Structure of the L5 Lipopolysaccharide Core Oligosaccharides of Neisseria meningitidis*

(Received for publication, October 2, 1989)

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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 (hepta- and octasaccharide) except for terminal glucose deletions from the longest antigen are structural replicas of the larger.

The meningococcal LPS1 has been implicated in the immune response to natural infection (1), and at least 11 serotypes (L1-L11) have been identified (2, 3). 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 (4). The LPS serotype epitopes are located in the glycan moieties of the LPS (5), 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 Kaw 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 2-galactose, 2-acetamido-2-deoxy-D-glucose, 2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, D-glycero-D-manno-heptose, and 3-deoxy-D-manno-2-octulosonic acid (KDO) in the molar ratio of 2:3:2:2:1. In addition the 1H 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 1H 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-galactosamine 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, 4.546, 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

* Portions of this paper (including "Experimental Procedures," Fig. 1, and Tables I-IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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be assigned the α-anomeric configuration.

Methylation analysis of 1 (Table II) indicated that it contained terminal nonreducing D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose residues. D-galactose linked at O-3, two D-glucose residues, and one 2-acetamido-2-deoxy-D-glucose residue linked at O-4. Both heptoses were branch points, one of them substituted at O-3 and O-4, the other one at O-2 and O-3. KDO was linked at O-5 as it is in the L3 determinant (7). In order to obtain information on the sequence, a number of chemical and enzymatic degradations were performed on 1. First, it was anticipated from the structural information obtained for the L3 core determinant and from the results of the methylation analysis of 1 that 1 would also contain the same lacto-N-neotetraose unit from which β-D-Galp-1→4β-D-GlcpNAc-1→3-D-Galp by methylation (Table II) and 1H NMR analysis (Table I).

As shown above, 3 is a product resulting from both the 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-Glepp 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 1H 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 coherence 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 e was linked to O-4 of the next L-glycero-D-manno-heptopyranosyl residue (f). Irradiation of H-1 (f) gave enhancements on H-2 (f) indicating that f is in the α-D-manno 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-α-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-D-manno-heptopyranosyl branch point residue (f). Finally when H-1 (i) was irradiated, enhancements on both H-2 (i) and H-2 (g) were observed consistent with the terminal 2-acetamido-2-deoxy-D-glucopyranosyl residue i being in the α-D-configuration and linked through O-2 of its adjacent L-glycero-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-deacetylated (~30%) on an yet undetermined position on the terminal 2-acetamido-2-deoxy-α-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 1H 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 (c) 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-deoxy-glucopyranosyl residue (i) was indeed linked to O-2 of residue g as previously indicated by 1H 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-β-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 β-D-glucopyranosyl internal residue (e) and an additional terminal α-D-glucopyranosyl residue (h) linked to O-2 of the heptopyranosyl side chain residue (g). The oligosaccharide is partially O-acetylated on residue (i) and does not contain phosphoryl substituents as in the case of the L3 core determinant (7). Finally the third product with K w 0.59 identified as 2 obtained from the acetic 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 β-D-Gal1→4α-D-GlcNAc disaccharide unit. The compositional analysis as well as the sequence of residues in oligosaccharides 1, 2, and 3 was totally (for the composition) and in part (for the sequence) confirmed by FAB-MS studies (24).

**Discussion**

The structures of the three oligosaccharides isolated from the L5 serotype LPS are shown in Fig. 2. The structure of the largest oligosaccharide has been previously reported (8) and has terminal lacto-N-neotetraose on its longest antenna. This structural feature has also been identified on the oligosaccharides obtained from the LPS of the L2 (8) and L3 (7) serotypes and the fact that the LPS from which they were obtained exhibit predominant serotype specificity (5) confirms that lacto-N-neotetraose, despite its large size, is not immunodominant. Thus the serotype epitopes of the meningococcal LPS reside in the heptose-containing inner core of its oligosaccharide moieties (8). The nonimmunogenicity of the lacto-N-neotetraose unit is probably due to immune tolerance because it is a known human and animal tissue antigen (25, 26). The identification of 0-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 O-acetyl groups could be partially removed under the mild hydrolytic conditions used to obtain the oligosaccharides from the LPS.

Acknowledgment—We thank Fred Cooper for the gas-liquid chromatography-MS analyses.

**References**

Supplemental material to:

Meningococcal LPS Oligosaccharide Epitopes


Table 1. H-NMR data for de-acyetylated LPS core oligosaccharides and derived products.

<table>
<thead>
<tr>
<th>Product</th>
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<th>δ2</th>
<th>δ3</th>
<th>δ4</th>
<th>δ5</th>
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<tbody>
<tr>
<td>LPS LPS</td>
<td>2.19</td>
<td>2.10</td>
<td>2.12</td>
<td>2.40</td>
<td></td>
</tr>
</tbody>
</table>

Glycose analysis

Sugar and methylation analysis were conducted essentially as previously described. Determined on an International Series 3 gas liquid chromatograph equipped with a 1-m column of 10% XAD-2 on 80/100 mesh Chromosorb W and a Packard chromatograph equipped with 3% SE-30 on 100/120 mesh Chromosorb W. The gases used were nitrogen and hydrogen, respectively. The column temperatures used were 140 °C and 20 °C for the gas liquid chromatograph and 250 °C and 200 °C for the Packard chromatograph. All sugar methylations were performed according to the procedure of Ziegler and coworkers (1973). The sugars were separated by HPLC on a Bio-Rad HPX-87H column and a Beckman 356 spectrophotometer. The mobile phase used was 0.01 M sulfuric acid at a flow rate of 0.6 ml/min.

Table 1: NMR analysis of oligosaccharides derived from the LPS lipooligosaccharide core.

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Meningococcal LPS Oligosaccharide Epitopes

Table III. Proton chemical shifts of some of the signals for borohydride reduced 3.

<table>
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<tr>
<th>Residue</th>
<th>H-1</th>
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<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>MAC</th>
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<tr>
<td>q</td>
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<td>4.19</td>
<td>4.74</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>q</td>
<td>5.36</td>
<td>3.99</td>
<td>3.70</td>
<td>3.05</td>
<td>3.70</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>5.38</td>
<td>3.96</td>
<td>3.79</td>
<td>3.56</td>
<td>3.64</td>
<td>2.12</td>
</tr>
<tr>
<td>e</td>
<td>5.90</td>
<td>4.80</td>
<td>4.00</td>
<td>4.70</td>
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<td>4.12</td>
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<td>3.63</td>
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<td>3.56</td>
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<tr>
<td>q</td>
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<td>3.53</td>
<td>3.62</td>
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</table>

* Measured at 500 and 100 MHz. Assignments made from data obtained by homonuclear 2D shift correlation (H-H), CCBF and two-step relayed COSY.

Table IV. Nuclear Overhauser enhancements for borohydride reduced 3.

<table>
<thead>
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<th>Saturated negative n.O.E.</th>
<th>Signal</th>
<th>% n.O.E.</th>
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</tr>
<tr>
<td>1-n</td>
<td>2-n</td>
<td>10</td>
</tr>
<tr>
<td>1-l</td>
<td>2-l</td>
<td>11</td>
</tr>
<tr>
<td>1-e</td>
<td>2-e</td>
<td>10</td>
</tr>
<tr>
<td>3-e</td>
<td>3-e</td>
<td>4</td>
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<tr>
<td>3-x</td>
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<td>11</td>
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<td>4-t</td>
<td>4-t</td>
<td>13</td>
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<td>5-t</td>
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<td>2-d</td>
<td>2</td>
</tr>
<tr>
<td>1,2,3,4,5,6</td>
<td>25</td>
<td></td>
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</tbody>
</table>

* Letters q-6 refer to residues of 3 as depicted in Fig. 2.

Fig. 1. Elution profile (Bio-Gel P-4) of the water soluble hydrolysate of the meningococcal envelope LPS.
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