Structure of the L5 Lipopolysaccharide Core Oligosaccharides of Neisseria meningitidis*

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Three different oligosaccharides were isolated by mild acid hydrolysis of the lipopolysaccharides, obtained from Neisseria meningitidis serotype 5, and their structures were elucidated by combined chemical and physical techniques. The use of 500-MHz 1H NMR in both one-dimensional and two-dimensional modes as well as nuclear Overhauser effect experiments were employed. To assist in the structural assignments the purified oligosaccharides were also degraded by chemical and enzymatic procedures to smaller fragments. The largest of the three original oligosaccharides is a triantennary partially O-acetylated decasaccharide in which the largest antenna terminates in a lacto-N-neotetraose unit. The smaller oligosaccharides (heptasaccharide and octasaccharide) except for terminal glucose deletions from the longest antenna are structural replicas of the larger.

The meningococcal LPS of Neisseria meningitidis has been implicated in the immune response to natural infection, and at least 11 serotypes (L1-L11) have been identified. There is no apparent correlation between meningococcal serogroup, designated by meningococci having a common capsular polysaccharide, and LPS serotype, except that the L10 and L11 serotypes are exclusively associated with serogroup A organisms. The LPS serotype epitopes are located in the glyco moieties of the LPS, the latter having been identified as low molecular weight oligosaccharides of the R-type (6, 7). By injecting rabbits with protein conjugates of the above oligosaccharides it has also been demonstrated (5) that they contain bactericidal epitopes. Structural studies (7, 8) on the largest of the oligosaccharides obtained from some individual meningococcal serotypes, including the one obtained from the L5 serotype (8), have identified regions of structural similarity and structural difference in them which are probably responsible for both the serotype specificity and cross-reactivity exhibited by meningococci (3, 5).

However, the above structural studies did not address the phenomenon of heterogeneity among the LPS oligosaccharides associated with individual meningococcal serotypes (9, 10), which is probably the basis of even further immunologic diversity. This heterogeneity is generated either by structurally similar oligosaccharides having phosphoethanolamine groups in differing locations (8) or by glucose deletions from the oligosaccharides. This latter phenomenon was hypothesized to explain the molecular size heterogeneity exhibited by the LPS of individual meningococcal serotypes when run in sodium dodecyl sulfate gels (11, 12), and this hypothesis has since been confirmed by chromatographic procedures on the isolated oligosaccharides (8, 13). The isolation and structural determination of three different sized but structurally related oligosaccharides from the meningococcal L5 serotype confirms the above hypothesis.

EXPERIMENTAL PROCEDURES

RESULTS

Isolation of Core Oligosaccharides—The heterogeneous nature of the core oligosaccharides of the L5 determinant was confirmed when 1% acetic acid hydrolysis of the LPS and gel filtration chromatography of the hydrolysate on Bio-Gel P-4 yielded three distinct products with K,, 0.44, 0.59, and 0.65, designated oligosaccharides 1, 2, and 3 in order of decreasing size (Fig. 1).

Structure of Oligosaccharides 1, 2, and 3—The structures of oligosaccharides 1, 2, and 3 are shown in Fig. 2. Sugar analysis of 1 indicated that it was composed of D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, L-glycero-d-manno-heptose, and 3-deoxy-d-manno-octulosonic acid (KDO) in the molar ratio of 2:3:2:2:1. In addition the 'H NMR spectrum of 1 indicated that it also contained O-acetyl groups (δ = 2.19 ppm) in a molar ratio of ~0.4. Following removal of these groups with sodium hydroxide, the 'H NMR of de-O-acetylated 1 (Table I) was in agreement with the sugar analysis, i.e. two signals at δ 2.041 and δ 2.119 ppm were indicative of N-acetyl groups assigned to the two D-glucosamine residues b and i, respectively. Also, nine signals in the proton anomeric region indicated that 1 contained at least 9 sugar residues with 5 of them, at δ 4.485, 4.756, 4.566, 4.541, and 4.576, having large 3J1,2 vicinal coupling constants (~7-8 Hz) indicating that they were in the β-anomeric configuration. The remaining 4 residues at δ 5.075, 5.424, 5.328, and 5.187 ppm (Table I) had small (~1-3 Hz) 3J1,2 coupling constants, and except for those having the manno configuration, could...
sequence, a number of chemical and enzymatic degradations were performed on 1. First, it was anticipated from the structural information obtained for 1, 2, and 3 by FAB-MS upon analysis of their partial hydrolysis (1% acetic acid) of the native L5 LPS and the endo-β-galactosidase treatment of oligosaccharide 1. Methylation analysis of 3 indicated that it contained all methylated sugars present in 1 (Table II) except for those corresponding to unit 6 and for the appearance of one terminal glucose unit and disappearance of the O-4-linked glucose residue present in 1. These results were in agreement with the known specificity of endo-β-galactosidase from E. freundii which cleaves the β-D-Galp-1→4β-D-Glcp linkage present in various glycosphingolipids (23).

The sequence and anomeric configurations of the individual residues of 3 (its O-deacetylated and NaBH₄, reduced form) were ascertained from 'H NMR (NOE) data. The chemical shifts of some of the protons associated with the individual residues of modified 3 were assigned by two-dimensional (H,H) COSY using both one-step (14) and two-step relayed coherent transfer (15) and are listed in Table III. Following assignments, the individual anomeric signals of each of the residues (g, h, i, f, e, d) in 3 (Fig. 2) were selectively irradiated, and the observed NOE values are listed in Table IV. Irradiation of H-1 (d) gave enhancements on its own H-3 and H-5 protons as well as on H-4 (e) indicating that the terminal glucopyranosyl residue (d) is in the β-D-configuration and is linked to O-4 of the adjacent D-glucopyranosyl residue (e). When H-1 (e) was irradiated it gave enhancements on H-3 (e) and H-5 (e) consistent with e being in the β-D-configuration and on H-4 (f) indicating that f is in the α-D-configuration; other enhancements were observed that could belong to the borohydride-reduced (open chain) form of the KDO residue (f), but we could not be certain of the assignments of these signals. When H-1 (h) was irradiated, it gave enhancements on H-2 (h) consistent with h being in the α-D-configuration and on H-3 (g) indicating that the terminal α-D-glucopyranosyl residue h was linked to O-3 of its adjacent L-glycero-a-D-manno-heptopyranosyl residue (g). Now when H-1 (g) was irradiated enhancements on both H-2 (g) and H-3 (f) were observed, indicating that g is in the α-D-manno configuration and that it is linked to O-3 of the L-glycero-a-D-manno-heptopyranosyl residue (f). Finally when H-1 (i) was irradiated, enhancements on both H-2 (i) and H-3 (g) were observed consistent with the terminal a-D-glucopyranosyl residue h being in the α-D-configuration and linked through O-2 of its adjacent L-glycero-a-D-manno-heptopyranosyl residue (g). The NOE data support the sequence of glycoses of 3 shown in Fig. 2. It is interesting to note that the structure of 3 is part of that of the L3 core determinant (7) except for an additional α-D-glucopyranosyl residue linked at O-3 of the heptopyranosyl side chain residue (g) and also for an additional β-D-glucopyranosyl residue linking the lacto-N-neotetraose unit to the inner core of 1. The "native" oligosaccharide 3 as well as the resulting digestion product of 1 by endo-β-galactosidase are both O-acetylated (~30%) on an as yet undetermined position on the terminal 2-acetamido-2-deoxy-a-D-glucopyranosyl residue (i); this chemical evidence was originally obtained for 1, 2, and 3 by FAB-MS upon analysis of their positive mode mass spectra (24). The final structure of 1, shown in Fig. 2, is of course the result of linking the reducing trisaccharide 6 to heptasaccharide 3. To confirm this we performed deamination studies on N-deacetylated 1. Following treatment with sodium nitrite in acetic acid, the products of deamination of N-deacetylated 1 were purified on Bio-Gel
P4, and two major products were obtained. The largest fragment identified as 4 (Fig. 2) was analyzed by methylation (Table II) and $^1$H NMR analyses (Table I). By comparison with the methylation analysis of 3, that of 4 contained one more additional terminal galactose residue, and in addition one of the original 2,3,4,6-tetramethylglucose residues found originally in 3 was transformed into a 2,3,6-trimethylglucose indicating that the terminal galactopyranosyl residue (e) is linked to O-4 of the glucopyranosyl residue (d). Also, the 4,6,7-trimethylheptopyranosyl residue present in the methylation analysis of 3 was now replaced by a 2,4,6,7-tetramethylheptopyranosyl residue indicating that the terminal 2-acetamido-2-deoxyglucopyranosyl residue (i) was indeed linked to O-2 of residue g as previously indicated by $^1$H NMR NOE data on 3. The smallest deamination product identified as 3 was shown to have the structure depicted in Fig. 2 following methylation and $^1$H NMR analyses.

Additional confirmatory evidence that terminal galactose residue (e) of 4 (Fig. 2) was linked to O-4 of the glucopyranosyl residue (d) was obtained when 4 was treated with a $\beta$-D-galactosidase. Indeed after treatment with the enzyme, 4 has its terminal $\beta$-D-galactose residue (e) removed, and a new product identified as 7 was characterized following methylation and $^1$H NMR analyses. It had the structure depicted in Fig. 2, where the O-4-linked glucose residue (d) in 4 had now become a terminal nonreducing end.

Based on the structural results obtained from both treatment of 1 with endo-$\beta$-galactosidase and deamination studies, a single structure for 1 as depicted in Fig. 2 was proposed. The entire structure differs from that of the L3 core (7) by having an additional O-4-linked $\beta$-D-glucopyranosyl internal residue (e) and an additional terminal $\alpha$-D-glucopyranosyl residue (h) linked to O-2 of the heptopyranosyl side chain residue (g). The oligosaccharide is partially O-acetylated on residue (h) and does not contain phosphoryl substituents as in the case of the L3 core determinant (7). Finally the third product with $K_{\text{m}} 0.59$ identified as 2 obtained from the acetate acid hydrolysis of the L5 LPS is an oligosaccharide whose structure depicted in Fig. 2 is an intermediate between that of 1 and that of 3. It differs from that of 1 by lacking the terminal $\beta$-D-Gal$\beta1\rightarrow4\beta$-D-GlcNAc disaccharide whereas glycose deletions from the inner heptose core would have produced oligosaccharides associated with LPS of different serotypes (8). Also, it is interesting to note that glycose deletions from the long antenna of the L5 oligosaccharide create new and the probably more immunogenic epitope due to the destruction of the lacto-N-tetraose unit. This is consistent with the fact that all the meningococcal serotype LPSs, only in the case of L5 serotype is the smallest component on SDS gels the major antigenic component (27).

All the three oligosaccharides obtained from the L5 serotype LPS are partially O-acetylated on their terminal 2-acetamido-2-deoxy-$\alpha$-D-glucopyranosyl residues. This is the first report of the presence of $\alpha$-acetyl substituents in the neisserial LPS, the identification of which suggests a further mechanism by which meningococci modulate their surface glycose structures. The identification of $\alpha$-acetyl substituents also raises the question as to whether in the L5 LPS the oligosaccharides are completely O-acetylated. Certainly it is reasonable to assume that $\alpha$-acetyl groups could be partially removed under the mild hydrolytic conditions used to obtain the oligosaccharides from the LPS.

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Supplemental material:

Structure of the L5 lipopolysaccharide core oligosaccharides of Neisseria meningitidis

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EXPERIMENTAL PROCEDURES

MATERIALS

Group B meningococcal meningitis strain R6 (serotype 16), was grown in a chemically defined medium (General Biologicals Inc., Chicago, Ill.) and the LPS was isolated previously described.

Gas-liquid chromatography (GLC) was performed on a Hewlett-Packard 1620A equipped with a flame ionization detector and a 30 m DB-1 glass capillary column (J and W Scientific Co., Rancho Cordova, Calif.). The temperature program was as follows: 240°C, 1 min; 240°C-260°C, 5°C/min; 260°C, 1 min. GLC analysis was performed using the following conditions: column temperatures of 240°C, 250°C, and 260°C for 2 min each, and a flow rate of 20 mL/min.

Isolation of oligosaccharides

The core oligosaccharides were obtained by heating the LPS (1 mg/mL) in 1M acetic acid for 2 h at 100°C. The insoluble residue was then dialyzed against deionized water and lyophilized. The residue was then treated with 0.1N acetic acid for 3 h at 60°C. The resulting solution was then applied to a DOWEX 50W-X2 (20-50 mesh, 200-400 mesh, 50 ml) column. The column was washed with deionized water and eluted with 1M acetic acid. The fractions were then concentrated and lyophilized.

Glycose analysis

Sugar and methylation analysis were conducted essentially as described by Neuhof et al. (1983). Following their desamination and conversion into amino sugars, 100-120 mg of oligosaccharide were saponified with 5M sodium ethoxide (0.5 ml) for 2 h at 70°C. The saponified material was then dialyzed against deionized water (12 ml) and 1 ml of 15% (v/v) acetic acid for 2 h at room temperature. The resulting solution was then applied to a DOWEX 50W-X2 (20-50 mesh, 200-400 mesh, 50 ml) column. The column was washed with deionized water and eluted with 1M acetic acid. The fractions were then concentrated and lyophilized.

Hexosamine analysis

Oligosaccharides were hydrolyzed in 1M acetic acid for 2 h at 120°C, then desalted with a Sep-Pak cartridge (Waters Associates) (19). Methylation was performed with 0.2M K2CO3 (1 ml) in 0.05M sodium acetate buffer pH 5.5 (1 ml). The resulting solution was then applied to a Sep-Pak cartridge and eluted with pyridine (1 ml). The eluate was then treated with 1M acetic acid for 2 h at room temperature. The resulting solution was then applied to a DOWEX 50W-X2 (20-50 mesh, 200-400 mesh, 50 ml) column. The column was washed with deionized water and eluted with 1M acetic acid. The fractions were then concentrated and lyophilized.

Preparation of LPS

Oligosaccharides were prepared from the L5 lipopolysaccharide cores of Neisseria meningitidis strain R6. The LPS was purified from the culture supernatant and further purified by chromatography on a DOWEX 50W-X2 (20-50 mesh, 200-400 mesh, 50 ml) column. The column was washed with deionized water and eluted with 1M acetic acid. The fractions were then concentrated and lyophilized.
Table III. Proton chemical shifts of some of the signals for borohydride reduced 2.

| Residue | H-1 | H-2 | H-3 | H-4 | H-5 | NAC |
|---------|-----|-----|-----|-----|-----|-----|
| q       | 5.48 | 4.19 | 4.74 | -   | -   | 0.7 |
| q'      | 5.36 | 3.99 | 3.70 | 3.48 | 3.70 | 0.5 |
| i       | 5.18 | 3.94 | 3.79 | 3.56 | 3.64 | 2.12 |
| e       | 5.45 | 4.20 | 4.00 | 4.18 | 4.19 | 4.20 |
| q       | 4.54 | 3.46 | 3.60 | 3.52 | 3.56 | 2.56 |
| q'      | 4.58 | 3.32 | 3.51 | 3.42 | 3.55 | 3.55 |

- Measured at 300K and 100 MHz. Assignments made from data obtained by homonuclear 2D shift correlated (H/H) COSY and two-step relayed copy.
- Separate signals due to two isomers of KDO-ol.

Table IV. Nuclear Overhauser enhancements for borohydride reduced 2.

| Saturated signal | Negative n.O.e. observed | Signal | % n.O.e. |
|------------------|--------------------------|--------|---------|
| 1-g              | 2-d                      | 11     | 11      |
| 1-h              | 2-b                      | 10     | 10      |
| 1-l              | 2-1                      | 11     | 10      |
| 1-e              | 2-d                      | 10     | 10      |
| 2-6              | 2-6                      | 4      | 4       |
| 2-7              | 2-7                      | 19     | 19      |
| 2-8              | 2-8                      | 13     | 13      |
| 2-d              | 2-d                      | 2      | 2       |
| 2-d,5,6-4e        | 25                       | 25     | 25      |

- Letters q - q refer to residues of 2 as depicted in Fig. 2.
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