Chemical Structure of the Lipid A Component of Lipopolysaccharides from *Fusobacterium nucleatum*

SUMIHIRO HASE, TOR HOFSTAD, AND ERNST T. RIETSCHEL*

Max-Planck-Institut für Immunbiologie, D-7800 Freiburg, Federal Republic of Germany; and Laboratory of Oral Microbiology, University of Bergen, Bergen, Norway*

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The lipid A component of lipopolysaccharides from *Fusobacterium nucleatum* Fev 1 consists of β-1',6-linked D-glucosamine disaccharides, which carry two phosphate groups: one in glycosidic and one in ester linkage. The amino groups of the glucosamine disaccharides are substituted by 3-hydroxyhexadecanolic acid. The hydroxyl groups of the disaccharide backbone are acylated by tetradecanoic, hexadecanoic, and 3-hydroxytetradecanolic acids. Part of the ester-bound 3-hydroxytetradecanolic acid is 3-O-substituted by tetradecanoic acid. Whereas a similar pattern of fatty acids was detected in lipopolysaccharides from two other *F. nucleatum* strains, the amide-bound fatty acid in *F. varium* and *F. mortiferum* was 3-hydroxytetradecanolic acid. The chemical relationships of lipid A from *Fusobacteria* and other gram-negative bacteria are discussed.

Lipopolysaccharides from *Fusobacterium* resemble those of aerobic gram-negative bacteria such as *Salmonella* in that they consist of a polysaccharide and a lipid component (14, 16). They appear in the electron microscope as structural particles (10) and possess O-antigenic specificity (15).

The endotoxic activity of *Fusobacterium* lipopolysaccharides appears to be comparable to that of *Salmonella* endotoxins (3, 4, 21). In *Salmonella* lipopolysaccharides (and those of other bacterial groups), the endotoxic activity is embedded in the lipid A component (3, 16, 21), the chemical structure of which has been studied (5, 6, 22).

The present communication deals with the chemistry of the lipid A component of *Fusobacterium nucleatum* lipopolysaccharides. It will be shown that its structure is related to that of *Salmonella* lipid A.

Cultures were grown in screw-cap bottles filled to the top with the following (in grams per liter): tryptone (Oxoid), 15; proteose peptone (Oxoid), 10; NaCl, 5; KH₂PO₄, 1.5; Na₂HPO₄, 2H₂O, 3.5; (NH₄)₂SO₄, 0.5; yeast extract (Oxoid), 3; l-cysteine·HCl, 1; and glucose, 2.5; pH 7.0. Cultures were grown for 48 h and washed two times with phosphate-buffered saline (pH 7.4).

Lipopolysaccharides. Washed organisms were extracted with phenol-water (25) at 20°C, and the lipopolysaccharide was purified by ultracentrifugation (100,000 × g, 90 min) and treatment with deoxyribonuclease and ribonuclease (9).

Reference compounds, chemicals, and enzyme. Tetradecanoic, hexadecanoic, and heptadecanoic acids were obtained from Serva (Heidelberg). 3-Hydroxytetradecanoic and 3-hydroxyhexadecanoic acids were synthesized by the Reformatsky reaction (22). 3,4,6-Tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol was prepared from methyl-N-acetylglosaminide by permethylation, followed by acid hydrolysis, reduction, and peracetylation (7). The optical antipodes (α- and β-forms) of the 3-hydroxy acids were prepared from the racemates with amphetamine according to Ikawa et al. (11). N-3-hydroxytetradecanoyl-d-glucosamine was obtained from G. van der Smiessen (Freiburg), N-acetylglosaminyl-α-1',4-N-acetylglosaminide from H. Paulsen (Hamburg), 8-N-acetylglosaminidase (EC 3.2.1.30; *Aspergillus oryzae* [18]) from Y. Matsushima and T. Mega (Osaka), and hydrazine from Roth (Karlsruhe).

**MATERIALS AND METHODS**

Bacteria. *F. nucleatum* strain Fev 1 (1) was kindly provided by S. E. Mergenhagen (Bethesda, Md.). The origin of the other strains (*F. nucleatum* F1 and ATCC 10953, *F. mortiferum* VIP 0473, and *F. varium* ATCC 8501) has been described previously (8, 13).
min, 100°C) and then with acid (0.02 N HCl, 35 min, 100°C). The precipitate formed, representing alkali-treated lipid A (lipid A-OH), was reduced with NaBH₄ (lipid A-OHred) and treated with hydrazine (40 h, 100°C). The reduced lipid A backbone consisting of a reduced glucosamine disaccharide (GlCN-GlcNred) was isolated by high-voltage electrophoresis and N-acetylated (GlCNAC-GlCNACred).

Methylation analysis of the reduced and N-acetylated backbone by gas-liquid chromatography as well as enzymatic analysis with β-N-acetylglucosaminidase was carried out as described previously (6, 7).

(ii) Fatty acids. Ester-bound fatty acids were released from lipopolysaccharides with sodium methy- late (0.25 N NaOCH₃, 10 h, 37°C), and amide-bound fatty acids were released from the de-O-acetylated preparation by strong alkaline hydrolysis (4 N KOH, 5 h, 100°C). For estimation of total fatty acids, lipopolysaccharide was treated with 4 N KOH (5 h, 100°C). The acids were analyzed by gas-liquid chromatography in the form of the methyl esters (22). Two types of columns (glass) were used: Castorwax (2.5% on Chromasorb G, 80 to 100 mesh) at 175°C and an ethylene succinate-methylsilicone copolymer (EGSS-X, 15% on Gas-Chrom P, 100 to 200 mesh) at 140°C. The nature of fatty acids was determined by comparison of their retention times (tₕ) obtained on both columns with those of authentic reference fatty acids and by their fragmentation pattern obtained on combined gas-liquid chromatography–mass spectrometry. n-Heptadecanoic acid methyl ester served as an internal standard for quantitative assays.

Configuration analysis of 3-hydroxy acids was performed by gas-liquid chromatography (3% OV-1 on Gas-Chrom Q, 100 to 200 mesh, 205°C) of their diastereomeric 3-methoxy-L-phenylethylamides (20).

Other methods of analysis. Colorimetric determinations of total phosphate and hexosamine (with and without hydrolysis), and other analyses including high-voltage paper electrophoresis, gas-liquid chromatography (Varian 1400), mass spectrometry (Finnigan 3200), and assays with an amino acid analyzer (Durrum, model D-500), were carried out as described previously (6, 7).

RESULTS

Backbone. To elucidate the structure of the lipid A backbone, essentially the procedure described previously was followed (6). F. nucleatum Fed 1 lipopolysaccharide (46 mg) was treated with alkalai (saponification of ester- bound fatty acids) and acid (hydrolysis of polysaccharide and glycosidically linked phosphate). The precipitate formed (lipid A-OH, 20% yield) contained glucosamine and phosphate in a molar ratio of 2.1:1.0. In the direct Morgan-Elson reaction (without hydrolysis), using N-3-hydroxytetradecanoyl glucosamine as a standard, the color yield corresponded to 0.74 mol of glucosamine (per 2 mol of glucosamine), indicating that part of the glucosamine in lipid A-OH is reducing. Lipid A-OH was then reduced (NaBH₄), yielding lipid A-OHred (73% yield). With the amino acid analyzer, glucosaminol (0.45 μmol/mg), glucosamine (0.17 μmol/mg), and glucosamine-phosphate (0.24 μmol/mg) could be detected. The molar ratio of glucosamine to phosphate was 1.03:1.0. As expected, lipid A-OHred was negative in the direct Morgan-Elson assay.

Lipid A-OHred was treated with hydrazine (40 h, 100°C) to remove amide-bound fatty acids and ester-linked phosphate groups, and the hydrazinolysate was subjected to high-voltage electrophoresis. With ninhydrin, one major fraction (Mglcn = 1.15), corresponding to a glucosaminyl-glucosaminol disaccharide (6, 7), and a second spot (approximately 10% of hydrazinolysate, Mglcn = 1.01), corresponding to glucosaminol, were detected. The disaccharide fraction was eluted with acid (0.01 N HCl) and N-acetylated (30% yield, relative to lipid A-OHred, based on glucosamine).

Preparation GlCNAC-GlCNACred (0.94 μmol) was methylated according to Stellner et al. (24). After hydrolysis, reduction, and peracylation of the methylated material, gas-liquid chromatography gave three peaks (Table 1). Their relative tₕ values indicated (7) that these peaks correspond to 1,3,4,5-tetra-O-methyl-6-O-acetyl-2-deoxy-2-(N-methylaceta-mido)-glucitol (peak 1, tₕ = 0.43), 3,4,6-tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol (peak 2, tₕ = 1.00), and 1,3,4,5-tetra-O-methyl-6-O-acetyl-2-deoxy-2-(N-acetylasa-mido)-glucitol (peak 3, tₕ = 1.10). The relative amounts of the peaks were 5% (peak 1), 53% (peak 2), and 41.8% (peak 3). It has previously been shown (7) that from 6-0-substituted glucosaminol after methylation, hydrolysis, reduction, and peracylation, both the N-meth-

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Identity</th>
<th>Relative tₕ*</th>
<th>Relative amt (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,3,4,5-Tetra-O-methyl-6-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol</td>
<td>0.43</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>3,4,6-Tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol</td>
<td>1.00</td>
<td>53.8</td>
</tr>
<tr>
<td>3</td>
<td>1,3,4,5-Tetra-O-methyl-6-O-acetyl-2-deoxy-2-(N-acetylasamido)-glucitol</td>
<td>1.11</td>
<td>40.8</td>
</tr>
</tbody>
</table>

* On gas-liquid chromatography.
* Based on peak 2 (tₕ = 1.00) on ECNSS-M.
Therefore, it was concluded that peaks 1 and 3 derive from the N-acetylglucosaminol residue and peak 2 from the nonreducing N-acetylgulcosamine residue of fraction GlcNAc-GlcNAcred. The fact that the compounds corresponding to peaks 1 and 3 are 6-O-acetylated shows that in fraction GlcNAc-GlcNAcred a 1′,6-linkage is present.

To study the anomeric configuration of glucosamine, fraction GlcNAc-1′,6-GlcNAcred was treated with β-N-acetylgulcosaminidase. The N-acetylgulcosamine released, as estimated by the direct Morgan-Elson reaction, was 64% after 10 min and 94% after 60 min of incubation. These results agree with those of previous studies (6). Thus, the nonreducing β-glucosamine residue in fraction GlcNAc-1′,6-GlcNAcred is present as the β-anomer. Control experiments with chitobitol and a reduced α-1′,4-linked glucosamine disaccharide gave significantly lower rates and no release of glucosamine, respectively (6).

Fatty acids. Gas chromatographic analysis of fatty acids released from lipopolysaccharides of F. nucleatum Feb 1 by alkali (4 N KOH) revealed the presence of tetradecanoic, hexadecanoic, 3-hydroxytetradecanoic, and 3-hydroxyhexadecanoic acids in amounts of 0.258, 0.037, 0.204, and 0.110 μmol per mg of lipopolysaccharide, respectively (Table 2). Also Δ²-tetradecanoic acid was detected. This acid, however, which was not present in acid hydrolysates, represents an artifact resulting from 3-hydroxytetradecanoic acid (22). The total amount of fatty acids was 0.61 μmol per mg of lipopolysaccharide (= 15 wt%). To distinguish between ester- and amide-bound fatty acids, lipopolysaccharide was first treated with sodium methyate. On gas-liquid chromatography of the methyl esters, the non-hydroxylated fatty acids (C₁₄, 0.03 μmol/mg; C₁₆, 0.03 μmol/mg) and 3-hydroxytetradecanoic acid (0.05 μmol/mg) were found (Table 2). In addition, 3-methoxytetradecanoic acid methyl ester (0.079 μmol/mg) was detected. Since this latter acid was not present in acid hydrolysates, it was concluded that it is an artifact resulting from a β-elimination reaction involving 3-O-substituted, ester-bound 3-hydroxytetradecanoic acid. The product of the β-elimination reaction is known to be Δ²-tetradecanoic acid methyl ester (as detectable in alkaline hydrolysates). Under the conditions of methyate treatment, however, a nucleophilic addition of methyate to the α,β-unsaturated acid ester takes place, yielding 3-methoxytetradecanoic acid methyl ester (22, 23).

The nature of the substituent at the 3-hydroxy group of 3-hydroxytetradecanoic was established in a similar way as described for Salmonella (22); e.g., part of the methanolsate was carbomethyated with diazomethane and compared with the nontreated methanolsate (Table 2, A and B). The peak corresponding to tetradecanoic acid was increased in chromatogram B as compared with the corresponding peak in chromatogram A. This finding shows that part of the tetradecanoic acid was present

### Table 2. Nature, relative retention times, fragments from mass spectrometry, and amounts of fatty acids present in lipopolysaccharides from Fusobacterium nucleatum Feb 1

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Relative t&lt;sub&gt;R&lt;/sub&gt; on gas-liquid chromatography&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mass spectrometry</th>
<th>Amt in hydrolysates&lt;sup&gt;b&lt;/sup&gt; (μmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Castor wax (175°C)</td>
<td>EGSS-X (140°C)</td>
<td>Base peak</td>
</tr>
<tr>
<td>Tetradecanoic (C&lt;sub&gt;14&lt;/sub&gt;)</td>
<td>1.00</td>
<td>1.00</td>
<td>74</td>
</tr>
<tr>
<td>Hexadecanoic (C&lt;sub&gt;16&lt;/sub&gt;)</td>
<td>2.38</td>
<td>1.96</td>
<td>74</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;3&lt;/sup&gt;-Hydroxytetradecanoic (C&lt;sub&gt;16&lt;/sub&gt;)</td>
<td>3.06</td>
<td>7.78</td>
<td>43</td>
</tr>
<tr>
<td>Δ&lt;sup&gt;3&lt;/sup&gt;-Hydroxyhexadecanoic (C&lt;sub&gt;16&lt;/sub&gt;)</td>
<td>7.20</td>
<td>15</td>
<td>43</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on tetradecanoic acid methyl ester (t<sub>R</sub> = 1.00).
<sup>b</sup> Calculated for fatty acid methyl ester.
<sup>c</sup> M, Molecular weight.
<sup>d</sup> Sum of 3-OH-C<sub>14</sub> and Δ<sup>2</sup>-C<sub>14</sub> (0.072 μmol/mg).
<sup>e</sup> Sum of 3-OH-C<sub>16</sub>, 3-OCH₂C<sub>14</sub> (0.079 μmol/mg) and Δ<sup>2</sup>-C<sub>14</sub> (0.009 μmol/mg).
<sup>f</sup> Not visible on mass spectrum.
in the free form, suggesting that it had been released from the lipopolysaccharide by β-elimination. It was concluded that 40% of the ester-bound 3-hydroxytetradecanoic acid in *F. nucleatum* Fev 1 lipopolysaccharide was 3-O-substituted by tetradecanoic acid. By analogy, it could be assumed that the smaller amounts of 3-hydroxytetradecanoic acid (0.05 μmol/mg) found in the methanolsate originated from 3-O-(3-hydroxytetradecanoyl)-tetradecanoic acid. This, however, was unlikely since the amount of 3-methoxytetradecanoic acid methyl ester (0.079 μmol/mg) detectable was lower than would be expected if the free 3-hydroxytetradecanoic acid had undergone β-elimination.

The (absolute) configuration of ester- and amide-bound 3-hydroxy fatty acids was determined by gas-liquid chromatography of diastereomeric derivatives (3-methoxy acid-L-phenylethylamides [30]). Gas-liquid chromatography of the derivatizes on OV-1 (205°C) gave two main peaks with tδ values of 1.24 (peak 1) and 2.41 (peak 2) relative to tetradecanoic acid-L-phenylethylamide, which had a tδ value of 1.00. Authentic Δ- and L-3-methoxytetradecanoic acid-L-phenylethylamides revealed tδ values of 1.23 and 1.32, respectively; authentic Δ- and L-3-methoxyhexadecanoic acid-L-phenylethylamides had tδ values of 2.41 and 2.61, respectively. Accordingly, both 3-hydroxy acids present in *F. nucleatum* Fev 1 lipopolysaccharides possess the Δ-configuration.

Comparative fatty acid analyses were performed on other *Fusobacterium* strains (Table 3). The three *F. nucleatum* strains investigated (Fev 1, F1, and ATCC 10953) exhibited a similar fatty acid pattern in that Δ-3-hydroxyhexadecanoic acid was amide bound and tetradecanoic, hexadecanoic, and 3-hydroxytetradecanoic acids were ester bound, and in that part of the ester-bound 3-hydroxytetradecanoic acid was 3-O-acylated by tetradecanoic acid.

In lipopolysaccharides of *F. varium* ATCC 8501 and *F. mortiferum* VIP 0473, tetradecanoic, hexadecanoic, and Δ-3-hydroxytetradecanoic acids were detected, but 3-hydroxyhexadecanoic acid was not. This latter fatty acid therefore appears to be a characteristic constituent of *F. nucleatum* strains. In *F. mortiferum* and *F. varium*, part of the 3-hydroxytetradecanoic acid was amide bound. The other part as well as tetradecanoic and hexadecanoic acids was ester bound. Again, the ester-bound 3-hydroxytetradecanoic acid was partially 3-O-substituted with tetradecanoic acid.

### DISCUSSION

According to the results of the present study, lipid A of *F. nucleatum* Fev 1 consists of β-1',6-linked β-glucosamine disaccharides, which carry two phosphate residues: one in glycosidic and one in ester linkage. This type of backbone structure has previously been identified in the majority of lipid A's from aerobic strains such as *Salmonella* (5, 6), *Escherichia coli* (6), *Shigella* (6, 17), and *Pseudomonas* (2). Lipid A of the anaerobic organism *Selenomonas ruminantium* also contains β-1',6-linked glucosamine disaccharides; phosphate, however, is absent (12).

The spectrum of fatty acids present in lipid A from *F. nucleatum* Fev 1 resembles that of enterobacterial and other lipid A's in that even-numbered, saturated, non-hydroxylated (C14,0 and C16,0) as well as 3-hydroxy acids (3-OH-C14,0 and 3-OH-C16,0) predominate. Of these, 3-hydroxyhexadecanoic acid is linked to the amino groups of the glucosamine residues. The other fatty acids are bound to the hydroxyl groups of the disaccharide backbone. Thus, like in most lipid A's studied, the 3-hydroxy acid with the longest chain is amide linked; the other hydroxy acids are present in ester bonds.

Part (approximately 40%) of the ester-bound 3-hydroxytetradecanoic acid was substituted by tetradecanoic acid. This structure, which is also present in enterobacterial lipid A's, was recognized by the fact that, with methylate, tetradecanoic acid is β-eliminated as the free acid and that concomitantly 3-methoxytetradecanoic

### TABLE 3. Nature and relative amounts of fatty acids present in lipopolysaccharides of various *Fusobacterium* strains

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Relative amt (mol%) of fatty acids in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>F. nucleatum</em> Fev 1</td>
</tr>
<tr>
<td>C14:0</td>
<td>39.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.2</td>
</tr>
<tr>
<td>Δ-3-OH-C14:0</td>
<td>34.6</td>
</tr>
<tr>
<td>Δ-3-OH-C16:0</td>
<td>20.1</td>
</tr>
</tbody>
</table>

* Values represent the sum of Δ-3-OH-C14:0 and Δ-3-OH-C16:0.

* Amide-bound fatty acids are underlined.
acid methyl ester is formed. In the methanolyt-
sate, small amounts of 3-hydroxytetradecanoic
acid were also found, the origin of which is not
well understood. That part of ester-bound 3-
hydroxytetradecanoic acid was 3-O-substituted
by 3-hydroxytetradecanoic acid appears to be
unlikely since the amount of 3-methoxytetra-
decanoic acid methyl ester formed (0.079 μmol/
mg) was too low to account for eliminated tetra-
decanoic acid (0.096 μmol/mg) plus 3-hydroxy-
tetradecanoic acid (0.054 μmol/mg).

Configuration analysis of the 3-hydroxy fatty
acids present in lipopolysaccharides of the vari-
ous *Fusobacterium* strains was investigated
revealed that all 3-hydroxy acids possess, as was
found in lipopolysaccharides of various other
bacterial groups (20, 21), the ω-configuration.
The fatty acid composition of other *F. nuclea-
tum* strains was similar to that of *F. nuclea-
tum* Fav 1 with regard to quantity, nature, and type
of linkage of fatty acids. In *F. varium* and *F.
mortiferum*, however, 3-hydroxyhexadecanoic
acid was absent. In these cases only 3-hydroxy-
tetradecanoic acid was present, which is, like in
*Enterobacteria*, partly ester and partly amide
bound. It should be mentioned that in lipopoly-
saccharides of two *F. necrophorum* strains, tetra-
and hexadecanoic acids were detected; 3-
hydroxy fatty acids, however, were absent (19).
The results of the present study indicate that
the chemical structure of lipid A of *F. nuclea-
tum* Fav 1 resembles that of lipid A of several
other, systematically quite remote bacteria (e.g., *Salmonella* [6], *Pseudomonas* [21], *S.
ruminantium* [12], and *Rhodopseudomonas ge-
latinoa* [6]) with regard to the glucomamine-
phosphate backbone and the nature and type of
linkage of the constituent fatty acids. It seems,
therefore, that lipid A’s among certain groups of
gram-negative bacteria exhibit close structural
relationships and that the lipid A component of
these lipopolysaccharides was not sub-
ject to major modifications during evolution.

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