Natural Phosphoryl and Acyl Variants of Lipid A from Neisseria meningitidis Strain 89I Differentially Induce Tumor Necrosis Factor-α in Human Monocytes

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The native lipooligosaccharide (LOS) from Neisseria meningitidis strain 89I was analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry and the spectrum compared with that of the LOS after O-deacylation and hydrogen fluoride treatment. The data are consistent with the presence of natural variations in the LOS, which include a triphosphorylated lipid A (LA) with and without a phosphoethanolamine group, and both hexa- and pentaacylated LA molecules. Thin-layer chromatography was performed on 89I LA produced by hydrolysis of the LOS, and the purified LA molecules were analyzed by MALDI-TOF and tested for their relative ability to induce the secretion of tumor necrosis factor-α by human mononuclear THP-1 cells and primary human monocytes. The potency of tumor necrosis factor-α induction varied by 2–10-fold, depending on the state of acylation and phosphorylation. The results highlight the significance of phosphorylation along with acylation of the LA component of LOS in stimulation of inflammatory signaling, and suggest that natural strain variation in these moieties may be a feature of meningococcal bacteria, which is of critical importance to the progression of the infection.

The lipid A (LA) portion of the lipopolysaccharide (LPS) or lipooligosaccharide (LOS) of Gram-negative bacteria is an inflammatory, pathogenic component of the bacterial outer membrane (1–3). Our interests lie in the Neisserial LA as it is implicated as a significant contributor to the pathogenesis of infections due to Neisseria meningitidis and Neisseria gonorrhoeae, which are of major public health concern around the world. In particular, N. meningitidis is the leading cause of epidemic meningitis and fatal sepsis in otherwise healthy individuals (4). On average more than 500,000 cases of meningococcal infection occur annually leading to ~50,000 deaths, and large epidemic outbreaks can cause periodic spikes in occurrence. N. gonorrhoeae is a major cause of sexually transmitted infections, which can lead to pelvic inflammatory disease in 10–20% of infected women who can suffer from chronic pain, infertility, and ectopic pregnancy as a result (5). In addition, a growing number of studies have shown that gonococcal infection can facilitate the transmission of HIV (6).

Numerous studies of LA signaling through the toll-like receptor 4 (TLR4) have increased our knowledge of the relationship between the LA structure and its inflammatory and immunogenic activity. The affinity of LA for monomeric binding to MD-2, which is a critical determinant in the agonist activity of LA for TLR4, has been found to be most potent in the hexaacylated compared with penta- or tetraacylated forms (7, 8). More recently, we and others have shown that triggering receptor expressed on myeloid cells-2 binds LOS and LPS in both myeloid and non-myeloid cells and initiates an inflammatory cytokine response (9, 10).

Neisserial LOS lacks the repeating O-antigens of the LPS of the Gram-negative enteric bacteria, and differs also in the position, number, and chain length of the acyl groups on the LA. There can be differences in the acyl groups on the LA moieties within individual strains as well as between strains and species of Gram-negative bacteria (11, 12). The tetraacylated lipid IVa, which is a precursor of Escherichia coli LA has been found to be a TLR4 antagonist in human cells (13).

In both LPS and LOS the number of phosphate (P) and phosphoethanolamine (PEA) groups on LA can vary (14), which impacts on the bioactivity of the molecule for innate immune responses. Recent work has shown that whereas hexaacyl monophosphoryl LA was restricted to the myeloid differentiation factor 88 (MyD88)-independent pathway, which resulted in T cell activation, the diposphoryl LA also engaged the MyD88-dependent pathway, which activated NF-κB resulting in the production of TNF-α and other inflammatory cytokines (15). Because of its immunogenic properties, monophosphoryl LA has been approved for use as an adjuvant in a hepatitis B vaccine in Europe (16).

We have observed major differences in the induction of proinflammatory cytokines through TLR4-mediated signaling.
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elicted by various LOS purified from meningococcal and gonococcal strains (17). Little is known about the extent and biological significance of the natural structural variation in the LA of LOS occurring within a Neisserial strain. Recently, we reported that structural analyses of native LOS from different Neisserial strains indicated that differences in both acylation and phosphorylation of LA correlated significantly with the potency of LA to induce inflammatory cytokines (18). In this study, we used thin-layer chromatography (TLC) and matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometry (MS) to investigate the heterogeneity and inflammatory activity of the structural variants of the LA from N. meningitidis strain 89I, the LOS of which we found previously to be the most potent inducer of TNF-α among a group of seven Neisserial strains studied (17).

EXPERIMENTAL PROCEDURES

Bacterial Strains and LOS Extraction—N. meningitidis serogroup C strain 89I is a clinical isolate that has been described previously (17, 19). The LOS was extracted and purified by a modification of the hot phenol-water method (20, 21). E. coli strain O55:B5 LPS was from Sigma.

O-Deacylation of Native LOS—A portion of the native or intact 89I LOS was O-deacylated prior to MS as reported previously (22). Briefly, LOS (~0.3 mg) was placed in a 1.5-ml polypropylene tube and 200 μl of anhydrous hydrazine was added. The sample was kept in a water bath at 37 °C for 20 min and intermittently vortexed. The solution was cooled, and 1 ml of chilled acetone (~20 °C) was added dropwise. The precipitated O-deacylated LOS was centrifuged at 12,000 × g for 20 min, and the supernatant was removed. The pellet was washed with cold acetone, centrifuged again, and the supernatant was removed. Water (200–500 μl) was added to the pelleted O-deacylated LOS, and then the sample was lyophilized.

Hydrogen Fluoride (HF) Treatment of Native LOS—Phosphoesters were partially removed by mild HF treatment to aid in distinguishing between N-acetylhexosamine (HexNAc; 203 Da), and phosphate (P or HPO₄²⁻; 80 Da) plus PEA (123 Da) substitutions. From 0.1 to 1 mg of native LOS was placed in a polypropylene tube and cold 48% aqueous HF was added to make a 5–10 mg/ml solution, which was then allowed to react at 4 °C for 16–20 h. Excess HF was removed using a SpeedVac (Thermo Savant, Asheville, NC) with an in-line trap.

Isolation of LA—To produce the hydrolyzed LA, LOS (~1–2 mg/ml) was dissolved in acetic acid (1%) solution and then stirred at 100 °C for 60 min. After cooling the sample to room temperature, five parts (v/v) of chloroform:methanol solution (2:1, v/v) were added to two parts of the acetic acid solution. The mixture was stirred for 1 min and then centrifuged at 5,000 × g for 20 min. After discarding the upper aqueous phase, the lower and middle phases were transferred into a screw cap vial and dried under a stream of nitrogen gas. The dry LA was stored at −20 °C.

Thin-layer Chromatography (TLC) of LA—Plates for TLC (LK6 Silica Gel 60 Å, 5 × 20 cm; Whatman, Piscataway, NJ) were prepared by baking at 110 °C for 60 min and then storing under vacuum for 15 min or until use. The dry LA was dissolved in chloroform:methanol (1:1, v/v) and spotted on a TLC plate using glass pipettes and the smallest drops reasonably possible allowing the solvent to dry before respotting. The plates were developed in a solution of chloroform:methanol:water:acetic acid (50:25:4:0.15 v/v). The areas on the plate containing lipid were visualized with iodine, and then scraped from the plate into a glass container combining similar spots from 3 plates. The lipids were eluted from the silica by stirring in the developing solution at 40 °C for 10 min with intermittent vortexing. After allowing the silica to settle for 5 min, the supernatants were collected into clean glass tubes. The silica was washed three times with chloroform:methanol (using ~20% volume of original eluant each time) at 1:1, 2:1, and 4:1 (v/v) ratios with vortexing, the silica was allowed to settle, and the supernatants removed. After combining, the supernatants were centrifuged for 10 min to pellet any remaining silica, removed, and then dried under a stream of nitrogen and stored at −20 °C.

Preparation of LOS and LA for MS Analysis—LOS was prepared for MS using a method previously described for analysis of native rough-type bacterial LPS (23), and recently reported for the analysis of native Neisserial LOS (18). Purified, HF-treated, or O-deacylated LOS was suspended (1–2 μg/μl) in a mixture of methanol:water, 1:1 (v/v), containing 5 mM EDTA. An aliquot was desalted with a few cation-exchange beads (Dowex 50WX8–200) that had been converted to the ammonium form. The desalted sample was mixed with an equal volume of dibasic ammonium citrate (20 mM) and 0.5 μl was deposited on the mass spectrometry sample plate on top of a thin layer of matrix. The matrix layer was formed by deposition of drops (0.3–0.9 μl) of a solution composed of 2,4,6-trihydroxyacetophenone (200 mg/ml; Sigma) in methanol, with nitrocellulose transblot membrane (15 mg/ml; Bio-Rad) in acetone:isopropyl alcohol (1:1, v/v) mixed in a 4:1 (v/v) ratio. For MS analysis of the LA mixture and TLC fractions 1–9, samples were dissolved in chloroform:methanol (1:1, v/v), and 2 μl of each mixture with 4 μl of a saturated solution of 5-chloro-2-mercaptobenzothiazole (Sigma) in chloroform:methanol (3:1, v/v) as described previously (24). After desalting as described above with cation-exchange beads, sample aliquots were analyzed by MALDI-TOF MS.

MALDI-TOF Mass Spectrometry—MALDI-TOF MS was performed in the linear mode on a Voyager-DE STR model TOF instrument equipped with a 337-nm nitrogen laser and delayed extraction. Spectra were obtained in the negative-ion mode with an average of 500 pulses per spectrum. The acceleration voltage was −20 kV. Spectra were processed with digital smoothing and baseline correction using Data Explorer software. External calibration was performed using the average masses of the molecular ions of the peptides porcine renin substrate, bovine insulin, and oxidized insulin chain B (Sigma).

After external calibration, internal calibration of the mass spectra was performed using the average masses of ions as described below. For the native LOS the following ion peaks were used: (i) the B-type fragment ions for the oligosaccharide corresponding to the previously reported structure containing lacto-N-neotetraose plus a single sialic acid group with a single PEA without an O-acetyl group (calculated m/z 2130.81), (ii) molecular ions for the intact LOS with a sialylated oligosaccharide with an O-acetyl group and a single PEA and a triphospho-
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Monophosphoryl LA moiety with a single PEA (calculated $m/z$ 4090.11), (iii) ions for the intact LOS minus the O-acetyl and one PEA group (calculated $m/z$ 3967.06), and (iv) ions for LA (calculated $m/z$ 1793.20 and 1738.28 for the triphosphorylated LA, and LA with a single P and a PEA group that has lost $H_2O$, respectively). For internal calibration of the HF-treated native LOS the following ions were employed: (i) the $B$-type fragment ions for the oligosaccharide component without $O$-acyl or PEA that has lost $H_2O$ (calculated $m/z$ 1898.75), (ii) the native LOS with a monophosphoryl LA and the oligosaccharide component without $O$-acyl or PEA, and minus one $H_2O$ (calculated $m/z$ 3624.00), and (iii) the mono- and diphosphoryl LA components. For the $O$-deacylated LOS the following two ion peaks were used: (i) the $O$-deacylated diphosphoryl LA (calculated $m/z$ 952.00) and (ii) the oligosaccharide component without $O$-acyl plus the $O$-deacylated diphosphoryl LA (calculated $m/z$ 3083.82).

After external calibration as described above for the spectra of LA, a 1- or 2-point internal calibration of the mass spectra of LA was performed using the average masses of 1 or 2 molecular ions with previously reported structural components: a diphosphoryl LA with a single PEA substituent (calculated $m/z$ 1836.27), monophosphoryl LA with a single PEA substituent and without $H_2O$ (calculated $m/z$ 1756.29), diphosphoryl LA (calculated $m/z$ 1713.22), monophosphoryl LA (calculated $m/z$ 1633.24), monophosphoryl pentaacyl LA with a single PEA substituent (calculated $m/z$ 1557.99), and monophosphoryl pentaacyl LA (calculated $m/z$ 1450.94).

Gas Chromatography (GC)-MS Analysis of Fatty Acids Derived from LA—Fatty acid standards (hydroxydecanoate, laurate, hydroxyxaurate, hydroxymyristate, stearate, and palmitate) were obtained from Sigma. From 1 to 2 mg of the standards, and portions of TLC fractions 1–9, and the LA mixture were dissolved in chloroform:methanol (50:50, v/v) and transferred to 2-ml Reacti-Vials with phenolic open-top screw caps and Teflon-lined natural rubber septa (Wheaton Science Products, Millville, NJ). Hydroxydecanoic acid (38 µg/sample) was dissolved in chloroform:methanol (50:50; 0.5 µg/ml) and added to each sample as an internal standard. After evaporation to dryness, each sample was treated with 0.5 ml of 10% (w/w) BF$_3$-methanol (Supelco, Bellefonte, PA) and heated at 100 °C for 6 h. Each sample was partitioned by 0.5 ml of saturated NaCl (0.5 ml) and hexanes (0.5 ml; Sigma) after cooling to room temperature. The aqueous layer was extracted again with 0.5 ml of hexanes. The organic layers from each fraction were evaporated to dryness under a stream of nitrogen after combination and back-extraction with 0.3 ml of $H_2O$.

For GC or GC-MS, the fatty acid methyl esters were dissolved in hexanes and analyzed in the electron impact mode using a CP-3800 GC with a CP-8400 autosampler interfaced with a Saturn 2000 mass spectrometer (Varian, Palo Alto, CA). Fatty acid methyl esters were separated on a 30-m × 0.25-mm BPX70 column with a 0.25-µm film thickness (SGE, Inc., Austin, TX) with helium as the carrier gas (10 pounds/in$^2$). The initial oven temperature was 100 °C for 5 min, followed by a temperature gradient from 100 to 220 °C at 4 °C/min. For quantitative analysis of the fractions by GC-MS, relative peak areas of the methyl esters of hydroxylaurate and hydroxymyristate were measured from the total ion chromatogram and normalized to the internal standard.

Proinflammatory Cytokine Response of Human Monocytes—Human monocyte-like cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA) and has been characterized previously (17). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum, and were differentiated by phorbol myristate acetate treatment (10 ng/ml) for 24 h prior to stimulation. Differentiated THP-1 cells were seeded at 10$^5$ cells per well in a 96-well plate and treated with fractions 1–6 by loading equal amounts based on quantification of fatty acids using GC-MS or with 100 ng/ml HF-treated or native LOS and LPS for 18 h. The supernatants were collected and stored at −70 °C for the subsequent cytokine assay. For human monocyte isolation, freshuffy coat (Blood Centers of the Pacific, San Francisco, CA) was diluted 1:1 with phosphate-buffered saline and layered onto Ficoll for centrifugation. The interphase containing the monocytes was collected, washed twice with phosphate-buffered saline, and then seeded in T75 cell culture flasks at 10$^6$ cells/ml in RPMI 1640 with 10% fetal bovine serum. After 2 h incubation, lymphocytes were washed away with phosphate-buffered saline and the adherent monocytes were trypsinized, reseeded into 24-well plates at 6.25 × 10$^5$ cells per well, and treated with LOS or LA fractions at the above concentrations for 18 h. Control wells were treated with culture media only. Supernatants were collected and analyzed for TNF-α expression. Cytokine levels were assayed using a bead-based singleplex human TNF-α kit (Bio-Rad). Samples were processed as recommended by the manufacturer and analyzed using a Bio-Plex 200 analytical system (Bio-Rad).

Statistical Analyses—Statistical analyses were performed using SigmaStat for Windows version 3.11 (Systat Software, San Jose, CA). Groups of data were analyzed by the Tukey test for multiple pairwise comparisons. Values of $p < 0.05$ were considered significant for all comparisons.

RESULTS

MALDI-TOF MS of Native 89I LOS—Our analysis of the native LOS from N. meningitidis strain 89I was performed using a thin layer of 2,4,6-trihydroxyacetophenone/nitrocellulose matrix as previously described for negative-ion MALDI-TOF (23). As shown in Fig. 1, the mass spectrum contained several apparent molecular ions ($M-H$) that differed by masses corresponding to loss of $P$, PEA, acetate ($Ac$), or sialic acid groups. A structure for the molecular ions ($M-H$) at $m/z$ 4090.2 is presented in Fig. 1, which is consistent with the spectrum and includes details such as the monosaccharide composition and sequence that were previously reported for the 89I L4 immunotype oligosaccharide (19), which could not be identified by the MALDI-TOF analysis.

In addition to the peak at 4090.2, there also are molecular ion peaks at $m/z$ 3967.5, which differs by 123 Da consistent with loss of PEA, and at 3870.7 for loss of PEA, $P$, and $H_2O$ (123, 80, and 18 Da, respectively). Loss of sialic acid from the molecular ion peak is represented by small peaks at $m/z$ 3797.7 and 3675.4, the latter with an additional loss of PEA. Peaks for molecular ions with loss of the $Ac$ group can be seen at $m/z$ 4047.7 and 3924.9, the latter reflecting loss of PEA also.
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**FIGURE 1.** Negative-ion MALDI-TOF mass spectrum and structure of LOS from *N. meningitidis* strain 89I. The structure of the LOS derived from the current and previously published data, and the prototypical Neisseria LA. The molecular ions (M−H)− at *m/z* 4090.2 have 2 PEA, 3 P, and 1 Ac and differ from the ions seen in the peaks in the *inset* at *m/z* 4047.7, 3967.5, and 3924.9 by an Ac (42 Da), a PEA (123 Da), and an Ac plus a PEA (165 Da), respectively. The locations of the third P and the PEA on the LA are not known and have been shown as R₁ and R₂. The ions for the peaks also seen in the *inset* at *m/z* 3870.7, 3797.7, and 3675.4 differ from those in the peak at *m/z* 4090.2 by a PEA and P plus H₂O (98 Da), sialic acid (291 Da), PEA and sialic acid, respectively. In the lower mass range (*m/z* < 2400) three oligosaccharide fragment ion peaks can be observed; one B-type at *m/z* 2172.6 and two related oligosaccharide peaks at *m/z* 2130.2 and 1839.6 for ions that differ from those ions at *m/z* 2172.6 by an Ac, and both an Ac and sialic acid (Neu5Ac), respectively. The ions in the LA peak at *m/z* 1915.6 have 3P and a single PEA, and differ from those in the peaks at *m/z* 1793.1 and 1738.4 by PEA, and 2P plus H₂O, respectively. At *m/z* 1617.8 is another peak for LA ions that are pentaacyl having lost lauric acid (182 Da), P, and two molecules of H₂O.

The proposed composition for the ions at *m/z* 4090.2 is consistent with peaks observed for Y-type ions retaining the oxygen of the glycosidic bond for the LA at *m/z* 1915.6 and for the corresponding B-type ions for the oligosaccharide at *m/z* 2172.6. The composition proposed for the ions at *m/z* 2172.6 is: sialic acid; 3 hexose; 2 HexNAc; 2 α-glycerophosphate; 2 3-deoxy-D-manno-octulosonic acid (Kdo), Ac, and PEA. This composition is in accordance with the structure previously reported for the 89I oligosaccharide (19). The oligosaccharide ions at *m/z* 2130.2 correspond to an additional loss of Ac.

A novel composition consistent with other peaks in the spectrum and that for the *m/z* 1915.6 lipid ions is a conserved hexaacylated LA with 3P plus a PEA. Other evidence for this includes the base peak in the spectrum at *m/z* 1793.1 which corresponds to loss of PEA and, therefore, a LA with 3P (*m/z* 1915 − 123 Da = *m/z* 1792). A prominent peak is observed at *m/z* 1738.4 that is likely due to a prompt in-source fragmenta-

The native 89I LOS was subjected to HF treatment and then analyzed by MALDI-TOF to illustrate the difference due to preferential cleavage of phosphoester bonds and help distinguish between HexNAc (203 Da) and P plus PEA (80 plus 123 Da) moieties. The LOS was analyzed in negative-ion mode on a thin layer of 2,4,6-trihydroxyacetonaphenone/nitrocellulose matrix. The resulting spectrum and a structure based on it and previously published analyses as described above are shown in Fig. 2.

The molecular ion peak at *m/z* 3641.1 is consistent with the loss of 2 PEA groups, 2P, and a single Ac compared with the molecular ion peak in Fig. 1 for the native LOS at *m/z* 4090.2. A single PEA oligosaccharide constituent was reported previously (19). The peak at *m/z* 3624.3 represents a loss of H₂O from the peak at *m/z* 3641.1. The most prominent peak in the molecular ion region is at *m/z* 3350.6 and corresponds to loss of sialic acid from the molecular ions at *m/z* 3641.1. The peak at *m/z* 3404.6 corresponding to loss of Kdo and H₂O.
also acquired to aid in assignment of the ions observed in the spectra of the native and HF-treated LOS and is shown in Fig. 3, along with a structure based on it and previously published analyses as described above. The molecular ions at \( m/z \) 3083.8 are consistent with the same oligosaccharide composition, but with an \( O \)-deacylated LA, as expected. As in the previous two spectra, peaks for both Y- and B-type fragment ions of the highest mass molecular ion peak can be observed at \( m/z \) 952.0 and 2129.8, respectively. The peaks at \( m/z \) 3083.8 and 2792.0 differ by 291 Da corresponding to the loss of a single sialic acid residue, as observed in the previous analyses.

Peaks for other ions observed represent loss of \( CO_2 \) (44 Da), loss of Kdo (220 Da), or loss of both or loss of one or both with sialic acid. Prompt fragmentation resulting in loss of these moieties is more common with \( O \)-deacylated LOS according to these results and consistent with our previous mass spectral analysis of Neisserial LOS (18).

TLC of 89I LA—The 89I LOS was subjected to mild acid hydrolysis to enable isolation and purification of different components of the LA by TLC. After spotting a solution of the lipid in chloroform:methanol on a line, the silica-coated glass plates were developed in chloroform:methanol:water:acetic acid solution. Once dry after removal from the solvent, the areas on the plate containing lipid were visualized with iodine. A photograph of the TLC with lanes stained with iodine is shown in Fig. 4. Five discrete well resolved bands were observed (numbers 2–6), in addition to a much broader, faintly stained band at the bottom of the plate near the loading line (number 1) and 3 dark bands (numbers 7–9) near the top that were not well separated. After development of the plates and staining with iodine, the bands were scraped and the samples eluted with solvent, and then dried. Aliquots of each sample were analyzed by MALDI-TOF MS. In addition, the relative amount of LA in each sample was quantified by GC-MS using hydroxydecanoic acid as an internal standard.

MALDI-TOF MS of LA Separated by TLC—to perform MALDI-TOF MS of the LA, the samples were dissolved in chloroform:methanol and then aliquots were mixed with a saturated solution of 5-chloro-2-mercaptobenzothiazole in chloroform: methanol as described previously (24). After desalting, the samples were analyzed by negative-ion MALDI-TOF. The spectrum of the unfraccionated 89I LA is presented in Fig. 5A; the analyses of fractions 1–6 are presented in Fig. 5, B–G, respectively. The proposed compositions of the ions observed are presented in Table 1.

The base peak in the spectrum of the unfraccionated lipid shown in Fig. 5A is at 1756.3, with peaks of comparable intensity occurring at \( m/z \) 1713.2 and 1633.2, corresponding to the LA with P-PEA, 2P, or P substituents, respectively. Much less abundant were the ions at \( m/z \) 1836.3 for the diphosphoryl LA (2P) with a single PEA group, at \( m/z \) 1557.8 for LA molecules with a P-PEA group lacking a hydroxyaurate moiety \( (C_{12}H_{22}O_{2}) \), and at \( m/z \) 1434.7 for molecules that have only a single P but also lack a hydroxyaurate group.

There are more molecular ion peaks in the analysis of fraction 1 as shown in Fig. 5B than in any of the spectra from fractions 2–6 as shown in Figs. 5, C–G. In Fig. 5B, there are peaks at
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Several apparent molecular ion peaks can be observed in the spectra of fractions 5 and 6 as shown in Fig. 5, F and G. In Fig. 5F, peaks for pentaacyl P LA that are lacking laurate, and hydroxylaurate moieties, respectively, can be observed at m/z 1450.9 and 1434.9. A much less abundant peak can be observed at m/z 1236.0, which could correspond to a tetraacyl P LA that is lacking both hydroxylaurate moieties. In Fig. 5G, the most prominent peak at m/z 1633.4 is consistent with a hexacyl P LA. Much less abundant are the ions at m/z 1417.2 and 1201.7 that are in accordance with a pentaacyl P LA that is missing hydroxylaurate and has lost H$_2$O as well, and a tetraacyl LA, which has lost both hydroxylaurate molecules and two molecules of H$_2$O, respectively.

The MALDI-TOF analyses of fractions 7–9 (data not shown) revealed a number of low mass (<1,000 Da) peaks. Thus, it is postulated that these fractions represent various fragments or components of the LA, and/or an assortment of small molecules produced by the bacteria whose solubility and lipophilicity are similar to LA and LOS.

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LA in fraction 3, the P-pentaacylated LA from fraction 5, and the hexaacylated P LA from fraction 6 compared with one another were not statistically significant.

To further confirm the importance of phosphorylation in inflammatory responses to LOS, we next tested the relative potency of the native 89I LOS and E. coli strain O55:B5 LPS in induction of TNF-α in THP-1 cells after preferential hydrolysis of phosphoryl groups by treatment with aqueous HF. HF treatment of the 89I LOS produced primarily the monophosphoryl LA, with some remaining diphasphoryl molecules as revealed by the MALDI-TOF analysis shown in Fig. 2. The results of the TNF-α assay are presented in Fig. 6B. Compared with untreated LOS, the activity for TNF-α induction of the HF-treated LOS was reduced almost 3-fold (*p < 0.001), and that of the LPS was reduced more than 4-fold (*p < 0.001). After HF treatment the residual activity of the LOS was almost twice that of the LPS (*p < 0.01). In addition, the amount of TNF-α induced in the THP-1 cells by native LOS was more than that of any of the LA fractions shown in Fig. 6A. However, the data does not allow direct comparison of the activity of the 89I LA with native LOS as the relative and not the absolute amount of LA was used to determine the dosing of the fractionated LA with the THP-1 cells, whereas the dosing of the LOS was by weight.

Last, we tested the ability of 89I LA from fractions 4 and 5 to induce TNF-α expression in primary human monocytes. As shown in Fig. 6C, the relative activity of the two fractions in the induction of TNF-α in monocytes was nearly identical to that observed in THP-1 cells as shown in Fig. 6A, thus further confirming our results as the same portion of each fraction was tested for both cell types. However, compared with the THP-1 cells, the monocytes were more sensitive to the LA in these two fractions as evidenced by their expression of nearly a 10-fold greater quantity of TNF-α.

**DISCUSSION**

The results of this study demonstrate that natural phosphoryl and acyl variants of LA from *N. meningitidis* strain 89I have differential ability to induce TNF-α in human monocytes. In addition, our data identifies specific LA variants, such as the P-PEA hexaacyl LA, which have greater TNF-α induction capability than related LA molecules such as the mono- and diphasphoryl LA. The expression of LOS with varying inflammatory potential may facilitate the survival of meningococci in the variety of environments that would be encountered in infected host tissues.

Previous reports including our own on the structure of LA from meningococcal LOS mainly describe a diphasphoryl LA with from 1 to 2 PEA groups (18, 26, 27). However, we detected a triphosphoryl variant with and without a single PEA substituent as the primary form of the LA. In general, the MALDI-TOF spectra of fractions 1–6 contained peaks for molecules corresponding to the expected LA moieties based on previously published studies of LA from *N. meningitidis* (26), with the exception of the prominent peaks at *m/z* 1793.1 and 1915.6 that were detected in the MALDI-TOF analysis of the native LOS shown in Fig. 1. These peaks are in accordance with the primary expression of a triphosphorylated LA. In addition, the MALDI-
TOF analyses of both the LOS and LA from 89I are consistent with the previously reported oligosaccharide structure (19).

The potential biological significance of the expression of a triphosphoryl LA with a PEA substituent is highlighted by our previous finding that the native 89I LOS induced more TNF-α production in THP-1 cells than either of the two other N. meningitidis strains tested, 7880 and 7889, any of four N. gonorrhoeae strains GC56, DOV, F62, or 1291, or the LPS from Salmonella minnesota strain Re595 (17). Only the LPS from E. coli strain 055:B5 showed a trend toward more TNF-α induction. A recent study focused on mass spectrometric analysis of LA also reported that pyrophosphoryl groups were present in several common Gram-negative bacteria (25).

The elution pattern on TLC of the postulated molecules was consistent with that expected for silica gel, as the more highly polar molecules containing a greater number of phosphoryl and PEA groups did not elute as far on the plate compared with less polar molecules such as the monophosphoryl LA, which migrated further with the solvent.

Comparing the spectra of the unfractionated LA shown in Fig. 5A with that of the fractions shown in Fig. 5 B–G, reveals the clear advantage of the TLC. Using TLC to purify the LA molecules enabled better detection of the individual components by MS. The only significant peak that occurred in more than one fraction was at m/z 1713.2, which can be observed in the spectra of both fractions 1 and 2.

| Calculated (M-H) | Entity | Observed (M-H) |
|-----------------|--------|----------------|
| 1200.6          | P LA–(C_{12}H_{22}O_4–H_2O)_2 | 1201.7 |
| 1236.6          | P LA–(C_{12}H_{22}O_4) | 1236.0 |
| 1416.9          | P LA–(C_{12}H_{22}O_4–H_2O) | 1417.2 |
| 1434.9          | P LA–C_{12}H_{22}O_4 | 1434.7/1434.9 |
| 1450.9          | P LA–C_{12}H_{22}O_4 | 1450.9 |
| 1514.9          | 2P LA–C_{12}H_{22}O_4 | 1514.2/1515.5 |
| 1530.9          | 2P LA–C_{12}H_{22}O_4 | 1531.5 |
| 1558.0          | PEA-P LA–C_{12}H_{22}O_4 | 1557.8/1558.0 |
| 1574.0          | PEA-P LA–C_{12}H_{22}O_4 | 1574.0 |
| 1615.2          | P LA–H_2O | 1615.4 |
| 1633.2          | P LA | 1633.2 |
| 1713.2          | 2P LA | 1713.2 |
| 1738.3          | PEA-P LA–H_2O | 1738.5 |
| 1756.3          | PEA-P LA | 1756.3 |
| 1793.2          | 3P LA | 1793.5 |
| 1836.3          | PEA 2P LA | 1836.3/1836.5 |

TABLE 1
Peaks from LA spectra

FIGURE 5. Negative-ion MALDI-TOF spectra of the unfractionated LA and the LA from fractions 1–6. A, in the spectrum of the unfractionated LA at m/z 1756.3, 1713.2, and 1633.2 are peaks corresponding to LA with P-PEA, 2P, or P substituents, respectively. At m/z 1836.5 is the peak for ions of diposphoryl LA with a single PEA group. Pentaacyl ions both lacking hydroxylauric acid at m/z 1557.8 are P-PEA LA, and at m/z 1434.7 is a peak for ions of P LA. B, in the spectrum of fraction 1 are four hexacyl LA ion peaks at m/z 1836.3 for 2P PEA LA, at m/z 1793.5 for 3P LA, at m/z 1738.5 for P-PEA LA that has lost H_2O, and at m/z 1713.2 for 2P LA. Two pentaacyl LA ion peaks can be seen at m/z 1531.5 and m/z 1515.6 that are lacking a lauric acid and hydroxylauric acid. C, the spectrum of fraction 2 contains a single prominent ion peak at m/z 1713.2 for the 2P LA. Smaller peaks at m/z 1615.4 and 1514.2 contain ions for P LA that has lost H_2O, and a pentaacyl 2P LA lacking a hydroxylauric acid group, respectively. D, in the spectrum of fraction 3 are peaks at m/z 1574.0 and 1558.0 for ions of pentaacyl P-PEA LA that do not have a lauric and hydroxylauric acid group, respectively. E, in the spectrum of fraction 4 there is a peak at m/z 1756.3 corresponding to ions of P-PEA hexacyl LA. F, peaks for ions of pentaacyl P LA from fraction 5 that are lacking a lauric and hydroxylauric acid moiety can be seen at m/z 1450.9 and 1434.9, respectively. The peak at m/z 1236.0 corresponds to ions of tetraacyl P LA. G, in the spectrum of fraction 6 the peak at m/z 1633.4 is in accordance with ions for the P LA, and the ions at m/z 1417.2 and 1201.7 are consistent with pentaacyl P LA, which is minus a hydroxylauric acid group and H_2O, and a tetraacyl LA, which has lost both hydroxylauric acid groups and two molecules of H_2O, respectively.
A few minor components that were minimally detected or undetectable in the MALDI-TOF spectrum of the mixture were readily observed in the spectra of the fractions. For example, those peaks seen in Fig. 5B at m/z 1836.3 and 1793.5 for the relatively highly phosphorylated 2P-PEA and 3P LA species were minimal in Fig. 5A. This is likely due to ion suppression occurring in the analysis of the mixture due to differences in mass, charge, and hydrophobicity (28). Some peaks prominent in Fig. 5B also may have been produced by particularly facile prompt fragmentation that occurred in the ion source. For example, the peak at m/z 1738.5 could be due to the loss of P with H_2O (98 Da) from the molecular ions at m/z 1836.5, and the peaks at m/z 1531.5 and 1515.5 could be from loss of laurate and hydroxylaurate, respectively, from the ions at m/z 1713.2. Peaks likely produced by similar fragmentation of the molecular ions at m/z 1713.2 can be observed in Fig. 5C at m/z 1615.4 for loss of 98 Da and at m/z 1514.2 for loss of hydroxylaurate. The TLC separation of the LA molecules enabled the testing of their relative ability to induce inflammatory cytokine signaling in cell culture. Interestingly, these data revealed that the hexacylated P-PEA LA of fraction 4 was significantly more active than either the di- or monophosphoryl LA molecules by a factor of 5- and 10-fold, respectively. This result suggests that both the nature and the position of the phosphoryl substitution on the LA molecule may be key structural determinants in inflammatory signaling by the LOS. Accordingly, but of less magnitude, was the greater TNF-α induction by the diphosphoryl LA of fraction 2 compared with that induced by the monophosphoryl LA of fraction 6.

The TNF-α levels detected after incubation of THP-1 cells with the HF-treated and untreated 89I LOS were in accord with the data from the LA fractions. The native 055:B5 E. coli LPS induced somewhat more TNF-α in the THP-1 cells than the native 89I LOS, which is in agreement with our previous results.
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Although HF treatment of the LPS and LOS affected TNF-α induction in the THP-1 cells similarly, the reduction of activity of the LPS was more pronounced, which is likely related to the structural differences in both the length and number of fatty acid groups in Neisserial LOS compared with E. coli LPS.

The response of the monocytes to incubation with LA fractions 4 and 5 was very similar to the relative response of the THP-1 cells to these fractions, and supports the validity of our results. However, ~10-fold more TNF-α was induced in the monocytes. Their much greater sensitivity to LOS implies that primary human monocytes may be found to have more relevance in analysis of LOS inflammatory signaling than cell lines such as THP-1.

Previously we reported detection of peaks in negative-ion MALDI-TOF analysis of native LOS from N. meningitidis strain 7889 consistent with the expression of a triphosphoryl LA, but these appeared to be minor components (18). In the same study, our analyses of three strains of N. gonorrhoeae also showed that their LA moieties were primarily either diphasphoryl or diphasphoryl with a single PEA group, consistent with other studies of Neisserial LA (26, 29), although peaks consistent with the presence of additional phosphoryl groups on the LA have been observed in gonococcal LOS (30, 31).

The relevance of LA phosphorylation in inflammatory signaling is indicated by the relative activity of the LA molecules in TLC fractions to induce TNF-α expression in THP-1 cells. The differences in activity of the lipid due to variation in phosphorylation were similar to those observed apparently due to differences in acylation alone. The reduction in TNF-α signaling with one less acyl chain on the pentaacylated P-PEA LA of fraction 3 compared with the hexaacylated P-PEA LA of fraction 4 mirrors the reduction observed with one less PEA on the hexaacylated P LA of fraction 6.

A number of earlier studies examined the physicochemical features of LA, which are critical to its endotoxic activity and found that there is a relationship between the three-dimensional shape of LA and its bioactivity (32–35). Factors affecting the three-dimensional conformation of LA are the length and number of acyl chains, their asymmetry, and the number and distribution of charged groups. LA molecules that adopt a conical shape with a larger cross-section of the hydrophobic than of the hydrophilic region, are highly active, whereas LA with a cylindrical shape with both cross-sections equal are less active in cytokine induction (36). For example, analysis of the induction of IL-6 in whole human blood by LPS, LA, monophosphoryl LA, and dephosphorylated LPS from the Re mutant of E. coli showed that the intact LOS was most active, followed by the LA, which was approximately an order of magnitude less active, and the monophosphoryl LA and dephosphorylated LPS were less active by almost 3 orders of magnitude (36). Interestingly, analysis of the ability of synthetic LA and that from rough mutants of S. minnesota and E. coli to induce IL-1 in primary human mononuclear cells showed that LA with a reducing terminal (1-position) phosphate was more highly inflammatory than LA with non-reducing terminal (4’-position) phosphate (37).

Monophosphoryl LA is much less inflammatory than the LPS from which it is derived (38, 39), and, nonetheless, induces immune reactions (16). A recent report on the recognition of LPS by the TLR4 dimeric complex with MD-2 found that signal transduction by hexaacyl monophosphoryl LA was restricted to the MyD88-independent TRIF-TRAM pathway, which resulted in T cell activation, whereas diphasphoryl LA also activated the MyD88-dependent pathway, which activated NF-κB, and subsequently TNF-α and other inflammatory cytokines causing inflammation (15).

In summary, our data show that significant variation occurs in both the phosphorylation and acylation of LA within a single strain of N. meningitidis and that the resulting heterogeneous mixture contains molecules that have significantly different inflammatory potential. Previously reported data support our results that diphasphoryl LA from strain 89I is more inflammatory than its monophosphoryl LA, and that the pentaacyl LA is less inflammatory than the hexaacyl (36). We also have shown that the relatively highly inflammatory 89I LOS predominantly expresses a triphosphoryl LA, and that the presence of a single PEA group on monophosphoryl 89I LA enhances its ability to induce TNF-α in monocytes relative to either the mono- or diphasphoryl LA. This result is in accord with our previous analyses of native LOS from both N. gonorrhoeae and N. meningitidis, which revealed that the occurrence of a PEA on diphasphoryl LA from either species was correlated with increased inflammatory cytokine signaling (18). In conclusion, a thorough characterization of Neisserial LOS structural heterogeneity is critical to understanding the innate immune responses to these molecules in the context of disease pathogenesis and host protection.

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