and 2426.85) (Fig. 5).

In an attempt to assign the PEA positions by ^{31}P NMR spectroscopy a series of 1D and 2D experiments were performed. The 1D ^{31}P NMR spectra of the mono-PEA core OS2 (Fig. 6A) showed several resonances at around ~1.05 ppm, and that of the di-PEA core OS1 (Fig. 6B) showed an additional cluster of resonances at ~0.4 ppm. 1D and 2D COSY ^{1}H NMR experiments were carried out on core OS2 to assign glycosyl anomeric and PEA-derived ^{1}H resonances. The 1D ^{1}H NMR spectrum of core OS2 (Fig. 6C) showed dominant α anomeric signals for HepI (δ_H 5.07), HepII (δ_H 5.48) and GlcNAc (δ_H 5.10). Also two characteristic PEA methylene (P-O-CH_2-C**H_2**-NH_2) resonances at δ_H 3.12 and 3.23 were observed, which indicated that there were at least two dominant and independent PEA moieties. Our efforts to obtain 2D correlation spectroscopy data

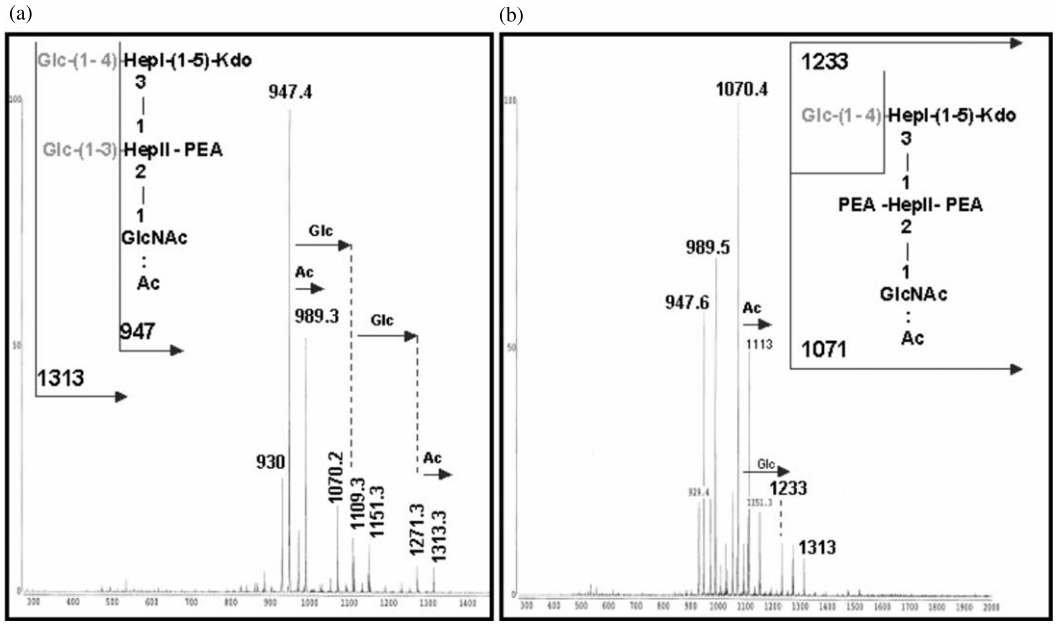

Fig. 4. The ES-MS spectra of (A) NMB-R6 core OS2 and (B) NMB-R6 core OS1.

in order to assign the PEA position on the heptose proved ineffective as both 2D $^{1}H-^{1}H$ and $^{1}H-^{31}P$ spectra were too complex to arrive at any solid conclusions. This convolution of ^{31}P and ^{1}H NMR signals was to be expected due to the fact that from the MS data described above (Fig. 4) it was observed that the inner-core region was heterogeneous in acetylation, glucosylation, and phosphorylation.

The linkage sites of the PEA units on HepII were deciphered by performing the following steps: (i) methy-

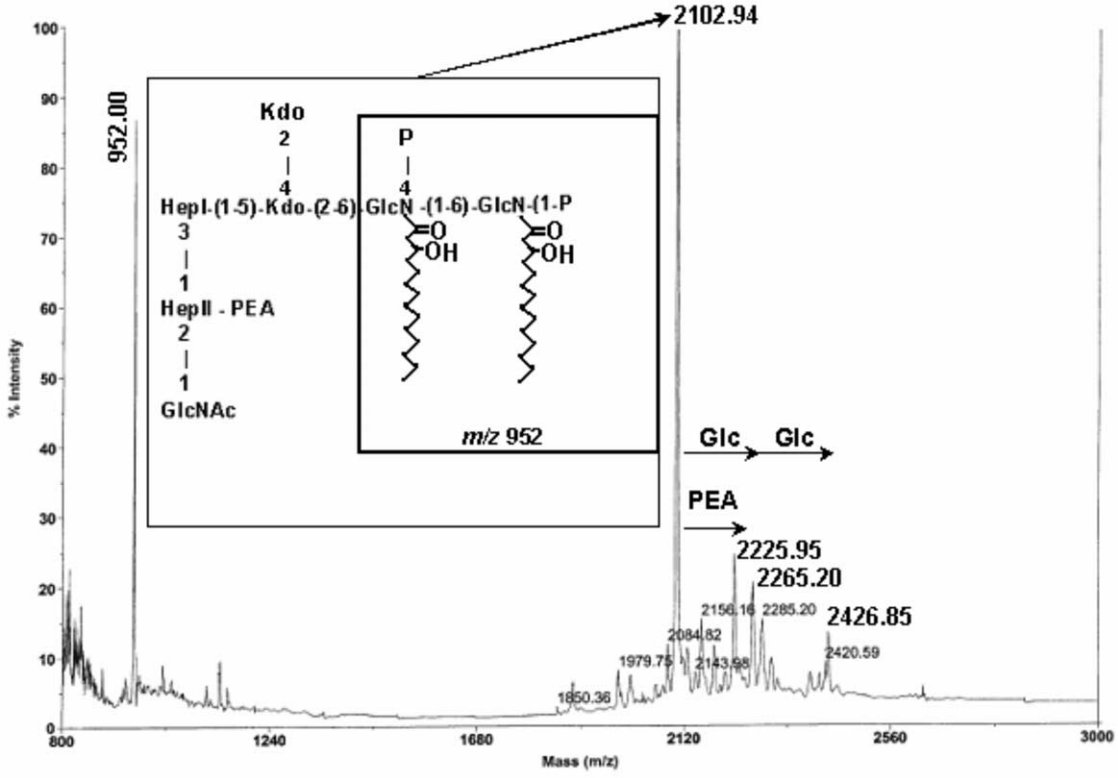

Fig. 5. The MALDI-TOF/MS of de-*O*-acylated NMB-R6 LOS.

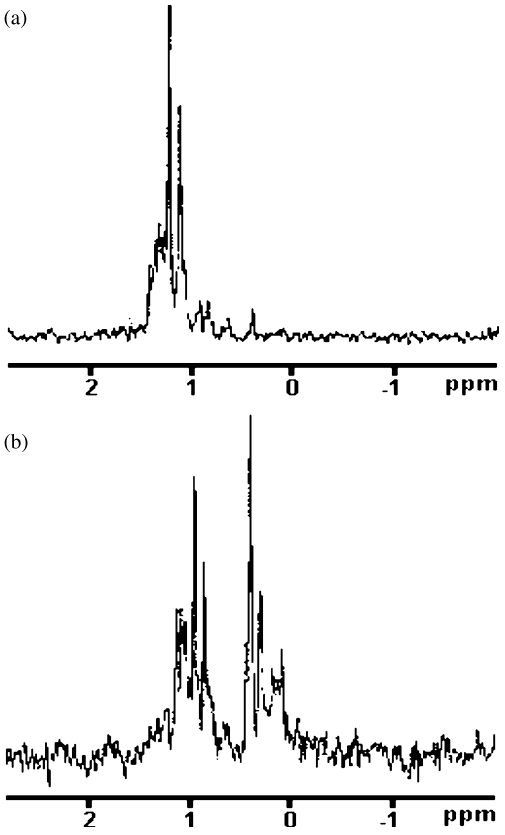

Fig. 6. The 1D spectra of (A) ^{31}P NMR of NMB-R6 core OS2, (B) ^{31}P NMR of NMB-R6 core OS1 and (C) ^{1}H NMR of core OS2.

lation of core OS (obtained from Bio-Gel P-6 fractionation) with CH_3I; (ii) removal of PEA(s) with 48% HF; (iii) methylation with CD_3I; (iv) hydrolysis; (v) reduction with $NaBD_4$; (vi) acetylation; and (vii) characterization of derivatives by GLC-MS. The linkage positions that carried PEA were substituted by CD_3. The complete GLC profile of the permethylated alditol acetate derivatives from the above-described manipulations can be seen in Fig. 7. As expected, the non-phosphorylated units, terminal Glc, terminal GlcNAc, 3-substituted HepI, and 3,4-disubstituted HepI were observed. More importantly, several derivatives of HepII were characterized that lead to the unambiguous assignment of the PEA moieties; those being small amounts of non-phosphorylated terminal HepII, 6(PEA)-substituted HepII, 7(PEA)-substituted HepII, which indicated that traces of HepII units did not carry PEA and some HepII residues were not substituted by GlcNAc. Derivatives of 2(GlcNAc),3(PEA),6(PEA)-trisubstituted HepII and 2(GlcNAc),3(PEA),7(PEA)-trisubstituted HepII showed the presence of a di-PEA HepII, and indicated that PEA could be simultaneously attached to O-3/O-6 or to O-3/O-7. Glucosylation at O-3 of a mono-phosphorylated HepII was also characterized by the derivatives of 2(GlcNAc),3(Glc),6(PEA)-trisubstituted HepII and 2(GlcNAc),3(Glc),7(PEA)-trisubstituted HepII. The detection of small quantities of terminal Gal and 4-substituted Glc (Fig. 7) pointed to tiny amounts of a Gal-(1→4)-Glc-(1→3)-HepI moiety. These analyses showed that NMB-R6 could produce LOSs with PEA at O-3, O-6 and O-7 of HepII, a structural feature that covers the PEA characteristics of all the *N. meningitidis* LOS immunotypes (Fig. 1). These variations in PEA linkage-sites in NMB-R6, detected by GLC-MS of the just described permethylated alditol acetate derivatives go hand-in-hand with the detection of several NMR resonances (Fig. 6) that related to independent PEA residues. No evidence for PEA migration in the methylation linkage procedure was detected, as similar controlled experiments with previously characterized LOSs containing PEA at a single position (PEA-6) did not afford any unexpected PEA substitution.

Collectively, the data presented here showed that the *N. meningitidis* Pgm mutant NMB-R6 manufactured truncated LOS molecules devoid of an outer core, and possessed an inner core with various degrees of PEA substitution and glycosylation (Fig. 8). The most important conclusion is that NMB-R6 produced LOSs with PEA at all the positions so far detected in previous analyzed *N. meningitidis* LOS immunotypes, namely, at O-3, O-6 and O-7 of HepII. N. meningitidis NMB wild type strain has only been structurally shown[22] to produce LOSs with PEA at O-6 and O-7 (Fig. 1). From these structural data, the following LOS biosynthetic observations can be put forth: (i) addition of PEA to O-6 or O-7 of HepII can take place prior to addition of GlcNAc at O-2 of HepII; (ii) no sole O-3 PEA was observed, and thus, most likely, addition of PEA at O-3 takes place after placement of PEA at the exocyclic HepII O-6 or O-7 positions; (iii) that upon interruption of Glc attachment through inactivation of the Pgm enzyme in NMB, PEA can be added to the newly accessible HepII O-3 position in NMB (L2) immunotype, which indicated that there are genes capable of adding PEA to this particular position; (iv) and biosynthetically L2 immunotypes may first place PEA at O-3, O-6 and O-7 of HepII and subsequently remove PEA from O-3 prior to addition of Glc to that position for a final L2 immunotype inner-core epitope; and (v) albeit in small amounts, Glc can still be produced and transferred to O-4 of HepI and O-3 of HepII and, subsequently, a Gal-(1→4)-Glc epitope is also produced in trace amounts, as in NMB wild type strain (Fig. 1).

N. meningitidis LOSs carrying two concurrently PEA units at HepII (PEA-3/6) have been described in two mutant strains expressing truncated meningococcal

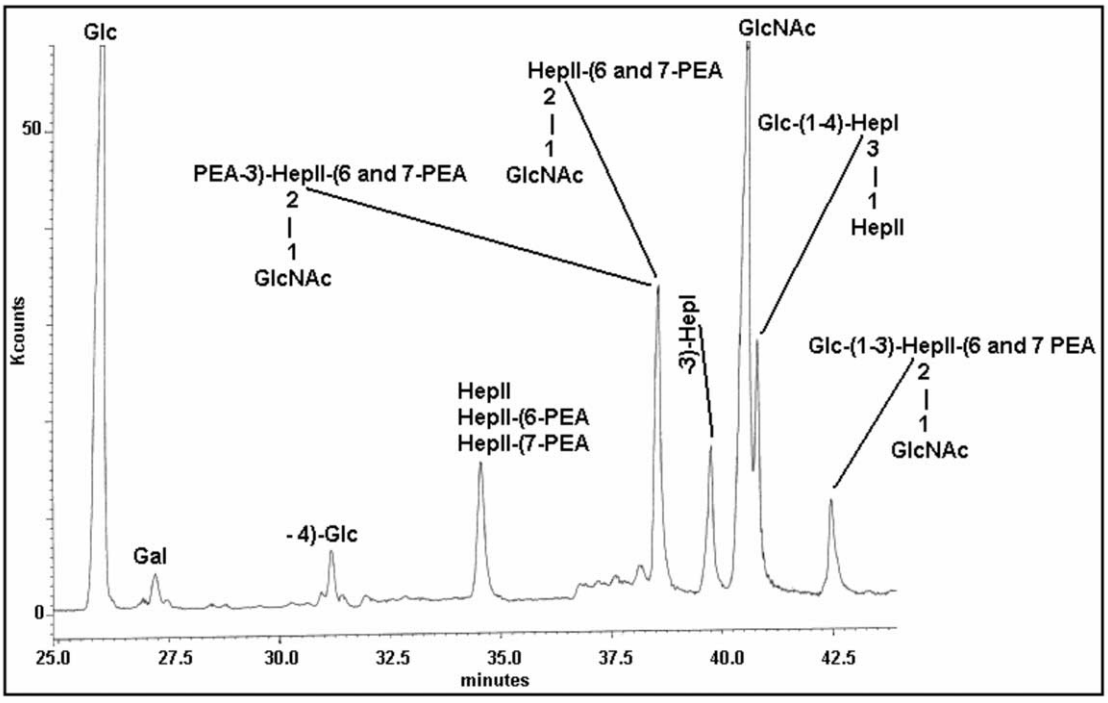

Fig. 7. The GLC profile of the permethylated alditol acetate derivatives from the modified methylation linkage analysis (CH_3I; 48% HF; CD_3I) to characterize PEA-substituted HepII units.

LOS.[27,28] The first case[27] described the LOS structure from the GlcNAc transferase ($rfaK_{NMB}$) mutant strain CMK1 and the other example was that of the LOS from strain BZ157 *galE* mutant.[28] The LOS structures of NMB-R6 described here displayed a high degree of phase variability (Fig. 8). The feasibility of a glycoconjugate vaccine composed of NMB-R6 LOSs is presently being evaluated in our laboratory.

1. Experimental

1.1. Generation of NMB-R6, bacterial growth, and LOS extraction and purification

The Pgm mutation of strain NMB was performed as described.[23] For this analysis NMB-R6 was further manipulated by removing its capacity to produce a

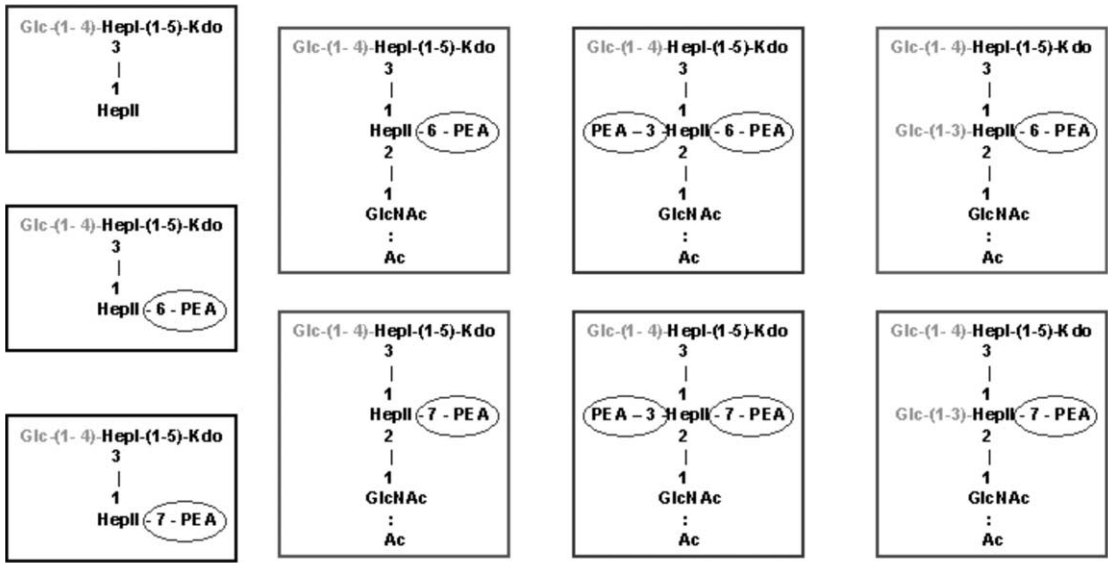

Fig. 8. The variable LOS core structures expressed by NMB-R6.

CPS through the mutation of replacement of the CPS biosynthesis genes *siaA-siaD* with an antibiotic resistance cassette. *N. meningitidis* NMB-R6 was grown using Morse's defined media for 6 h. Cells were heat-killed.

LOS isolation and purification was done as described by Wu et al.[29] and Gu et al.[30] with some modifications. Cell pellets were suspended in 40 mM phosphate buffer containing 5 mM EDTA and 0.02% NaN_3 and digested with lysozyme (2 mg/mL) for 16–18 h at 4 °C followed by incubation (37 °C) with nucleases (100 μg/mL) for 3 h. Crude LOS was isolated by hot phenol–water extraction.[31] The LOS was precipitated from the aqueous phase with NaOAc (5 mg/mL) and two volumes of acetone kept at 4 °C overnight. The LOS pellet was washed with 70% EtOH to remove traces of phenol and retreated with nucleases as described above followed by proteinase K treatment (0.35 mg/mL) at 60 °C for 16–18 h. After three ultracentrifugations at 105,000 g for 3 h at 5 °C, the purified LOS was solubilized in sterile water and lyophilized.

1.2. De-*O*-acylation of LOS, gel-electrophoresis, and core OS preparation

LOS (10 mg) was de-*O*-acylated using 1 mL of anhydrous hydrazine for 3 h at 37 °C.[32] The suspension was cooled on ice and added drop wise to 5 mL of cold acetone. Precipitated de-*O*-acylated LOS was solubilized with water and purified through a Bio-Gel P6 column. Selected fractions monitored by UV 206 nm were pooled, assayed for Kdo and lyophilized.

LOS gel-electrophoresis was carried out on a 4–20% polyacrylamide gel, and visualized by silver stain as described by Morrissey.[33] Core OS was released by mild acid hydrolysis with 1% AcOH at 100 °C for 3 h. The supernatant was passed through a Bio-Gel P-6 (water eluent) or Bio-Gel P4 column equilibrated with 0.05 M pyridinium acetate buffer pH 5.23.

1.3. Sugar composition analysis and linkage site analysis

Sugar composition analysis was performed by the alditol acetate method. The hydrolysis was done in 4 M-trifluoroacetic acid at 100 °C for 4 h followed by reduction in H_2O with $NaBD_4$ and subsequent acetylation with Ac_2O and with residual NaOAc as the catalyst. Alditol acetate derivatives were analyzed by GLC-MS using a Hewlett–Packard chromatograph equipped with a 30-m DB-17 capillary column (210 °C (30 min) → 240 °C at 2 °C/min), and MS in the electron-impact mode was recorded using a Varian Saturn II mass spectrometer. Enantiomeric configurations of the individual sugars were determined by the formation of the respective 2-(*S*)- and 2-(*R*)-butyl chiral glycosides. Methylation linkage analysis was carried out by the $NaOH–Me_2SO–CH_3I$ procedure and with characterization of permethylated alditol acetate derivatives by gas–liquid chromatography–mass spectrometry in the electron impact mode (DB-17 column, isothermally at 190 °C for 60 min).

1.4. Mass spectrometry and nuclear magnetic resonance spectroscopy

The ES-MS experiments were carried out on a Thermo Finnigan LCQ Deaxp instrument. An electrospray stainless-steel needle (27 gauge) was butted against the low dead volume tee and enabled the delivery of the sheath solution to the end of the capillary column. The separations were obtained on about 90-cm length bare fused-silica capillary using in deionized water. A voltage of 20 kV was typically applied at the injection. Mass spectra were acquired with dwell times of 3.0 ms per step of 1 m/z unit in full-mass scan mode. The de-*O*-acylated LOS was analyzed in a Perseptive Biosystems Voyager instrument with 2,5-dihydroxybenzoic acid as matrix.

^1H and ^{31}P NMR spectra of core OSs were recorded on a Bruker AMX 500 spectrometer at 300 K using standard Bruker software. Prior to performing the NMR experiments, the samples were lyophilized three times with D_2O (99.9%). The HOD peak was used as the internal reference at δ_H 4.786 in the ^1H NMR spectrum, and orthophosphoric acid (δ_P 0.0) was used as the external reference in the ^{31}P NMR experiments.

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