Fatty Acid Composition of Lipopolysaccharides of Vibrio cholerae 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag)

KAZUHITO HISATSUNE,1* SEICHI KONDO,1 TOMIO KAWATA,2 AND YASUO KISHIMOTO2

Department of Microbiology, Josai University School of Pharmaceutical Sciences, Sakado, Saitama 350-02, Japan;1 Department of Food Microbiology, Tokushima University School of Medicine, Tokushima 770, Japan;2 and The John F. Kennedy Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received for publication 5 October 1978

Considerable amounts of odd-numbered fatty acids, such as non-hydroxy C15 and C17 and 3-hydroxy C11 and C13 acids, were found in lipopolysaccharides from Vibrio cholerae 35A3 (Inaba).

The fatty acid composition of lipopolysaccharides (LPS) of many gram-negative bacteria has been well investigated (11), but relatively little work has been done with Vibrio cholerae. Armstrong and Redmon (1) studied V. cholerae 569B (Inaba), and Raizuddin and Kawasaki (13) and Raizuddin (12) used V. cholerae El Tor (Inaba) as well as V. cholerae 569B (Inaba). Rietschel et al. (17) have studied the nature and linkages of the fatty acids present in LPS from Vibrio metchnikovii and Vibrio parahaemolyticus.

Using gas-liquid chromatography, we made a comparable study of the fatty acid composition of LPS isolated from the cell walls of selected strains of V. cholerae, strains 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag). V. cholerae 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag) (type 03 according to the classification of Sakazaki and Tamura [19]) were cultured in 1% glucose-peptone medium, pH 8.0, at 30°C for 16 h. The cells, except strain 4715 (Nag), were treated with 1% phenol for 12 h at room temperature and then washed with cold distilled water.

The cell walls were prepared, as described previously (6), by shaking the cells in a Bühler cell homogenizer (Edmund Bühler, Tübingen, West Germany) with glass beads (diameter, 0.11 to 0.12 mm).

LPS were isolated and purified from either acetone-dried crude cell walls or lyophylized crude cell walls, essentially by the phenol-water technique of Westphal et al. (20) and repeated ultracentrifugation (105,000 × g) and treatment with RNase (20 μg/ml) in 0.025 M Tris-hydrochloride buffer, pH 7.4.

About 3 mg of LPS was hydrolyzed with 0.5 ml of 4 N HCl for 4 h at 100°C and extracted with ether. The extract was evaporated to dryness under a flow of nitrogen, yielding residue 1 (total lipid fraction). Fatty acids present in residue 1 were esterified at room temperature for 1 h by the method of Hoshi et al. (7) in a solution containing 0.2 ml of 20 mM methanolic Cu(OAc)2, 0.2 ml of CHCl3, and 1 ml of 0.5 N methanolic HCl. Fatty acid methyl esters were extracted with hexane, and the extract was washed with water and then evaporated to dryness under nitrogen, yielding residue 2 (total fatty acid methyl ester fraction).

Examination by infrared spectrometry of residue 2 confirmed that the 3-hydroxy fatty acids present in residue 1 were completely esterified. Residue 2 was therefore subjected to preparative thin-layer chromatography to isolate normal fatty acid methyl esters and 3-hydroxy fatty acid methyl esters (Fig. 1). In addition to these two fractions, which were well separated from each other, some unknown materials (Fig. 1, bands A, B, A', and B') were detected in residue 2 from both strain 35A3 (Inaba) and strain 4715 (Nag). There was one additional unknown band (Fig. 1, band C) in residue 2 from strain NIH 90 (Ogawa). The ratio of the amount of nonhydroxy fatty acid esters to the amount of 3-hydroxy fatty acid esters was estimated to be approximately 1:1 in each strain. Each of the two fractions was extracted with hexane.

Total lipid and fatty acid content of LPS from the cell walls of the three strains are shown in Table 1. The total lipid content was between 25 and 34% of the LPS. The total fatty acid content recovered as methyl esters was unexpectedly low (7%) in NIH 90 (Ogawa) LPS, approximately ½ that of both strain 35A3 (Inaba) and strain 4715 (Nag) LPS.

The results of the analysis of the nonhydroxy and 3-hydroxy fatty acid fractions by gas-liquid chromatography are presented in Table 2. The major nonhydroxy fatty acid components of all three strains were found to be C14:0 and C16:0. In addition, small but significant amounts of C18:0,
C_{16:1}, and C_{18:1} were observed in the LPS of all three strains. It is of particular interest that considerable amounts of odd-numbered fatty acids were found in the LPS of only strain 35A3 (Inaba); these were C_{15:0} and C_{17:0}.

With regard to 3-hydroxy fatty acid composition, C_{12:0} and C_{14:0} were found to be present in all three strains. Furthermore, substantial amounts of odd-numbered fatty acids were also observed, particularly in the LPS of strain 35A3 (Inaba); they were C_{11:0} and C_{13:0}. Gas-liquid chromatography-mass spectrometry data, which were obtained with an LKB model 9000S instrument (LKB Instruments Inc., Uppsala, Sweden), confirmed the presence of the above-mentioned fatty acid methyl esters. Thus, fatty acid compositions of the LPS from the three selected strains were relatively similar, with the exceptions of (i) a minor deviation in the relative proportions of the component fatty acids, and (ii) the presence of odd-numbered normal and 3-hydroxy fatty acids, particularly in LPS from strain 35A3 (Inaba). Although C_{12:0} was previously reported to be the most abundant 3-hydroxy fatty acid in LPS from both 569B (Inaba) and E1 Tor (Inaba) (1, 13), the quantity of C_{14:0} was slightly greater than that of C_{12:0} in the three strains studied here.

Of the two O serotypes of cholera vibrios, Inaba and Ogawa, only the Inaba type has been investigated with regard to the fatty acid composition of endotoxic LPS; almost identical results have been obtained by Armstrong and Redmond (1) and Raziuddin and Kawasaki (13), with the exception of a minor quantitative deviation. They reported that in the LPS from both 569B (Inaba) and E1 Tor (Inaba), C_{14:0} and C_{16:0} are the main nonhydroxy fatty acid components and C_{12:0} and C_{14:0} are the main 3-hydroxy fatty acid components. Our results with

### Table 1. Lipid fractions of LPS of V. cholerae

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amt (mg) in the following strains:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35A3 (Inaba)</td>
<td>NIH 90 (Ogawa)</td>
<td>4715 (Nag)</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>3.47</td>
<td>3.67</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>Residue 1 (total lipid)</td>
<td>1.06 (31)*</td>
<td>0.93 (25)</td>
<td>1.15 (34)</td>
<td></td>
</tr>
<tr>
<td>Residue 2 (esterified fatty acid)</td>
<td>0.61 (18)</td>
<td>0.23 (7)</td>
<td>0.61 (18)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses are percentages of total LPS.

### Table 2. Fatty acid composition of lipid A of LPS of V. cholerae

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Normal acids</th>
<th>C_{12:0}</th>
<th>C_{14:0}</th>
<th>C_{16:0}</th>
<th>C_{18:0}</th>
<th>C_{20:0}</th>
<th>C_{22:0}</th>
<th>C_{24:0}</th>
</tr>
</thead>
<tbody>
<tr>
<td>35A3 (Inaba)</td>
<td>27.0</td>
<td>1.1</td>
<td>10.9</td>
<td>3.0</td>
<td>3.0</td>
<td>2.5</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>NIH 90 (Ogawa)</td>
<td>21.5</td>
<td>1.1</td>
<td>4.9</td>
<td>2.5</td>
<td>2.5</td>
<td>2.0</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>4715 (Nag)</td>
<td>36.9</td>
<td>1.3</td>
<td>2.5</td>
<td>2.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* An F and M model 7624 A gas-liquid chromatograph equipped with flame ionization detectors and a digital integrator (model 3370B, Hewlett-Packard) was used for the analytical gas-liquid chromatography of fatty acid methyl esters. A glass column (2 m by 4 mm), packed with 3% Silicone OV-1 coated on Chromosorb W with 25% diethylene glycol succinate on the same support, was used with temperature programming from 150°C at 1°C/min. The identities of the fatty acid methyl esters were based on retention times compared with those of authentic standards. The fatty acids were recovered as methyl esters. Values are the averages of duplicate determinations.

* Values for normal acids are percentages of the total normal fatty acid fraction.

* Values for 3-hydroxy acids are percentages of the total 3-hydroxy acid fraction.
Ogawa- and Nag-type strains, as well as with Inaba-type strain, coincided with their observation.

The amino groups of the glucosamine residues of lipid A of LPS are generally substituted by D-3-hydroxy fatty acids through amide linkages (4, 14, 16). This amide-linked 3-hydroxy acid is present in the LPS of all gram-negative bacteria so far investigated, with the exception of Brucella (2, 9–11, 16). If several 3-hydroxy fatty acids do occur, the one with the longest chain is amide linked (11, 16). It has been shown by Rietzschel (16) that in LPS from V. cholerae strain 569B (Inaba) C14h-0 is an amide-bound fatty acid. This has also been observed by Raziuddin with LPS from V. cholerae E1 Tor (Inaba) as well as 569B (Inaba) (12). In this study, we have shown that C14h-0, which is the most usual amide-bound fatty acid in many gram-negative bacteria (15, 16), is the longest-chain 3-hydroxy fatty acid in the LPS from all of the three selected strains of V. cholerae, 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag). Of particular interest is the finding that unexpectedly high amounts of both odd-numbered nonhydroxy and 3-hydroxy fatty acids, i.e., C15o, C17o, C11h0, and C13h0, are detected in the LPS of 35A3 (Inaba), whereas very little of these was found in the LPS of the other two strains. The occurrence of the odd-numbered fatty acids C13 and C15 in LPS has been reported in Veillonella (3, 5), C17 has been reported in Brucella (2, 9), C15 and C17 have been reported in Myxobacter (18), and C19 has been reported in Selenomonas ruminantium (8). It would be of interest to know whether the odd-numbered 3-hydroxy fatty acids C11h0 and C13h0 found in LPS from strain 35A3 (Inaba) are involved in amide linkage, as is LPS from Veillonella, where C13h0 and C14h0 are amide linked (3, 5). This question is at present under detailed investigation.

This investigation was supported in part by Public Health Service research grant NS-13559 from the National Institutes of Health.

Kenji Takeya, Department of Bacteriology, School of Medicine, Kyushu University, is gratefully acknowledged for his support and encouragement.

LITERATURE CITED