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# Lipoteichoic Acid from *Listeria monocytogenes*

NICHOLAS W. HETHER† AND LARRY L. JACKSON*

*Biochemistry, Johnson Hall, Montana State University, Bozeman, Montana 59717*

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A lipoteichoic acid (LTA) was extracted from *Listeria monocytogenes* (serotype 1) by phenol-water partition and isolated by gel-filtration chromatography. The LTA exhibited amphiphilic properties by changes in gel-filtration mobility in the presence of detergent buffers and after mild base hydrolysis. In a hemagglutination assay, *Listeria* LTA bound antibody prepared against a known LTA from *Streptococcus* spp. *Listeria* LTA inhibited the binding of anti-LTA antibody to a *Lactobacillus* LTA in a hemagglutination inhibition assay. The *Listeria* LTA contained glucose, galactose, fatty acids, glycerol, and phosphate with molar ratios of 0.05, 0.07, 0.21, 0.94, and 1.0 to phosphate, respectively. Adjacent glycerols were linked between the C-1 and C-3 positions by phosphodiesters (structural type 1). The average chain length was 19 ± 2 (standard deviation) glycerol-phosphate repeating units. Approximately one glycosyl side chain was present per LTA molecule. The side chain was a galactose-containing disaccharide. The lipid portion of the LTA was a galactose- and glucose-containing glycolipid which may have been a phosphoglycolipid, but the structure was not confirmed. Major fatty acids of LTA and the glycolipid were 17:anteiso, 15:anteiso, 16:iso, 16:n, and 18:n. *L. monocytogenes* contained cell wall products typical of gram-positive bacteria which is in contrast to the reports by others of the presence of lipopolysaccharides from *L. monocytogenes*.

Extracts obtained from *Listeria monocytogenes* contain apparent amphiphilic polysaccharides and possess biological activities which play a role in the pathogenicity of this increasingly important bacterium. The monocytosis-producing agent from serotype 1 cells is present in a high-molecular-weight fraction containing phosphate, lipid, and sugars (15). Polysaccharide and lipid material extracted from serotype 1 cells has the biological activity of lipopolysaccharide (LPS) endotoxin but lacks the biochemical markers (2-keto-3-deoxyoctonate, heptose, and hydroxy fatty acids) associated with endotoxins (26). An amphiphilic polysaccharide from serotype 4 cells has both biochemical markers and biological activities strikingly similar to those of endotoxins (10, 33, 37).

Teichoic acids are phosphate-containing polymers associated with the gram-positive bacteria. Included in this class of bacterial polymers are capsular, cell wall, and membrane polymers containing glycerol phosphate or ribitol phosphate (20, 23, 39). *Listeria* serotype 4b does not appear to have teichoic acids (34), whereas serotype 1 cells contain abundant quantities of ribitol (18). Ribitol in serotype 1 cells suggests the presence of teichoic acids. Glycerol teichoic acids act as bacterial heterophile antigens (20). *Listeria* cells cross-react with antisera prepared against glycerol teichoic acids (20, 27). Soluble material extracted from *Listeria* serotype 4b adsorbs antibody directed against lipoteichoic acids (LTAs) of *Lactobacillus* (1). This study was undertaken to isolate and examine LTAs from *L. monocytogenes*.

## MATERIALS AND METHODS

**Bacteria.** The *L. monocytogenes* (serotype 1) cell preparation was a gift from P. A. Campbell, National Jewish Hospital, Denver, Colo. and was prepared in her laboratory. It was composed of sonically disrupted, lyophilized cells and retained much membrane material as described previously (18, 29).

**LTA extraction and isolation.** Lyophilized cell preparations were extracted with organic solvents to remove lipids (14, 38). Dried residual cell material was then extracted with hot (65°C) 45% aqueous phenol (37). Aqueous phases of the phenol extracts were dialyzed against 3 mM $NaN_3$ in distilled water. Dialysates were concentrated, partitioned with a Folch wash (14), applied in 0.5-ml volumes to a column (0.7 by 115 cm) of Sepharose 6B (Pharmacia, Inc., Piscataway, N.J.), and eluted with 0.1 N ammonium acetate (pH 6.8) which was 3 mM in $NaN_3$ (buffer A). Column fractions (1.0 ml) were analyzed for phosphate, total sugars, and absorbance at 260 nm. Tubes under the peaks were pooled, concentrated, and analyzed again on the same column. Fractions isolated by gel filtration were dialyzed, lyophilized, weighed, dis-

† Present address: Department of Medicine, National Jewish Hospital and Research Center, Denver, CO 80206.

solved in distilled water (10 mg/ml), and analyzed for chemical constituents. Partial deacylation of LTA was done in methanolic KOH (38). Deacylated LTA (dLTA) was analyzed by gel-filtration chromatography as described above. A sample of LTA was also purified by ion-exchange chromatography in detergent buffers with DEAE-Sephadex A-25 (2).

**Gel filtration in detergent.** LTA in 0.5 ml of buffer A was applied to a column (0.7 by 75 cm) of Sepharose 6B previously equilibrated with buffer A. LTA was eluted with buffer A, and the fractions (1.0 ml) were analyzed for phosphate to determine the elution volume of the LTA. Tubes under the phosphate peak were pooled, reduced in volume, and dialyzed. The column was then equilibrated with five column volumes of 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in buffer A. The LTA was dissolved in 0.5 ml of the same detergent buffer, applied to the column, and eluted with the detergent buffer. Fractions were analyzed for phosphate. The experiment was then repeated with 0.5% Triton X-100 in buffer A as the eluent.

**Immunological methods.** Passive hemagglutination and hemagglutination inhibition were done with sheep erythrocytes (1). The rabbit anti-LTA serum used was a gift from Robert W. Jackson, Southern Illinois University, Carbondale, Ill., and was prepared against the polyglycerol phosphate backbone of an LTA from *Streptococcus pyogenes* (13, 30). *Lactobacillus fermenti* LTA was a gift from Anthony J. Wicken, University of New South Wales, Kensington, Australia (38).

**TLC.** Lipids were analyzed by thin-layer chromatography (TLC) in (A) hexane-diethylether-acetic acid (85:15:1 [vol/vol/vol]) (18), hydroxy fatty acids in (B) hexane-diethylether (1:1 [vol/vol]) (18), mono- and diacylglycerols in (C) hexane-diethylether-acetic acid (60:40:4 [vol/vol/vol]) (21), and glycolipids in (D) chloroform-acetone-methanol-acetic acid-water (80:20:10:10:4 [vol/vol/vol/vol/vol]) (21) on silica plates of Absorbosil 2 (Applied Science, State College, Pa.). Partial hydrolysates of LTA were analyzed by double development in (E) *n*-propanol-ammonium hydroxide-water (6:3:1 [vol/vol/vol]) (3) and in (F) 95% ethanol-ammonium hydroxide-water (6:3:1 [vol/vol/vol]) (N. W. Hether and L. L. Jackson, submitted for publication) on sheets of Baker Flex silica gel 1B (J. T. Baker Chemical Co., Phillipsburg, N.J.). Sulfuric acid-potassium dichromate and alkaline permanganate (35) were the general detection sprays. Reducing substances were visualized with alkaline silver nitrate (17), phosphate esters with ammonium molybdate (25), and 1,2 diols with either periodate-benzidine (8) or periodate-Schiff (32) sprays. Standards, obtained from either Applied Science or Sigma were run concurrently on all plates. An authentic glycolipid standard was not available for comparison to the glycolipid obtained from *Listeria* LTA.

**Partial hydrolysis of LTA.** Samples of LTA (100 to 1,000 μg) were hydrolyzed in 1.0 N HCl or 1.0 N NaOH (3). Hydrolysates were analyzed by TLC in solvents E and F. TLC sheets were sprayed for reducing substances, 1,2 diols, and phosphate. Standards of glycerol-1,2-diphosphate, 1,4-anhydroribitol, and ribitol-1-phosphate were prepared, respectively, from cardiolipin (24), ribitol (6), and ribose-5-phosphate (7) which were obtained from Sigma.

**NMR spectroscopy.** Natural abundance broad band proton-decoupled carbon-13 nuclear magnetic resonance (NMR) spectra were obtained on a Bruker WM-250 MHz spectrometer at 62.83 MHz in a Fourier transform mode. Chemical shifts were determined relative to a tetramethylsilane external standard in a coaxial system. Samples were dissolved in deuterated water (25 to 100 mg/ml), and 2,000 to 15,000 scans were accumulated for each spectrum. Carbon assignments were verified with gated decoupled experiments.

**Chain length estimation.** LTA chain length was estimated by a periodate consumption method (1). Periodate consumption was measured at intervals by an iron-oxidation method (5). After 24 h, formaldehyde was also measured at intervals (11). After removal of terminal glycoaldehyde groups, terminal phosphomonoesters were hydrolyzed with a phosphomonoesterase (bovine intestine alkaline phosphatase, type VII-s; Sigma), and $P_i$ release was measured to provide an additional estimation of chain length.

**HF hydrolysis.** Hydrofluoric acid (HF) hydrolysis of LTA was done by the method of Koch and Fischer (21). Lipids were extracted from hydrolysates with a Folch wash (14, 21). Water-soluble material was concentrated and applied to a column (0.7 by 120 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Inc., Richmond, Calif.). The column was eluted with 1.0 mM $NaN_3$, and the fractions (1.0 ml) were assayed for sugars. Material eluted from the column was desalted on a 1.0-ml column of Amberlite MB-1 (Sigma) and analyzed for chemical constituents. Glycoside 1 thus isolated was oxidized with periodate (5), and formaldehyde release was determined (11). Lipids extracted from HF hydrolysates were analyzed by TLC in solvents A, B, C, and D. Lipid fractions were separated by preparative TLC in solvent D and were analyzed for chemical constituents. Fatty acids were released from the glycolipid by hydrolysis in 0.2 N KOH at 45°C for 4 h, extracted into hexane-diethylether (1:1 [vol/vol]), and analyzed separately. Water-soluble material (glycoside 2) was neutralized and treated as described above. The Bio-Gel P-2 column was precalibrated with mono-, di-, tri-, and tetrasaccharides (Sigma).

**Sugar analysis.** Sugars were analyzed by gas-liquid chromatography (GLC) as the alditol acetates (18). Inositol (0.5 μmol/μmol of sugar) was the internal standard. A fatty acid internal standard was also included. After hydrolysis, free fatty acids were extracted into hexane-diethylether (1:1 [vol/vol]) and analyzed separately. In the preparation of alditol acetates, the sequence of reactions is hydrolysis, reduction, and acetylation. Reversal of the first two steps or omission of the reduction step gave structural information. GLC conditions were as described previously (18), and all GLC work was done on a Varian model 3700 gas-liquid chromatograph equipped with a Hewlett Packard 3380 A electronic integrator. Sugar standards, including heptose and 2-keto-3-deoxyoctonate, were obtained from Sigma.

**Fatty acid analysis.** An internal standard of methyl dodecanoate was added to all samples analyzed for fatty acids. LTA samples had 0.1 μmol internal standard per μmol of phosphate, and the glycolipid had 0.5 μmol internal standard per μmol of sugar. Fatty acids obtained from the various hydrolyses were examined by TLC in solvents A, B, and C and by argentation

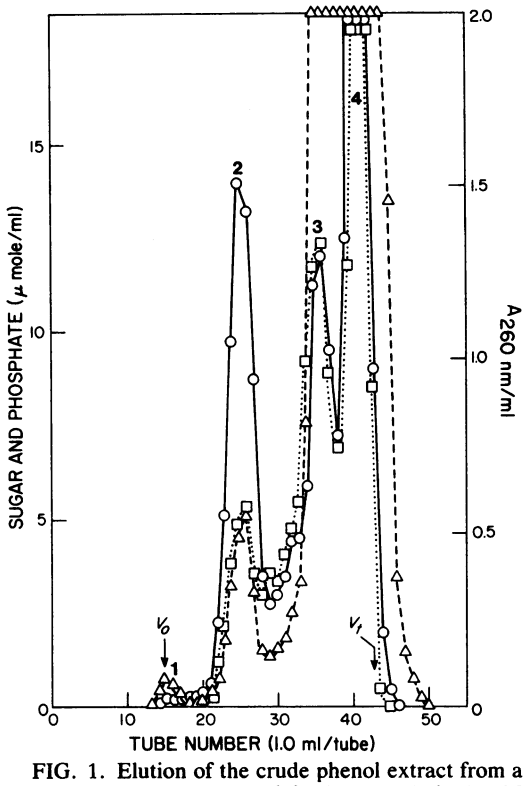


FIG. 1. Elution of the crude phenol extract from a column (0.7 by 115 cm) of Sepharose 6B in 0.1 M ammonium acetate–3 mM $NaN_3$ (pH 6.8). Fraction 2 had a $V_e/V_o$ of 1.6, and fraction 3 had a $V_e/V_o$ of 2.3. Column fractions (1.0 ml) were analyzed for phosphate (○), sugars (□), and absorbance at 260 nm (△).

TLC in solvent A. LTA and other fractions were also hydrolyzed, as described previously (18), to release amide-linked fatty acids. Fatty acids were analyzed by GLC as the methyl esters (18), and hydroxy fatty acids were analyzed as the methyl ester trimethylsilyl ether derivatives (18). GLC was done on a 10-m SE-30 fused silica capillary column described previously (18). Iso and anteiso fatty acid isomers were resolved on a 15-m bonded phase methyl phenyl silicone-fused silica capillary column employing the conditions described for the SE-30 column (18). Fatty acid standards were obtained from Applied Science.

**Other analytical methods.** Amino acids, amino sugars, organic phosphate, $P_i$, and neutral sugars were analyzed as described previously (18). Rhamnose was estimated by the cysteine-sulfuric acid method (12). The method of Wells and Dittmer (36) was used for glycerol. Nucleic acid content was estimated by absorbance at 260 nm (38).

## RESULTS

**LTA isolation.** A typical gel filtration profile of the crude phenol extract is given in Fig. 1. A small peak (peak 1, Fig. 1) of 260 nm of absorbing material emerged from the column in the void volume. Similar peaks have been observed by others (9) and found to be artifacts which could be precipitated by low-speed centrifugation. Peak 1 behaved similarly and was not further examined. Material with high absorbance at 260 nm and rich in phosphate and sugar (peak 4, Fig. 1) eluted near the bed volume. Peak 4 had components typical of nucleic acids and was not further characterized. Peak 3 of Fig. 1 emerged from the column with an elution volume to void volume ratio ($V_e/V_o$) of 2.3, and had a high sugar to phosphate ratio. It contained high amounts of phosphate, glucosamine, rhamnose, and ribitol with smaller quantities of glycerol, alanine, glutamic acid, and diaminopimelic acid.

The material of interest (LTA), eluted from the column under peak 2 in Fig. 1 with a $V_e/V_o$ of 1.6. LTA, had a low sugar to phosphate ratio and low absorbance at 260 nm. LTA was isolated as a single peak with no change in gel filtration characteristics (Fig. 2A). A total of 101 mg of LTA was obtained from 4.9 g of cells, which was a yield of 2.1%. Mild base hydrolysis of LTA released 0.08 μmol of fatty acid per μmol of phosphate, and dLTA shifted to lower apparent molecular weight by gel filtration with a $V_e/V_o$ of 2.3 (Fig. 2B). A sample of LTA was further purified by ion-exchange chromatography (Fig. 3). LTA eluted from the ion-exchange column between 0.3 and 0.5 M salt. Some material did not elute from the column, and the yield of phosphate was ca. 70% of the original sample. No additional phosphate-containing material eluted from the column at higher salt or detergent concentrations.

**Gel-filtration chromatography in detergent.** Three experiments were conducted to examine the gel-filtration properties of *Listeria* LTA in detergent buffers. A sample of LTA was applied to a column of Sepharose 6B and eluted from the column with an ammonium acetate buffer containing no detergent. The experiment was then repeated twice with the same buffer made 0.2 and 0.5%, respectively, in detergent as the eluent. The elution profiles of the three experiments are given in Fig. 4. With no detergent in the eluent buffer, LTA eluted as a broad peak with a $V_e/V_o$ ratio of 1.5 (peak A, Fig. 4). With an eluent buffer made 0.2% in detergent, a $V_e/V_o$ ratio of 1.8 was obtained (peak B, Fig. 4), whereas the 0.5% detergent buffer produced a sharp peak with a $V_e/V_o$ ratio of 2.0 (peak C, Fig. 4).

**Immunological assays.** *Lactobacillus* and *Listeria* LTAs were adsorbed to sheep erythrocytes at concentrations ranging from 10 to 1,000 μg/ml of 10% sheep erythrocytes. Anti-LTA serum prepared against *Streptococcus* LTA agglutinated cells with either *Lactobacillus* LTA or *Listeria* LTA adsorbed on the cells. Titers of 256 were obtained for both *Lactobacillus* and *Lis-*

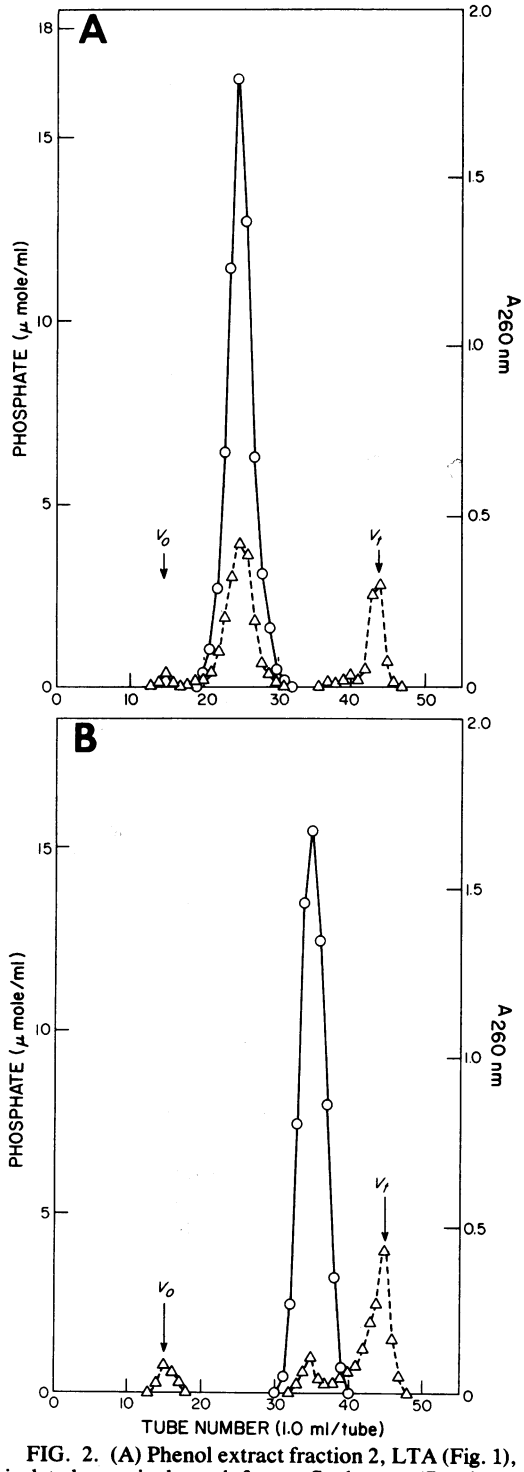


FIG. 2. (A) Phenol extract fraction 2, LTA (Fig. 1), isolated as a single peak from a Sepharose 6B column (0.7 by 115 cm) with a $V_e/V_o$ of 1.6. (B) dLTA (0.1 N methanolic KOH, 15 min, 37°C) eluted from the same column with a $V_e/V_o$ of 2.3. Free fatty acids (0.08 μmol/μmol of phosphate) were released during hydrolysis. Column fractions were assayed for phosphate (○) and absorbance at 260 nm (△).

*teria* LTAs, and concentrations of 100 and 1,000 μg/ml of cells gave the clearest patterns for both LTAs in this assay system. No agglutination was observed for cells treated with dLTA, phenol extract fractions 3 or 4 (Fig. 1), or buffers only, and normal rabbit serum did not agglutinate cells sensitized with either *Lactobacillus* or *Listeria* LTA. In hemagglutination inhibition assays, *Listeria* LTA or dLTA (200 ng per well) inhibited the agglutination of cells sensitized with *Lactobacillus* LTA (1,000 μg/ml of 10% cells). No inhibition was produced by phenol extract fraction 4 (Fig. 1). Phenol extract fraction 3 (Fig. 1) gave inhibition of high concentrations which indicated the presence of dLTA.

**LTA composition.** The chemical compositions of three LTA preparations isolated as single peaks by gel filtration (Fig. 2A) are given in Table 1. The major components were always phosphate, glycerol, fatty acids, galactose, and glucose. Galactose was always present in excess of glucose. Minor components ranged from 4 to 10% by mass. No heptose or 2-keto-3-deoxyoctonate was detected in any of the LTA preparations. Residual nucleic acid contaminants were not removed by treatment with nucleases, although control samples of nucleic acids were readily hydrolyzed. Greater than 90% of the amino acids in all cases were present as alanine, glutamic acid, and diaminopimelic acid. Deacylation of LTA and ion-exchange chromatography reduced contaminants to 3% or less by mass. Table 2 gives the molar ratios of the major components to phosphate for the LTA preparations given in Table 1 as well as for dLTA and LTA purified by ion-exchange chromatography. Fatty acid compositions of the LTA prepara-

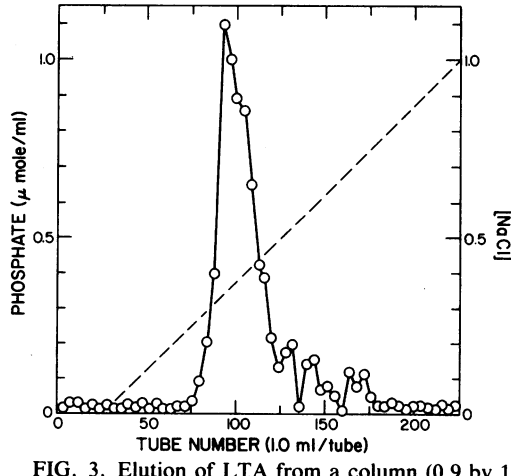


FIG. 3. Elution of LTA from a column (0.9 by 10 cm) of DEAE-Sephadex A-25 with a linear gradient from 0 to 1.0 M NaCl in 200 ml of distilled water which was 0.5% in Triton X-100. LTA eluted between 0.3 and 0.5 M salt. Every third tube was assayed for phosphate (○).

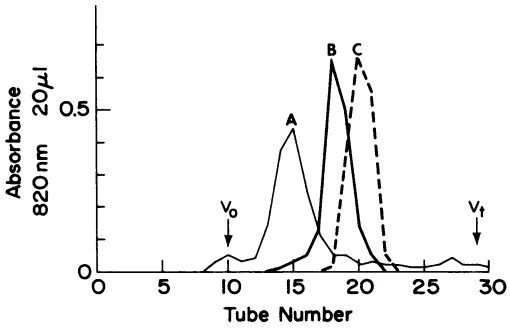


FIG. 4. Elution of LTA from a column (0.7 by 75 cm) of Sepharose 6B in buffer A containing various concentrations of Triton X-100. Peak A, buffer A with no detergent; $V_e/V_o$ = 1.5. Peak B, buffer A with 0.2% Triton X-100; $V_e/V_o$ = 1.8. Peak C, buffer A with 0.5% Triton X-100; $V_e/V_o$ = 2.0. Samples (20 μl) of each column fraction (1.0 ml) were analyzed for phosphate, and absorbance was determined at 820 nm.

tions were essentially the same, and Table 3 gives a typical fatty acid distribution. Anteiso-17 represented approximately half the fatty acid composition. A small quantity of 18:1 was detected by argentation TLC but insufficient amounts were present to locate the position of the double bond. No hydroxy fatty acids were detected.

**LTA partial hydrolysis.** Results of the TLC analysis of partial hydrolysis products of *Listeria* LTA obtained under acidic and basic conditions are summarized in Table 4. A nonreducing glycoside with TLC mobility similar to lactose was found in base hydrolysates but not in acid hydrolysates. Only trace quantities of glycerol-2-phosphate were present in acid hydrolysates, whereas base hydrolysates contained large quantities of glycerol-2-phosphate as judged by spot intensities. Glycerol-2-phosphate in the acid hydrolysates was detected only after heavy initial sample spotting.

TABLE 1. Composition of the LTA from *Listeria* spp.[a]

| Component | Amt in prepn no.: 1 μg/mg | 1 μmol/mg | 2 μg/mg | 2 μmol/mg | 3 μg/mg | 3 μmol/mg |
|---|---|---|---|---|---|---|
| Phosphate | 343 | 3.5 | 323 | 3.3 | 285 | 2.9 |
| Glycerol | 294 | 3.2 | 283 | 3.1 | 227 | 2.5 |
| Fatty acids | 207[b] | 0.81 | 179[b] | 0.70 | 164[b] | 0.64 |
| Galactose | 45 | 0.25 | 52 | 0.29 | 40 | 0.22 |
| Glucose | 26 | 0.14 | 33 | 0.18 | 28 | 0.15 |
| Rhamnose | 14 | 0.09 | 22 | 0.13 | 25 | 0.15 |
| Glucosamine | 18 | 0.10 | 30 | 0.17 | 35 | 0.20 |
| Ribitol | 6 | 0.04 | 13 | 0.08 | 15 | 0.10 |
| Amino acids | 25 | | 39 | | 31 | |
| Nucleic acids | 4 | | 4 | | 4 | |

[a] Averages of triplicate determinations.
[b] Calculated from the micromole yield as palmitic acid.

TABLE 2. Molar ratios of LTA components to phosphate

| Component | LTA prepn from Table 1: 1 | 2 | 3 | dLTA[a] | LTA[b] |
|---|---|---|---|---|---|
| Phosphate | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Glycerol | 0.91 | 0.94 | 0.86 | 0.98 | 0.96 |
| Fatty acids | 0.23 | 0.21 | 0.22 | 0.10[c] | 0.17 |
| Galactose | 0.07 | 0.09 | 0.08 | 0.07 | 0.08 |
| Glucose | 0.04 | 0.05 | 0.05 | 0.05 | 0.06 |

[a] See legend to Fig. 2B.
[b] LTA purified by ion-exchange chromatography (Fig. 3).
[c] Fatty acids extracted from acid hydrolysates done for GLC sugar analysis and remaining after partial deacylation.

**NMR spectroscopy.** Natural abundance carbon-13 NMR spectroscopy of *Listeria* LTA revealed two major broad signals at 66.6 and 69.9 ppm, respectively. The signal at 66.6 ppm was coupled to phosphate ($^2J_{^{31}P-^{13}C}$ = 5.5 Hz). The resonance at 69.9 ppm was also coupled to phosphate, but the signals were not well enough resolved to determine the coupling constant. The LTA carbon resonances are compared with those of glycerol monomers in Table 5. In a gated decoupled spectrum, the signal at 69.9 ppm split into two signals ($J_{^{13}C-H}$ = 144 Hz), and the signal at 66.6 ppm split into three signals ($J_{^{13}C-H}$ = 145 ± Hz).

**LTA chain length.** The intact LTA polymer had few, if any, terminal phosphomonoesters because less than 0.5% of the phosphate was released as $P_i$ by a phosphomonoesterase. In two periodate consumption experiments, the ratios of glycerol to periodate consumed were 15 and 21. The ratios of glycerol to formaldehyde produced in the same periodate consumption experiments were 17 and 18. After removal of terminal glycoaldehyde groups produced by periodate oxidation of the terminal glycerols, the ratios of total phosphate to $P_i$ released by a phosphomonoesterase were 19 and 21 in two experiments. The average chain length of the LTA from *Listeria* spp. was 19 ± 2 (standard deviation) glycerol phosphate units.

**HF hydrolysis.** *Listeria* LTA was hydrolyzed in HF for various time periods. By 48 h, ca. 90% of the phosphate was released as $P_i$ and 48 h was used as the standard hydrolysis time in subsequent experiments. The major products of HF hydrolysis were always $P_i$ and glycerol.

After extraction of lipids from HF hydrolysates, a water-soluble glycoside (glycoside 1)

TABLE 3. Fatty acid composition of the *Listeria* LTA[a]

| Fatty acid | % of total fatty acids |
|---|---|
| 14:iso[b] | 0.7 |
| 14:0 | 0.5 |
| 15:anteiso[c] | 13 |
| 15:0 | 0.9 |
| 16:iso[b] | 9.8 |
| 16:0 | 22 |
| 17:anteiso[c] | 49 |
| 18:x[d] | 0.6 |
| 18:0 | 2.1 |
| 20:iso[b] | 1.2 |

[a] Averages of triplicate determinations. Data presented from preparation no. 1 (Table 1). Trace was considered to be >0.1% but <0.5%.
[b] Trace quantities of anteiso isomers were also present.
[c] Trace quantities of iso isomers were also present.
[d] 18:x, Sum of the 18 carbon-branched chain and unsaturated fatty acids.

remained in the aqueous phase. Glycoside 1 eluted from a calibrated Bio-Gel P-2 column with a molecular weight of 440. Glycoside 1 contained galactose and glycerol in 2.1 to 1 respective molar ratios. No reducing end was present in the induct glycoside, and glycerol was the only alditol present. No formaldehyde was produced upon oxidation of glycoside 1 with periodate. LTA containing 60 μmol of phosphate gave a total yield of 2.7 μmol of glycoside 1.

The major components in the lipid extracts of HF hydrolysates of LTA as determined by TLC in solvents A, C, and D were a glycolipid and a diacylglycerol. Minor components included free fatty acids, a monoacylglycerol, and a second glycolipid. The presence of free fatty acids indicated that some acyl ester hydrolysis had occurred. The minor glycolipid had a lower $R_f$ than the major glycolipid and may have been the deacylated major glycolipid. Insufficient quantities of the minor glycolipid were present for further characterization.

The major glycolipid contained glucose, galactose, fatty acids, and glycerol with respective molar ratios to glycerol of 1.1:1.0:2.2:1.0. The fatty acid distribution found in the glycolipid was similar to that of the LTA (Table 6). Glycoside 2, isolated after deacylation of the glycolipid, eluted from a calibrated Bio-Gel P-2 column with an apparent molecular weight of 440. Glycoside 2 contained equal molar quantities of glucose, galactose, and glycerol. Glycerol was the only alditol found, and the intact glycoside had no reducing end. Formaldehyde (0.9 μmol/μmol of glycerol) was produced upon periodate oxidation of glycoside 2. A total of 2.9 μmol of glycoside 2 were isolated from *Listeria* LTA containing 60 μmol of phosphate.

## DISCUSSION

A variety of amphiphilic molecules from bacteria are known, and included in this class of bacterial products are LTAs and lipopolysaccharides (LPS) (39). LTAs are generally associated with the gram-positive bacteria, whereas LPS are associated with the gram-negative bacteria (39). Amphiphiles such as LTA and LPS appear to have high molecular weights by gel-filtration chromatography because they form micelles to exclude water from the hydrophobic regions of the polymers (39). Deacylation or gel filtration in the presence of detergents disrupts the micellar structure and results in a shift to lower apparent molecular weight (39). The material isolated from *Listeria* spp. exhibited such amphiphilic properties (Fig. 2 and 3).

The glycerol phosphate backbone of glycerol teichoic acids acts as a gram-positive bacterial heterophile antigen (20). The material isolated in this study bound anti-LTA antibody prepared against a known polyglycerol phosphate polymer, and it inhibited the binding of the antibody to a known LTA. The material isolated in this study had the antigenic characteristics of LTA which was in agreement with serological work done by others (1, 27).

TABLE 4. TLC analysis of LTA hydrolysates

| Compound | Standard $^{R}$GLC[a] | Acid-hydrolyzed LTA | | Base-hydrolyzed LTA | |
|---|---|---|---|---|---|
| | | Compound detected | $^{R}$GLC[a] | Compound detected | $^{R}$GLC[a] |
| Glycerol | 1.2 | + | 1.2 | + | 1.2 |
| Glucose | 1.0 | + | 1.0 | − | |
| Galactose | 0.84 | + | 0.84 | − | |
| Glycerol-2-phosphate | 0.64 | ± | 0.63 | + | 0.64 |
| Glycerol-1-phosphate | 0.55 | +[b] | 0.53[b] | +[b] | 0.56[b] |
| Lactose | 0.45 | − | | +[c] | 0.48[c] |
| Glycerol-1,3-diphosphate | 0.12 | + | 0.13 | + | 0.12 |

[a] Average values calculated from five thin-layer sheets. $^{R}$GLC, Relative GLC.
[b] Mixtures of *sn*-glycerol-1-phosphate and *sn*-glycerol-3-phosphate were indistinguishable by TLC.
[c] A nonreducing glycoside with TLC mobility similar to lactose.

TABLE 5. Carbon-13 chemical shifts of *Listeria* LTA and glycerol monomers

| Compound | Chemical shift of glycerol carbons | | |
|---|---|---|---|
| | C-1 | C-2 | C-3 |
| Glycerol | 63.8 | 73.3 | 63.8 |
| Glycerol-2-phosphate | 62.5 (3.8)[a] | 75.1 (5.0)[b] | 62.5 (3.8)[b] |
| Glycerol-3-phosphate | 62.8 | 71.6 (6.4)[a] | 65.2 (4.8)[b] |
| *Listeria* LTA | | 69.9[c] | 66.6[d] (5.5)[b] |

[a] $^3J$ for $^{31}P$-$^{13}C$.
[b] $^2J$ for $^{31}P$-$^{13}C$.
[c] Coupled signal, no coupling constant determined.
[d] Signal for both C-1 and C-3 of glycerol.

To be classified as a teichoic acid, a bacterial polysaccharide must contain an alditol in the linear sequence of repeating units, and repeating units must be linked by phosphodiesters (4, 23). The presence or absence, as well as linkage details, of short glycosyl moieties interposed between alditols in the polymer sequence are the major differences among the three known teichoic acid structural types (3). The low sugar to phosphate ratios of *Listeria* LTA (Tables 1 and 2) suggested that adjacent glycerols were linked directly by phosphodiesters. Phosphodiester hydrolysis in base requires an unsubstituted adjacent hydroxyl group, because the hydrolysis proceeds by a migration of the phosphate to the free hydroxyl (4). In acid, a free adjacent hydroxyl is not required for phosphodiester hydrolysis, although phosphate migration can occur (4). The presence of much larger quantities of glycerol-2-phosphate in base hydrolysates of *Listeria* LTA than occurred in acid hydrolysates indicated that the glycerols were 1,3 linked by phosphodiesters, because the phosphate migrated from the primary glycerol hydroxyl to the secondary hydroxyl during the base hydrolysis.

In carbon-13 NMR spectroscopy of sugars, α-phosphorylation results in a 2 to 5 ppm downfield shift of a given carbon resonance, whereas neighboring carbon resonances (β to the phosphate) are shifted 1.5 to 2 ppm upfield (31). In low-molecular-weight compounds, signals of α and β carbons are split owing to respective two-bond and three-bond spin-spin coupling to phosphorus, and in polymers additional signal broadening occurs (31). In *Listeria* LTA, the signal at 66.6 ppm resembled the signal for the α carbon of a glycerol phosphate monomer (Table 5), and the carbon-phosphorus coupling constant ($^2J_{^{31}P-^{13}C}$ = 5.5 Hz) was within the range of such two-bond couplings (31). The signal at 69.9 ppm was 3.4 ppm upfield from the C-2 of unsubstituted glycerol, and the magnitude of the shift suggested a glycerol C-2 β to two phosphates. The results of the gated decoupled carbon-13 NMR experiments confirmed the carbon assignments. Because only two carbon resonances were observed with *Listeria* LTA, two of the glycerol carbons were in similar, if not identical, chemical environments as would be expected in a 1,3-linked polymer. We concluded that *Listeria* LTA was a structural type 1 polymer in which adjacent glycerols were linked between the C-1 and C-3 positions by phosphodiesters.

Cold concentrated hydrofluoric acid hydrolyzes primarily, if not exclusively, phosphate esters and leaves glycosidic linkages and acyl esters largely intact (21). Glycerol teichoic acids may contain glycosyl side chains (4, 20, 39). Because no formaldehyde was produced by periodate oxidation of glycoside 1, the galactose dissaccharide was linked to the C-2 of glycerol which is the expected linkage position for substituents of a 1,3-linked glycerol phosphate polymer. Such glycosyl side chains frequently are the major antigenic determinant sugars for glycerol teichoic acids (20). A chemically uncharacterized phenol extract from *Listeria* serotype 4b had galactose as the major immunodeterminant sugar (1). The quantity of glycoside 1 isolated from *Listeria* LTA indicated that on the average one or slightly less than one glycosyl side chain occurred per LTA molecule.

The lipid moiety of LTA is usually a glycolipid or a phosphoglycolipid of the type normally present in the membrane (39). The glycolipids of *Listeria* spp. have not been extensively characterized, but an *O*-α-D-galactopyronosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→1)-diglyceride has been described (22). The glycolipid isolated in this study from *Listeria* LTA is consistent with such a structure, although structural detail remains to be determined. The absence of diacylglycerols in HF hydrolysates of LTAs is sufficient to preclude the presence of a phosphogly-

TABLE 6. Fatty acid composition of the glycolipid[a]

| Fatty acid | % of total fatty acids |
|---|---|
| 14:iso[b] | 0.7 |
| 15:anteiso[c] | 16 |
| 15:0 | 0.8 |
| 16:iso[b] | 6.0 |
| 16:0 | 18 |
| 17:anteiso[c] | 52 |
| 17:0 | 0.5 |
| 18:x[d] | 1.0 |
| 18:0 | 4.0 |
| 20:iso[d] | 0.8 |

[a] Averages of triplicate determinations. Trace was considered to be >0.1% but <0.5%.
[b] Trace quantities of anteiso isomers were also present.
[c] Trace quantities of iso isomers were also present.
[d] 18:x, Sum of the 18 carbon-branched chain and unsaturated fatty acids.

colipid as the lipid moiety of an LTA (21). A diacylglycerol was present in HF hydrolysates which suggested that the lipid group of *Listeria* LTA was a phosphoglycolipid. However, the diacylglycerol could have arisen from contaminating phospholipids. Whether *Listeria* spp. has phosphoglycolipids in the plasma membrane is unknown. The fatty acid composition of the LTA and the glycolipid was typical of *Listeria* spp. and of gram-positive bacteria in general (18, 28). The material isolated from *L. monocytogenes* serotype 1 by phenol extraction fit the definition of a teichoic acid and was clearly an LTA.

LPS are known which contain single glycerolphosphate or ribitol-phosphate units as side chains to the O-antigen polysaccharide (16, 19). Glycolipids are frequent contaminants of LTA extracts, and some gram-positive bacteria have glycolipids which contain biochemical sugar markers commonly associated with LPS (40). Although such occurrences are rare, they do point to the need for exercising caution when inferring the presence of particular structures based on composition data alone. In this study, *Listeria* spp. was found to be rich in LTA, which is the characteristic gram-positive amphiphile. None of the biochemical markers associated with LPS was detected. Others (10, 33, 37) have found products from *Listeria* spp. to be strikingly similar to LPS. The apparent presence of two such disparate amphiphiles in the same bacteria warrants continued careful evaluation of the cell wall polysaccharides of *L. monocytogenes*.

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