Structural Study of Lipomannan and Lipoarabinomannan from Mycobacterium chelonae

PRESENCE OF UNUSUAL COMPONENTS WITH α1,3-MANNOPYRANOSE SIDE CHAINS*

Received for publication, May 6, 2002, and in revised form, June 12, 2002

Yann Guérandel‡, Emmanuel Maes‡, Elisabeth Elass‡, Yves Leroy‡, Philippe Timmerman‡, Gurdyal S. Besra‡‡, Camille Locht*, Gérard Strecker‡, and Laurent Kremer**

From the ‡Laboratoire de Glycobiologie Structurale et Fonctionnelle, CNRS UMR8576, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d’Ascq Cedex, France, and **School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT United Kingdom, and Laboratoire des Mécanismes Moléculaires de la Pathogénie Microbienne, INSERM U447, Institut Pasteur de Lille IBL, 1 Rue du Pr. Calmette, BP245–39019 Lille Cedex, France

Lipomannan (LM) and lipoarabinomannan (LAM) are major glycolipids present in the mycobacterial cell wall that are able to modulate the host immune response. In this study, we have undertaken the structural determination of these important modulins in Mycobacterium chelonae, a fast growing pathogenic mycobacterial species. One-dimensional and two-dimensional NMR spectra were used to demonstrate that LM and LAM from M. chelonae, designated CheLM and CheLAM, respectively, possess structures that differ from the ones reported earlier in other mycobacterial species. Analysis by gas chromatography/mass spectrometry of the phosphatidylyl-myo-inositol anchor, which is thought to play a role in the biological functions of these lipoglycans, pointed to a high degree of heterogeneity based on numerous combinations of acyl groups on the C-1 and C-2 positions of the glycerol moiety. Characterization of the mannan core of CheLM and CheLAM revealed the presence of novel α1,3-mannopranosyl side chains. This motif, which reacted specifically with the lectin from Galanthus nivalis, was found to be unique among a panel of nine mycobacterial species. Then, CheLM and CheLAM were found to be devoid of both the mannooligosaccharide cap present in Mycobacterium tuberculosis and the inositol phosphate cap present in Mycobacterium smegmatis and other fast growing species. Tumor necrosis factor-α and interleukin-8 production were assessed from human macrophages with LAM preparations from different species. Our results suggest that the inositol phosphate capping may represent the major cytokine-inducing component of LAMs. This work not only underlines the diversity of LAM structures among various mycobacterial species but also provides new structures that could be useful to dissect the structure-function relationships of these complex molecules.

Mycobacterium species are responsible for important human diseases including tuberculosis and leprosy. Infection and immuneopathogenesis of these diseases widely implicate the mycobacterial cell wall (1) which is abundantly composed of mannoconjugates, notably polysaccharides and lipoglycans. The latter consist mainly of phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM), and the structurally related lipoarabinomannan (LAM). LM is a major cell wall component and is considered as a modulin through its various immunoregulatory and anti-inflammatory effects, which favor the survival of the mycobacteria within the infected host. These effects include suppression of T lymphocyte proliferation through interference with antigen processing (2), inhibition of macrophage activation by interferon-γ (3, 4), and scavenging of oxygen-derived free radicals (5). LAM is not only a virulence factor responsible for macrophage deactivation, but it is also implicated in phagocytosis of mycobacteria into phagocytic cells (6). In addition, PIMs that are believed to be precursors of LM and LAM have recently been proposed to recruit NK T cells, which play a primary role in the granulomatous response (7, 8).

The biosynthetic relationship of phosphatidylinositol (PI), PIMs, LM, and LAM has recently been supported by biochemical (9, 10) and genetic studies (11, 12), but the details of this pathway remain highly speculative. However, the structures of LAM from several species including Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis BCG, and Mycobacterium smegmatis have been extensively described during the last decade. LAM is a complex glycolipid composed of β-mannan and α-arabinan attached to a PI moiety that anchors the glycolipid in the mycobacterial cell wall (13). The biosynthesis of LAM involves the addition of manno-2-β-linked Manp backbone substituted at C-2 by single Manp units.

‡‡ Lister Institute-Jenner Research Fellow and supported by the Welcome Trust and the Medical Research Council.

** To whom correspondence should be addressed. Tel.: 33-3-20-87-11-54; Fax: 33-3-20-87-11-58; E-mail: laurent.kremer@ibl.fr.

* This work was supported in part by the CNRS (UMR 8576, Glycobiologie Structurale et Fonctionnelle, Director Dr. Jean-Claude Michalski), the Ministère de l’Enseignement Supérieur et de la Recherche, and by INSERM. The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Lister Institute-Jenner Research Fellow and supported by the Wellcome Trust and the Medical Research Council.

†‡ To whom correspondence should be addressed. Tel.: 33-3-20-87-11-54; Fax: 33-3-20-87-11-58; E-mail: laurent.kremer@ibl.fr.

1 The abbreviations used are: PIMs, phosphatidyl-myo-inositol mannosides; AraF, arabinofuranosyl; ConA, lectin from Canavalia ensiformis; COSY, correlation spectroscopy; CI, chemical ionization; EI, electron impact; GC/MS, gas chromatography/mass spectrometry; Gro, glycerol; GNA, lectin from Galanthus nivalis; HMBC, Heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; Ins, inositol; LM, lipomannan; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; LAM, lipoarabinomannan; MALDI MS, matrix-assisted laser-desorption ionization mass spectrometry; Manp, manno-2-β-linked Manp backbone substituted at C-2 by single Manp units
(16). The mannan size and the degree of branching can vary depending on the species. The arabinin consists of a linear α,5-linked arabinofuranosyl (Araf) backbone punctuated by branching produced with 3,5-O-linked α-d-Araf residue. The lateral chains are organized either as linear tetra-arabinofuranosides β-(1→2)-α-(1→3)-α-D-Araf as a biantennary hexa-arabinofuranosides [β-(1→2)-α-(1→3)-α-D-Araf-(1→3)-3 and 5]-a-(1→3)-α-D-Araf (17, 18). Comparativ analyses of LAMs from different mycobacterial species have shown that the non-reducing termini of the arabinosyl side chains are differentially modified. *M. tuberculosis* and *M. leprae* modify the termini with Manp residues, thereby yielding “ManLAM,” whereas the rapidly growing species *M. smegmatis* uses inositol phosphate, generating “AraLAM” (19). It is thought that these modifications are responsible for the marked differences in the biological activities of ManLAM and AraLAM (15, 19, 20). However, as mentioned above, all available LAM structures are derived from a very limited panel of mycobacterial species, and whether these structures are invariably present in most species remains to be investigated. Awareness that subtle differences among LAMs may affect their biological properties prompted us to establish the structure of LM/LAM from *Mycobacterium chelonae*, a rapidly growing pathogenic mycobacterium that is found in soil and fresh water throughout the world (21). *M. chelonae* infection typically causes localized skin lesions, often following penetrating trauma or injections. Disseminated disease usually occurs in patients with a significant immune compromised state that is most commonly attributable to exogenous steroid use (22, 23).

We report here the detailed structure of LM/LAM from *M. chelonae* and provide evidence for important differences such as the acylation composition of the PI, branching of the LM/LAM from *AraLAM* (15, 19, 20). However, as mentioned above, all available LAM structures are derived from a very limited panel of mycobacterial species, and whether these structures are invariably present in most species remains to be investigated. Awareness that subtle differences among LAMs may affect their biological properties prompted us to establish the structure of LM/LAM from *M. chelonae*, a rapidly growing pathogenic mycobacterium that is found in soil and fresh water throughout the world (21). *M. chelonae* infection typically causes localized skin lesions, often following penetrating trauma or injections. Disseminated disease usually occurs in patients with a significant immune compromised state that is most commonly attributable to exogenous steroid use (22, 23).

**Experimental Procedures**

*Strain and Culture Conditions*—All mycobacterial species used were grown on plates containing Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco) or in liquid Sauton medium. Except for *M. chelonae* (ATCC 19536), which was grown under shaking in Sauton medium at 30 °C for several days, all other species were grown at 37 °C. Confirmation of the identity of the *M. chelonae* strain was done by analyzing its mycolic acid profile, which is rather unusual because it consists of 60% of α-myrcales and 40% of α'-myrcales (24, 25).

**Purification of CheLAM and CheLM—**Extraction of CheLM and CheLAM was adapted from Nigou et al. (26) based on the Triton X-114 phase partitioning. Briefly, cells were harvested, washed in PBS (20 mM K2HPO4, pH 7.5), 0.15 M NaCl, and resuspended in lysis buffer (8% v/v Triton X-114 in PBS, 0.5 mM EDTA, 10 mM MgCl2). Cells were then heat-inactivated, disrupted using a French pressure cell and stirred overnight at 4 °C. Cellular debris were removed by centrifugation (27,000 × g, 30 min, 4 °C), and phase separation was induced at 37 °C. Lipoglycans present in the lower phase were precipitated by adding 5 volumes of cold ethanol and collected by centrifugation (27,000 × g, 30 min, 4 °C). The pellet was dissolved in water, and proteinase K was added to a final concentration of 10 μg/ml for 20 min at 55 °C. Proteins were extracted twice by adding saturated phenol. Combined aqueous phases containing lipoglycans were dialyzed for 72 h against water, lyophilized, and resuspended in Tris deoxycholate buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 mM NaCl, 0.25% deoxycholate). CheLAM and CheLM were then separated by gel filtration on a Sephacryl S-200 (Amersham Biosciences) column (80 × 10 cm) in the same buffer. The eluted samples were monitored by 13% SDS-PAGE in the same buffer for carbohydrates according to Tsai and Frash (27). The appropriate LAM and LM fractions were pooled and dialyzed for 48 h against 10 mM Tris-HCl (pH 8.0) and then for 48 h against water prior to lyophilization. The endotoxin content of all reagents was measured in a chromogenic Limulus lysate assay (BioWhittaker). The LAM preparations contained insignificant amounts of endotoxin (< 25 pg/ml).

**NMR Analysis**—Prior to NMR spectral analysis, LM (15 mg) and LAM (5 mg) were repeatedly exchanged in H2O (99.97% purity, Euriso-top, CEA Saclay, France) with intermediate freeze-drying and then dissolved in 250 μl of Me2SO-d6 (Euriso-top). Chemical shifts were expressed as parts per million downfield from the signal of the methyl group of Me2SO-d6. TMS was 2.5 ppm, 3P/TMS = 40.98 ppm at 343 K. The samples were analyzed in 200 × 5 mm BMS-005-B Shimadzu® tubes on a Bruker ASX-400 spectrometer (Centre d’Analyses RMN, Villeneuve d’Ascq (H, 400.33; 13C, 100.66, 31P, 162.5 MHz) equipped with a double resonance (1H/31P) Band Block Inverse z-gradient probe head. All NMR data were recorded without sample spinning.

For the one-dimensional homonuclear (1H-1H) spectra (COSY-ROESY-TOCSY) were measured using standard Bruker pulse programs. ROESY spectra were acquired with various mixing times (50, 100, 200, and 400 ms) and acquired in States mode according to Bax and Davis (29), whereas both COSY and relayed COSY were acquired in the magnitude calculation mode. Moreover, the two-dimensional TOCSY spectrum was recorded using a MLEV-17 mixing sequence of 120 ms. The spin lock field strength corresponded to a 90° pulse width of 35 μs. The spectral width was 4000 Hz in both dimensions. 512 spectra of 4096 data points with 32 scans per r1 increment were recorded giving a spectral resolution of 0.9 Hz/point in F2 and ~8 Hz/point in F1. Heteronuclear experiments (1H-31P and 1H-31P) were obtained with standard Bruker pulse sequences such as HMBC (inv4tp), HMQC-HOHAHA (inv4mltp), and HMBC (inv4lml). HMQC and HMQC-HOHAHA were acquired in the phase-sensitive increment time proportionate phase increment (TPPI) method, whereas HMBC was recorded in the magnitude mode calculation. All parameters (pulse widths, pulse powers, and delays) were optimized for each experiment. Acquisitions and processing signals are expressed in the figure legends.

**Gas Chromatography Techniques**—Monosaccharides were analyzed as alditol acetates. Lipoglycans were hydrolyzed in 4 N trifluoroacetic acid for 4 h at 100 °C and reduced with NaBH4 in 0.05 × NH4OH for 4 h. Reduction was stopped by dropwise addition of acetic acid until the pH reached 6, and borate salts were co-distilled by repetitive evaporation in dry methanol. Per-acetylation was performed in acetic anhydride at 100 °C for 2 h, and derivatives were analyzed in GC on a BPX70 12 m × 0.22 mm ID column (Chrompak). Lipoglycans were methylated and monolyglycans were hydrolyzed in 6 N hydrochloric acid constant boiling (Pierce), at 110 °C for 24 h, and analyzed as TMS derivatives (30) using GC on a DB-1 60 m × 0.25 mm inner diameter by on-column injection.

Linkage analyses of monosaccharides were achieved by two steps of permethylation according to Ciucanu and Kerek (31) and followed by derivatization with acetyl groups with acetic anhydride. Acylglycerols were analyzed according to Nigou et al. (26) after cleavage of the phosphodiester bond by acetylation. Briefly, 100 μl of lipoglycans were treated with 400 μl of anhydrous acetic acid/acetonic anhydride, 3.2 (v/v), at 110 °C for 12 h. Acetylated acylglycerols were extracted by cyclohexane and analyzed by GCMS on a WCOT fused silica column 30 m × 0.25 mm inner diameter column (Chrompak).

Fatty acids were analyzed from intact lipoglycans as well as from extracted acetylated acylglycerol as pyrroline derivatives. They were released by methyl esterification with 0.5 M HCl in anhydrous methanol at 80 °C for 20 h, extracted with heptane, and derivatized with 200 μl of pyrrolidinelineic acid, 9:1 (v/v), at 80 °C for 2 h. Pyrroline-derivatized fatty acids were repetitively extracted with CHCl3/H2O/LiCl (1:1:1 v/v) and analyzed by GCMS on a WCOT fused silica 30 m × 0.25 mm inner diameter column (Chrompak).

**MALDI-Time-of-Flight Mass Spectrometry**—The molecular mass of the lipoglycans was measured by matrix-assisted laser desorption ionization on a Vision 2000 time-of-flight instrument (Finngan Mat) equipped with a 337-nm UV laser. Samples were dissolved in water at a concentration of 100 pmol/μl. One μl of the solution was mixed with MALDI matrix.
and stained with silver nitrate. The gel was treated with periodic acid.

lanes 3, 2, and 1 represent purified LMs and LAMs or on crude mycobacterial lysates. For purified LM and LAM samples, the sugar content was estimated by GC, and the equivalent of 0.5 μg of mannose for each sample was analyzed by 13% SDS-PAGE. For crude lysates, mycobacterial cells were harvested, resuspended in 0.8 ml of PBS, and disrupted for 10 min with a Branson Sonifier 450. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce). Equal amounts of proteins (30 μg) were then separated by 13% SDS-PAGE and then transferred onto a Hybond-C Extra membrane (Amersham Biosciences). Membranes were then saturated with 5% bovine serum albumin in PBS, 0.1% Tween 20, and probed overnight with either ConA DIG or GNA DIG (Roche Molecular Biochemicals, dilution 1:1000). After washing, membranes were subsequently incubated with anti-DIG antibodies conjugated to alkaline phosphatase (Roche Molecular Biochemicals, 1:1000 dilution).

Cytokine Production—The human promonocytic THP-1 cell line was grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 2 × 10−5 μM β-mercaptoethanol in an atmosphere of 5% CO2 at 37°C. THP-1 cells were induced to express CD14 by treatment with 50 ng 1,25-dihydroxyvitamin D3 (Calbiochem) for 48 h. Cells were then washed twice with RPMI 1640 and cultured in 96-well plastic culture plates at a density of 2 × 105 cells/well in RPMI 1640 supplemented with 10% fetal calf serum and glutamine. The purified molecules LAMs from *M. chelonae*, *M. tuberculosis* Erdman, and *M. smegmatis* were added at a final concentration of 10 μg/ml, and triplicates were performed in order to measure cytokine release. Culture supernatants were collected after 6 or 24 h for TNF-α or IL-8 production, respectively. Specific enzyme-linked immunosorbent assays commercial kits were used according the manufacturer’s instructions. Human IL-8 and TNF-α kits were purchased from Bender Med systems Diagnostic and R & D Systems, respectively. Cytokine production was quantified with a microtiter plate reader in comparison with a standard curve generated with recombinant human cytokines.

RESULTS

Purification of CheLM and CheLAM

The experimental protocols used to extract LM and LAM from *M. chelonae* are based on successive differential and phenol extractions, leading to the recovery of nucleic acid-, protein-, and lipid-free materials. Purity of the preparation was assessed by GC/MS and SDS-PAGE. CheLAM and CheLM were finally resolved on an agarose gel S200 column. Fig. 1a represents the elution profile of each compound and shows that CheLM was present in higher amounts than CheLAM. This was confirmed by SDS-PAGE analysis of the total Triton X-114 extract (Fig. 1b). The CheLM/CheLAM ratio (w/w) was also determined by routine monosaccharide analysis and revealed that CheLM was three times as abundant as CheLAM. This result was found for several independent extractions (data not shown). It is noteworthy that the relative abundance of these two compounds largely differs from the ones reported previously for other mycobacterial species, in which LAM represents the major component. For instance, an approximate LM/LAM ratio (w/w) of 1:2.5 was found in *M. bovis* BCG (26) compared with the 3:1 ratio observed in *M. chelonae*.

Structural Analysis

Because LM is thought to be a biosynthetic precursor of LAM, we first undertook the structural elucidation of LM, and we subsequently determined the structure of the more complex LAM molecule.

Structure of CheLM—The molecular weight of CheLM was investigated by MALDI analysis. It showed a broad unresolved peak centered at 6900 Da (data not shown). Quantitative analysis of the aldito-acetate derivatives of CheLM led to an average composition of 35 mannose, 1 arabinose, and 1 myo-inositol residues. The exact quantification of TMS derivatives by GC using on-column injection demonstrated the presence of a 1.07 unit of glycerol per molecule of myo-inositol, which establishes the ratio of mannose/arabinose/inositol/glycerol as 35:1:1:1. Analysis of partially methylated aldito-acetate derivatives revealed the presence of three major components (Table I) identified on the basis of their retention times and fragmentation patterns as t-Manp, 6-Manp, and 3,6-Manp. Another product, different from a mannitol-acetate, was characterized by (M + NH4+)1+ at m/z 338, indicative of a di-acetylated, tetramethylated inositol. EI/MS fragments at m/z 200, 191, and 75 identified this compound as a 2,6-Ac2-1,3,4,5-Me4-Ins, which is in agreement with an earlier published report (16). An (M + NH4+)2+ ion at m/z 366 corresponding to a tri-acetylated, trimethylated inositol was detected as a minor component. However, because its fragmentation pattern was unclear, the detailed structure of this product was not analyzed further.

In order to establish the main features of both the polysaccharide and the putative GPI anchor of LM from *M. chelonae*, an exhaustive NMR-based study was conducted. NMR experiments were recorded successively in D2O and in Me2SO. As shown previously (26), an improved resolution was obtained in Me2SO, which was used for most experiments.

Polysaccharide Moiety—1H and 13C NMR parameters of the polysaccharide moiety from CheLM were assigned using one-dimensional 1H and 13C experiments as well as two-dimensional 1H-1H correlation experiments. Attributed 1H and 13C
NMR parameters from CheLM are summarized in Table II.

The anomeric proton region is dominated by two signals at δ 4.94 and δ 4.71 ppm (Fig. 2a). The former signal correlates in the COSY 90 experiment with a single H-2 signal at δ 3.84 ppm, whereas the other correlates with two largely distinct H-2 signals at δ 3.94 ppm and δ 3.68 ppm (Fig. 2d), suggesting the presence of two anomeric protons of distinct origins at δ 4.71 ppm. The configuration of these three spin systems was unambiguously attributed to Manp in accordance with TOCSY and NOESY experiments. Moreover, the magnitude of the 1H-1H COSY (Fig. 2b), HMQC-HOHAHA (data not shown), COSY and HMBC experiments (Fig. 2a), all the carbon parameters of these two units could be assigned. In particular, two distinct C-3 signals were identified, one at δ 72 ppm corresponding to an unsubstituted C-3, and one deshielded at δ 80.3 ppm indicative of a substituted C-3. On the other hand, a single deshielded C-6 resonance at δ 66.6 ppm, and a single slightly shielded C-5 resonance at δ 72.42 ppm could also be observed. These parameters confirm that the two mannosyl units are 6-O-substituted and 3,6-O-substituted. Altogether, these NMR data support the fact that the major 1H signal at δ 4.71 corresponds to the anomeric proton of both 6- and 3,6-substituted α-Manp residues, labeled VI and VIII, respectively.

Through 1J1H,C-C signal assignments, HMBC experiment (Fig. 2a) allowed us to define intra- and inter-residual correlations originating from the anomeric proton of t-Man (IV), 6-Man (VI), and 3,6-Man (VIII) residues. For t-Man, intra-residual correlations H-1/C-3 and H-1/C-5 were observed at δ 71.4 and δ 74.26 ppm, respectively, whereas an extra-residual 1H-7/II-VIII correlation was observed at δ 80.8 ppm. This unambiguously confirmed the attachment of t-Man residues to the C-3 of 3,6-Man residues. As observed above, the C-3 chemical shift is the only parameter significantly differing between 6-Man and 3,6-Man residues. Thus, the 1J1H,C signals starting from anomeric proton at δ 3.67 ppm were attributed as follows: the signal at δ 72 ppm was attributed to the intra-residue connectivities 1-VII/III; signals at δ 80.31 and δ 72.42 ppm to intra-residue connectivities 1-VIII/III and 1-VII/IV-III, respectively; the signal at 66.56 ppm was attributed to inter-residue correlations between the anomeric protons of VI/VIII units and C-6s of VI/VIII units.

These signals demonstrate that VI and VIII residues are connected to each other through 6-O-substitutions. In addition, an intense signal at δ 70.5 ppm was also attributed to the 1-VI/2-VI correlation. Altogether, these data deriving from methylation and NMR analyses are consistent with the occurrence of a linear backbone of 6-substituted α-Manp residues partially substituted in C-3 by single α-Manp residues.

GI Anchor—The structure of the GPI anchor of CheLM was first investigated through the use of 1H-31P HMQC and 1H-31P HMQC-HOHAHA experiments (Fig. 3). This method has been successfully applied to study the GPI anchors of LM and LAM isolated from M. bovis BCG by Nigou et al. (33). Coupled to HOHAHA experiments, they allow assigning glycerol, inositol, and mannose residues that constitute the GPI anchor.

The 31P spectrum of LM in MeSO showed only two sharp peaks at δ 1.65 and δ 1.85 ppm, indicative of the presence of two distinct GPI anchors. Integration of these signals gave us a P1/P3 ratio of 73:27. Assuming that the phosphorus atom of the GPI anchor is linked to both glycerol and myo-inositol, we used the 1H-31P HMQC experiment to assign Gro H-3 and myo-Ins H-1 (Fig. 3b). As expected, each phosphorus atom correlated with a single C-1 signal at 80.3 ppm indicative of the presence of two different sets of signals that were identified by analogy to the literature as myo-Ins H-1 (at δ 4.03 and δ 3.93 ppm, respectively) and Gro H3/H3’ (at δ 3.80 and δ 3.85 ppm, respectively). Then 1H-31P HMQC-HOHAHA experiments (Fig. 3a) permitted to assign the proton spin systems of myo-Ins and Gro linked to each phosphorus atom. The assignment of these signals was confirmed by the use of sequential multirelayed 1H-1H COSY and HOHAHA (data not shown) sequences, using a mixing time of 120 ms. It appeared that the glycerol protons derived from both phosphorus atoms presented very similar parameters: H-1 at δ 4.32–4.35 ppm; H-1’ at δ 4.09–4.11 ppm; H-2 at δ 5.07–5.09 ppm; and H3/H3’ at δ 3.80–3.85 ppm (Fig. 3d). Their most relevant feature was the presence of a very deshielded H-2 proton at δ 5.07–5.09 ppm, characteristic of a 1,2-di-acylated glycerol (34). On the other hand, the two myo-inositosil exhibited distinct 1H NMR parameters; in particular, the H-3 signals differed by about 1.4 ppm at δ 3.68 and δ 3.58 ppm. Such a deshielding is in agreement with an acylated position at C-3 occurring on one of the two myo-inositol types (Fig. 3c). Sets of parameters of Gro and myo-Ins matched perfectly with some of the previously analyzed GPI anchors (33, 35). By using the nomenclature proposed by these authors, we could typify the two phosphorus atoms at δ 1.65 ppm as P1 and at δ 1.85 ppm as P3, where P1 containing GPI is characterized by the presence of a diacylglycerol and a C-3-acylated myo-inositol, whereas P3 containing GPI is characterized by the presence of a diacylglycerol and non-acylated myo-inositol.

Four minor deshielded anomeric protons were clearly observed in the 1H spectrum at δ 5.15, δ 5.13, δ 5.19, and δ 5.11 ppm (Fig. 4). On the basis of their C-1 parameters at δ 98.7, δ 98.7, δ 100.96, and δ 101.5 ppm, respectively, as observed on the 1H-13C HMQC spectrum (Fig. 5), and their H-2 parameters at δ 3.61, δ 3.58, δ 3.79, and δ 3.78 ppm as observed on the HOHAHA spectrum (Fig. 4c), these four residues were tentatively identified as Manp residues. On the ROESY and NOESY spectra (Fig. 4, a and b), all four anomers showed intra-residue correlations with their respective H-2, which substantiate their attribution as α-Manp residues. Moreover, starting from H-1 signals at δ 5.19 ppm, NOESY and ROESY spectra showed intense NOE effect with H-2 myo-Ins P1 at δ 4.22 ppm, whereas starting from signal at δ 5.11, NOESY and ROESY showed NOE effect with H-2 myo-Ins P3 at δ 4.19 ppm. A weaker correlation signal was also observed on the NOESY spectrum between anomeric proton at δ 5.19 ppm and H-3 Ins P1 at δ 4.57 ppm. These data clearly demonstrate the glycosylation of...
This is in agreement with the 1H-31P HMQC NMR experiments analyzed. They included hexadecenoic acid (C16:1), octadecenoic acid (C18:1), and nonadecanoic acid (C19)-substituted glycerol. Partial methylation analysis of CheLM through the observation of P1- and P3-type phosphorus signals and substituted mannose residues was specifically investigated by derivatization with heptafluorobutyric anhydride (36). Acetolysis products appeared as being predominantly constituted of pyrrolidine derivatives by GC/MS. The occurrence of hydroxylated fatty acids was tentatively attributed to two peaks showing a constant ratio of 1:5. These fatty acid analysis based on acetolysis products indicated that the octadecenoic acid was present as a mixture of C16-C18:1 Gro, and C17-C18:1 Gro. Each of these compounds was tentatively attributed to two possible positions of fatty acids, but the ions resulting from the fragmentation between C-1 and C-2 of the glycerol were not intense enough to unequivocally localize the acyl group on each carbon and thus to determine the predominant form. Similarly, the remaining peaks were attributed to a very heterogeneous family of diacylglycerols, representing less than 10% of the total products. They were attributed to a very heterogeneous family of diacylglycerols, representing less than 10% of the total products. The acylation state of the glycerol moiety was subsequently studied by GC/MS by liberating the intact acylglycerol by acetolysis and analyzed as pyrrolidine derivatives by GC/MS. The occurrence of hydroxylated fatty acids was specifically investigated by derivatization with heptafluorobutyric anhydride (36). Acetolysis products appeared as being predominantly constituted of diacylglycerols. Quantification of each form showed that monoacylglycerols represented less than 1% of the total diacylglycerols analyzed. They included hexadecanoic acid (C16:0), octadecenoic acid (C18:1), and nonadecanoic acid (C19:0)-substituted glycerol. This is in agreement with the 1H-2P HMQC NMR experiments that only showed the presence of a diacylglycerol unit in CheLM through the observation of P1- and P3-type phosphorus atoms. The region of the EI total ion current chromatogram profile corresponding to the diacylated products exhibited 12 peaks that were all assigned by EI and CI/MS (Fig. 6a). The three most abundant species were assigned as 1/2-tetradecanoyl-1/2-nonadecanoyl-3-acytylglycerol, 1/2-hexadecanoyl-1/2-octadecenoyl-3-acytylglycerol, and 1/2-hexadecanoyl-1/2-nonadecanoyl-3-acytylglycerol, which represent 45, 34 and 13%, respectively, of the total acylglycerols isolated from CheLM. They were easily characterized because of the (M + NH4)+ ions at m/z 642, 654, and 670, respectively, and to the fragment ions resulting from the loss of each acyl group (m/z 327 and 397 for C14-C19 Gro, m/z 355 and 381 for C16-C18:1 Gro, and m/z 355 and 397 for C16-C19 Gro). Each of these compounds was attributed to two peaks showing a constant ratio of 1:5. These were tentatively attributed to the two possible positions of fatty acids, but the ions resulting from the fragmentation between C-1 and C-2 of the glycerol were not intense enough to unequivocally localize the acyl group on each carbon and thus to determine the predominant form. Similarly, the remaining peaks were attributed to a very heterogeneous family of diacylglycerols, representing less than 10% of the total products. They include C14-C18:1, C16-C18:1, C16-C16, C16-C17:1, C15-C19, C16-C18:1, C16-C19:1, and C17-C18:1 di-substituted glycerols. Further fatty acid analysis based on acetolysis products indicated that the octadecanoic acid was present as a mixture of C16:0 and C18:0 and allowed the identification of nonadecanonic acid as tuberculostearic acid (10-methyloctadecanoic acid).

**Structure of CheLAM**—The structure of CheLAM was elucidated by the same experimental protocols used for CheLM. Analysis of CheLAM by MALDI showed a broad peak centered on m/z 17,000 Da and ranging from 9000 to 23,000 Da. It is noteworthy that in identical experimental conditions CheLM gives a much more intense response in MALDI analysis than CheLAM. Composition analysis of this compound showed a mannose/arabinose/inositol/glycerol ratio of 31:80:1:1. Analysis of partially methylated and acetylated monosaccharides residues indicated the presence of t-Man, 6-Man, 3,5-Ara, 2-Ara, and 3,5-Ara (Table I). These experiments led to a first insight into the nature of the capping of LAM isolated from *M. chelonae*. Indeed, both CheLM and CheLAM showed a sim-
The presence of a single myo-inositol residue per molecule of LAM suggests that no other myo-inositol residue than the one incorporated in the GPI anchor is present in the molecule, and consequently that arabinan chains are not terminated by phospho-
myo-inositol groups as observed in fast growing Mycobacterium sp. (19) including M. smegmatis (38). Both observations were confirmed by multiple NMR experiments, as shown below.

Attributed $^1$H and $^{13}$C NMR parameters from CheLAM are summarized in Table III. Comparison of the anomeric region of $^1$H-$^{13}$C HMQC spectra from CheLM and CheLAM (Fig. 5) allowed us to identify signals from the mannose core of CheLAM. On this basis, the intense $^{13}$C resonance at $\delta$ 103.1 ppm was tentatively identified as the t-Manp anomeric carbon (IV), whereas the $^1$H resonance at $\delta$ 100.55 was identified as the 6-α-Manp and 3,6-α-Manp anomeric carbons (VI and VIII). Two-dimensional $^1$H-$^{13}$C homonuclear experiment confirmed that the $^1$H/$^{13}$C signal at $\delta$ 4.92/103.1 ppm correlated with a single spin system characteristic of a t-Manp residue, whereas the $^1$H/$^{13}$C signal at $\delta$ 4.70/100.55 ppm correlated with two distinct spin systems characteristic of 6-Manp and 3,6-Manp units (data not shown). Assignment of their respective $^{13}$C resonances owing to $^{13}$C-$^1$H heteronuclear experiment confirmed this observation. Furthermore, as observed in CheLM, an HMBC experiment showed inter-residual correlations between IV H-1 and VI C-3, VI H-1 and VII-VIII C-6, and between VIII H-1 and VI/VIII C-6 (data not shown). These data confirm that the mannose cores of CheLM and CheLAM are similar.

Fig. 5 shows that four minor signals previously assigned to anomeric carbons of α-Man-1P1 and P3 and α-Man-2P1 and P3 in CheLM were also clearly observed in the CheLAM spectrum. The remaining anomeric signals of the spectrum were attributed by comparison with previous spectral NMR data of LAM from M. tuberculosis (32) and M. smegmatis (38). Spin systems deriving from each anomere was identified owing to $^1$H-$^1$H homonuclear and $^1$H-$^{13}$C-$^1$H heteronuclear experiments. This way the remaining signals in the anomeric region of the spectra were all attributed to the anomeric signals of the arabinan core of CheLAM, in agreement with the methylation analysis: 3,5-α-Araf (I), 5-α-Araf (VI to VIII), 2-α-Araf (III1 to III4), and t-β-Araf (V1 and V2). No additional mannose residues could be identified. Most types of Ara units showed multiple anomeric signals presenting similar spin systems. This multiplicity of signals was previously attributed to the different positions that each unit may take within the arabinan core (14). HMBC spectrum showed a complex pattern of $^1$H/$^{13}$C correlations that was entirely resolved, except for the region between $\delta$ 4.95 and 5 ppm where all intra-residue connectivities from III1 to III4, and III1 to III2, and II1 to II2 could not be unambiguously attributed because of the very close chemical shift of their anomeric protons (not shown). Nevertheless, this shed some light onto the general sequence of the arabinan core. In particular all the anomeric protons of 3,5-α-Araf residues (I) and 5-α-Araf residues (VI to VIII) showed intense $^1$H/$^{13}$C correlation signals with a substituted C-5 at $\delta$ 6.79 ppm, unambiguously attributed as inter-residue connectivities with C-5 I and C-5 II. Similarly, both H-1 of t-β-Araf residues (V1 and V2) showed inter-residue connectivities with a very deshielded carbon at $\delta$ 88.29 ppm attributed to the C-2 of 2-α-Araf, confirming the attachment of all t-β-Araf at C-2 of 2-α-Araf residues. On the other hand, it is noteworthy that no other connectivity with the C-2 of 2-α-Araf residues could be observed suggesting that no arabinose residues other than t-β-Araf residues are linked to 2-α-Araf units. HMBC experiment also enables us to distinguish III1 to III4 residues from III1 to III2 and III1 to III3 based on their respective $^1$H/$^{13}$C connectivities. Indeed, whereas H-1 III1/III2 showed an intense inter-residue correlation with C-5 I/IIa at $\delta$ 67.9 ppm, H-1 III3 did...
not show any correlation at δ 67.9 ppm but an intense and broad signal around δ 83.5 ppm attributed to C-4-III, and to C-3-I. These data establish that III and III, residues are linked to C-5 of 3,5- or 5-α-Araf residues, whereas III, is linked to C-3 of 3,5-α-Araf. Therefore, NMR data arising from the arabinan chain of CheLAM show very little discrepancy with published NMR data of LAM from M. tuberculosis (32) and M. smegmatis (38), strongly suggesting that a similar arabinan core is shared by the three mycobacterial species. On the other hand, methylation and composition analyses have previously suggested that M. chelonae differs in its capping status from M. tuberculosis and M. smegmatis. Indeed, as already mentioned, homonuclear 1H-1H and heteronuclear 1H-13C NMR experiments showed no evidence of the presence of other mannose residues than the one found in the mannan core, suggesting the absence of oligomannosyl caps on the arabinan side chains as observed in M. tuberculosis and M. bovis. Furthermore, it was observed on the HMBC spectra

Fig. 3. NMR analysis of LM phosphate substituents in Me$_2$SO-d$_6$ at 343 K. a and b, expanded regions (δ$^1$H, 5.20 to 3.00, and δ$^{31}$P, 2.40 to 1.20) of the heteronuclear $^{31}$P-decoupled, $^1$H-detected HMQC-HOHAHA and HMQC spectra, respectively. HMQC-HOHAHA was recorded using MLEV-17 mixing sequence of 120 ms. The data matrix for (a and b) experiments were 2048 × 64 time proportional phase increment (TPPI) points with 256 scans for (a) experiment or 64 scans for (b) experiment per $t_1$ value. In both cases, the spectral window was 2592 Hz in F1 ($^3$P) dimension and 4003 Hz in F2 ($^1$H). The original data matrix were expanded to 4096 × 512 real matrix moreover for processing a sine-bell window shifted by 4003 Hz in F2 ($^1$H). The original data matrix were expanded to 4096 × 512 (states) points with 32 scans per $t_1$ increment and was expanded to 4096 × 1024.
that no additional $J_{\text{H},\text{C}}$ correlation occurred between any anomeric protons of mannose residues (IV, V, and VIII) and any carbon of arabinose residues. The possibility of the occurrence of phospho-myoinositol type capping motifs is discussed below.

In order to study the GPI anchor of CheLAM isolated from \textit{M. chelonae}, we used an experimental approach used above for CheLM. The $^{31}$P spectrum of CheLAM showed two sharp peaks at $\delta$ 1.65 and $\delta$ 1.85 ppm. Similarly, $^1$H-$^{31}$P HMQC-HOHAHA experiments established that glycerol and myo-inositol residues substituting both phosphorus types showed $^1$H-NMR parameters identical to those of CheLM. Thus, it was concluded

---

**FIG. 4.** Part of NMR spectra from CheLM analysis in Me$_2$SO-$d_6$ at 343 K. \(a\) and \(b\), expanded regions ($^1$H, 4.70–3.00 and 5.23–4.8 ppm) of 400-ms ROESY spectrum and 300-ms NOESY spectrum, respectively. \(c\) and \(d\), expanded regions ($^1$H, 4.70–3.00 and 5.23–5.07, and $^1$H, 4.70–3.00 and 3.24–3.02) of 120-ms TOCSY spectrum. \textit{Man-1} corresponds to O-6 linked mannosyl and \textit{Man-2} to O-2 linked mannosyl on inositol unit.
that GPI of CheLAM and CheLM shared identical acylation states, characterized by a mixture of P1 and P3 phosphates, in similar ratios. Detailed analysis of the acetolysis products of CheLAM by GC/MS showed again that diacylglycerols of CheLM and CheLAM present the same heterogeneity, reinforcing the hypothesis that CheLM and CheLAM share strictly identical GPI anchors.

It is noteworthy that the $^1$H-31P HMQC spectrum did not reveal any other type of phosphorus than those attributed to the GPI anchors of the lipopolysaccharide. In particular, no evidence for the presence of an additional phospho-myoinositol group substituting the arabinan side chains was found, as observed in LAM from $M$. $^{smegmatis}$ (38). Altogether, with the results of the composition analysis showing a myo-inositol/glycerol ratio of 1:1, the NMR data confirmed that CheLAM does not possess a phospho-myoinositol capping motif.

Mapping with Lectins

Occurrence of a new type of mannan core in lipoglycans isolated from $M$. $^{chelonae}$ was investigated by screening purified LM and LAM from various mycobacterial species with several mannose-recognizing lectins. The most relevant results were obtained with lectins isolated from Canavalia ensiformis (ConA) and from Galanthus nivalis (GNA). Although ConA is relatively unspecific toward mannose-containing glycoconjugates, GNA is known to be highly specific for non-reducing terminal $\alpha$-1,3-linked mannose residues (39). As expected, ConA interacted with purified LM from $M$. $^{chelonae}$, $M$. $^{bovis}$ BCG, and $M$. $^{smegmatis}$ (Fig. 7a). This suggests that ConA does not differentiate mannan cores branched at C-2 ($M$. $^{bovis}$ BCG and $M$. $^{smegmatis}$) from mannan cores branched at C-3 ($M$. $^{chelonae}$). Surprisingly, this lectin only recognized purified LAM from $M$. $^{tuberculosis}$ (Fig. 7a), but not LAM from $M$. $^{chelonae}$ or LAM from $M$. $^{smegmatis}$, although LM and LAM share common mannan cores. The absence of reactivity between ConA and LAM from $M$. $^{chelonae}$ or $M$. $^{smegmatis}$ may be explained by steric hindrance of the mannan core by the arabinan polymer. Specific interaction observed between ConA and LAM from $M$. $^{tuberculosis}$ would originate from the presence of an oligomannosyl-type capping only in this species. These results are in agreement with findings from Prinzis et al. (40) who reported that ConA recognizes purified LAM from $M$. $^{bovis}$ BCG and from $M$. $^{tuberculosis}$ Erdman but not from a rapidly growing strain.

GNA exhibited a much more restricted specificity than ConA. As observed in Fig. 7b, GNA recognizes exclusively CheLM. Considering the described specificity of GNA toward a Man($\alpha$-1–3) determinant, this result is in total agreement with direct structural studies in this report that distinguish the mannan core of CheLM from those of other mycobacterial species on the basis of their respective branching positions. As observed for ConA, GNA did not bind to CheLAM, again suggesting that arabinan masks the $\alpha$-1,3-Manp side chains of the mannan core.

Given the specificity of GNA for the Man($\alpha$-1,3)-substituted mannan core of CheLM, we addressed the question whether this lectin may be particularly useful to rapidly detect the presence of similar structures in various other mycobacterial species, including rapid, slow growing, pathogenic, and non-pathogenic species. Crude lysates of $M$. $^{bovis}$ BCG, $M$. $^{smegmatis}$, $M$. $^{chelonae}$, Mycobacterium sorofulaceum, Mycobacterium thermoresistibile, Mycobacterium fortuitum,
Mycobacterium xenopi, Mycobacterium gastri, and Mycobacterium malmoense were transferred to a membrane, incubated with GNA-DIG, and revealed with anti-DIG antibodies. Western blot analysis shows that Man(1,3)-substituted mannan core was only present in M. chelonae (Fig. 7c). Interestingly, GNA did not interact with LM from M. fortuitum, known to be a M. chelonae related species. This indicates that GNA constitutes a useful tool for a rapid screening of LM and LAM structures with 1,3-linked mannose residues without the need of a purification step. It also suggests that among the nine mycobacterial strains analyzed, the structure of M. chelonae appears to be unique.

Fig. 8 summarizes all the data collected from the structural analysis of CheLAM and highlights the main structural features and differences between CheLAM and LAMs from the already studied species M. tuberculosis/M. bovis and M. smegmatis.

**TNF-α and IL-8 Secretion by Various LAM Preparations**

To investigate the consequences of the structural differences established between CheLAM, ManLAM, and AraLAM, we compared TNF-α and IL-8 secretion by differentiated human macrophages stimulated with LAMs isolated from M. tuberculosis, M. smegmatis, and M. chelonae. As shown in Fig. 9, incubation THP-1 cells with LAM isolated from M. smegmatis resulted in a sharp increase of both TNF-α and IL-8 secretion. In contrast, LAMs from M. chelonae and M. tuberculosis were not able to induce cytokine production.
Previous studies (38, 41, 42) have been reported that mannose-capped LAM from \textit{M. tuberculosis} was inactive toward TNF-α and IL-8 secretion, whereas phosphoinositol-capped LAMs from a fast growing \textit{Mycobacterium} species and from \textit{M. smegmatis} were strong cytokine inducers. Considering the fact that CheLAM is neither capped by oligomannosyl nor by phosphoinositol groups, our results strongly suggest that TNF-α and IL-8 secretion by LAM was very likely modulated by the presence of the phosphoinositol-type capping. This also suggests that the mannose type capping does not influence cytokine induction.

\section*{DISCUSSION}

LM and LAM are two polysaccharides of the mycobacterial cell wall that display strong antigenicity and that are widely distributed in \textit{Mycobacterium} sp. LAM has been shown to participate in the virulence and immunopathogenesis of tuberculosis (43). Extensive studies performed on LAM from \textit{M. bovis} BCG or \textit{M. tuberculosis} have demonstrated that important biological effects are linked to the degree and chemical nature of capping functions and other substituents on the arabinan portion of LAM. Arabinans of LAMs from \textit{M. bovis} BCG, \textit{M. tuberculosis}, \textit{M. leprae}, and \textit{M. avium} (44) are mannose-capped (ManLAM) to varying degrees. Studies based on a fast growing \textit{Mycobacterium} sp. and on \textit{M. smegmatis} demonstrated that the arabinan termini are uncapped (AraLAM), whereas a minor portion terminates with inositol phosphate (19, 38, 40).

So far, LAM structures from only a restricted panel of mycobacterial species have been determined. The relatively low structural diversity encountered in these molecules may limit the possibilities of studying relationships between structure and biological activities. The aim of the present work was to decipher new sources of structural variability in LM and LAM in order to generate new models for such studies. We therefore used \textit{M. chelonae} as a model. \textit{M. chelonae} is a nontuberculous mycobacterium that is usually encountered in water sources.
and soil. Although it has originally been proposed to be Friedmann’s turtle tubercle bacillus (45), it sometimes causes disease in humans. *M. chelonae* presents also the advantage of being a rapidly growing species. Furthermore, we have recently shown that it can be transformed, thus making it attractive for future genetic studies (24). Considering the intrinsic polydispersity of mycobacterial lipoglycans, structural studies were undertaken, consisting of an independent comparison of each structural feature of these molecules with their homologs that have been studied in other species.

The first observation was that LAM from *M. chelonae* shares a similar overall structure with other LAMs, composed of an arabinan chain and a mannan chain linked to a GPI anchor. However, it shows unique features that unequivocally distinguishes it from other LAMs. Significant differences observed in *M. chelonae* are illustrated in Fig. 8. Considering the growing inter-specific heterogeneity observed in LAMs and, as proposed by Khoo et al. (19), we referred to lipoglycans isolated from *M. chelonae* CheLM and CheLAM, according to their origin, rather than to their structural features.

In this study, we demonstrate for the first time that a virulent mycobacterium species exhibits a LAM structure that is devoid of mannose and inositol phosphate capping motifs (Fig. 8). Therefore, both CheLM and CheLAM represent suitable molecules to use along with ManLAMs or AraLAMs to evaluate the role of mannose and inositol phosphate capping in relation to various biological functions attributed to LAM. As an example, it has been observed for a long time that LAMs extracted from avirulent mycobacterial species were more potent inducers of TNF-α than those extracted from virulent species (46). A correlation was rapidly made between the absence of an oligomannosyl capping and the potency to trigger TNF-α release. It was also reported that the activity of LAM from the avirulent strain *M. smegmatis* was diminished after alkali treatment (38), suggesting the importance of alkali-labile phosphoinositol groups in eliciting cytokine production. The present study confirms this observation by showing that a LAM naturally lacking mannose and phosphoinositol caps does not induce cytokine secretion.

In addition, our results demonstrate that both CheLM and CheLAM possess an unusual mannan core characterized by the presence of α1,3-Manp side chains, rather than the α1,2-Manp substitution as found in all mycobacterial LAM structures reported so far (Fig. 8). Analysis by Western blotting using GNA, an α1,3-Manp-specific lectin, revealed that α1,3-Manp side chains containing LM were only present in *M. chelonae* but not in other mycobacterial species. Whether these substitutions are important for biological activities of CheLM or CheLAM
remains to be investigated. Another substantial difference arising from the present study concerns the LM/LAM ratio. It is generally assumed that LM is a precursor of LAM and is usually present in smaller amounts than LAM (26). We observed that *M. chelonae* contains a LM/LAM ratio of 3:1 (w/w), corresponding to 10 times more molecules of CheLM than CheLAM. The presence and the possible physiological impact of larger amounts of CheLM than CheLAM in *M. chelonae* is presently unknown. This difference in the CheLM/CheLAM balance may arise from a deficiency in arabinosyltransferase(s) expression in *M. chelonae* compared with *M. tuberculosis* and *M. bovis* BCG. On the other hand, arabinosyltransferase(s) involved in arabinan polymerization may be less active in *M. chelonae* than in *M. tuberculosis*. At present, this remains very difficult to investigate because no arabinosyltransferases involved in LAM biosynthesis have been clearly identified.

Another unexpected difference between CheLAM and ManLAMs concerns the lipid part of the PI anchors. Nigou et al. (26) have demonstrated that cellular ManLAM anchors from *M. bovis* BCG mainly contain 1- or 2-palmitoylglycerol, 1- or 2-tuberculostearoylglycerol, 1,2-dipalmitoylglycerol, and 1-tuberculostearoyl-2-palmitoylglycerol. Surprisingly, the same authors found that the PI anchor of parietal ManLAM from *M. bovis* BCG is composed of a single type of acylglycerol consisting of 1-[(2-O-(methoxypropanoyl))-12-hydroxystearoyl]-glycerol. In addition, parietal ManLAM was found to be a better inducer of TNF-α and IL-8 by human dendritic cells than cellular LAM, thus suggesting that the unusual 1-[(2-O-(methoxypropanoyl))-12-hydroxystearoyl]-glycerol part is the major cytokine-regulating component of the ManLAMs (26). Moreover, the fact that biological activities of ManLAMs are abrogated after deacylation and that ManAMs devoid of the PI anchor are unable to stimulate cytokine production are consistent with the assumption that the lipid part of the PI anchor is essential for the immunological properties of the ManLAMs. The present study shows that the lipid part of the PI anchor of CheLAM is fundamentally different from that of previously described PI anchors because it presents a very large heterogeneity, due to a combination of fatty acids ranging from myristic acid to tuberculostearic acid with eventual unsaturations (Fig. 8). This heterogeneity generates a large pool of molecules with various PI anchors, which may influence the biological activities of the whole molecule. Whether this heterogeneity is due to numerous acylglycerol acyltransferases with different specificities in *M. chelonae* remains an open question. It is also noteworthy that CheLM and CheLAM show perfectly identical mixtures of diacylglycerols. This observation substantiates the hypothesis that LM is a biosynthetic precursor of LAM.

Mycobacteria represent a major source of microbial antigens that are presented by CD1 molecules. Most of the lipids that can be presented by CD1 molecules have two hydrophobic tails and a polar head group. This led to the suggestion that each hydrophobic tail fits into a pocket of the CD1 groove (47). Binding studies also revealed that the lipid portion of the
antigen is required for CD1 binding (48, 49). Sieling et al. (50) have shown that ManLAMs from *M. tuberculosis* Erdman and *M. leprae* can be presented in the context of CD1 molecules and are able to stimulate CD4/CD8 double negative T cells. How different CD1 molecules bind, in particular LM/LAM, is not known. Available data suggest that human CD1d and CD1d molecules require a minimal hydrocarbon chain length but that they are not as selective with regard to the type of lipid they will bind (48, 49). Therefore, it would be interesting to study the PI anchors of CheLM or CheLAM in relation to CD1 binding for presentation of these glycolipids to T cells. Given their unusual structures, CheLM and CheLAM constitute new tools to compare the biological activities of LMs and LAMs such as for antigen presentation via CD1 molecules, cell adhesion, or T cell activation and should help to clarify their roles in mycobacterial virulence.

REFERENCES

1. Daffe, M. & Draper, P. (1998) *Adv. Microb. Physiol.* 39, 131–203
2. Moreno, C., Mehlert, A. & Lamb, J. (1988) *Clin. Exp. Immunol.* 74, 206–210
3. Sibily, L. D., Hunter, S. W., Brennan, P. J. & Krabbenhöhl, J. L. (1998) *Infect. Immun.* 66, 1232–1236
4. Sibily, L. D., Adams, L. B. & Krabbenhöhl, J. L. (1990) *Clin. Exp. Immunol.* 80, 141–148
5. Chan, J., Fan, X. D., Hunter, S. W., Brennan, P. J. & Bloom, B. R. (1991) *Infect. Immun.* 59, 1755–1761
6. Schleisinger, L. S., Hult, S. R. & Kaufman, T. M. (1994) *J. Immunol.* 152, 4070–4079
7. Apostolou, I., Takahama, Y., Belmant, C., Kawano, T., Huerre, M., Marchal, G., Cui, J., Taniguchi, M., Nakasuhi, H., Fournie, J. J., Kourilsky, P. & Gachelin G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 5141–5146
8. Gilleron, M., Ronet, C., Mempel, M., Monsarrat, B., Gachelin, G. & Puzo, G. (2001) *J. Biol. Chem.* 276, 34896–34904
9. Khoo, K. H., Dell, A., Morris, H. R., Brennan, P. J. & Chatterjee, D. (1995) *Glycobiology* 5, 117–127
10. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J. & Brennan, P. J. (1997) *J. Biol. Chem.* 272, 18460–18466
11. Schaeffer, M. L., Khoo, K. H., Besra, G. S., Chatterjee, D., Brennan, P. J., Belisle, J. T. & Inamine, J. M. (1999) *J. Biol. Chem.* 274, 31625–31631
12. Kremer, L., Gurcha, S. S., Bifani, P., Hitchen, P. G., Bauld, A., Morris, H. R., Dell, A., Brennan, P. J. & Besra G. S. (2002) *Biochim. Biophys. Acta* 1563, 437–447
13. Hunter, S. W. & Brennan, P. J. (1990) *J. Biol. Chem.* 265, 9272–9279
14. Venisse, A., Berjeaud, J. M., Chaurand, P., Gilleron, M. & Puzo, G. (1993) *J. Biol. Chem.* 268, 12401–12411
15. Chatterjee, D., Lowell, K., Riveire, B., McNeil, M. R. & Brennan, P. J. (1992) *J. Biol. Chem.* 267, 6234–6239
16. Chatterjee, D., Hunter S. W., McNeil, M. & Brennan, P. J. (1992) *J. Biol. Chem.* 267, 6228–6233
17. Chatterjee, D., Bozig, C. M., McNeil, M. & Brennan, P. J. (1991) *J. Biol. Chem.* 266, 9652–9660
18. Chatterjee, D., Khoo, K. H., McNeil, M., Dell, A., Morris, H. R. & Brennan, P. J. (1993) *Glycobiology* 3, 497–506
19. Khoo, K. H., Dell, A., Morris, H. R., Brennan, P. J. & Chatterjee, D. (1995) *J. Biol. Chem.* 270, 12280–12289
20. Roach, T. I., Barton, C. H., Chatterjee, D. & Blackwell, J. M. (1993) *J. Immunol.* 150, 1886–1896
21. Wolinsky, E. (1979) *Am. Rev. Respir. Dis.* 119, 107–159
22. Wallace, R. J., Jr., Brown, B. A. & Onyi, G. O. (1992) *J. Infect. Dis.* 166, 405–412
23. Ingram, C. W., Tanner, D. C., Dursak, D. T., Kernodle, G. W., Jr. & Corey, G. B. (1985) *Clin. Infect. Dis.* 1, 463–471
24. Kremer, L., Dever, L., Carrere, S., Nampoothiri, M., Lesjean, S., Brown, A., Brennan, P. J., Minnikin, D. E., Locht, C. & Besra, G. S. (2002) *Biochem. J.* 364, 423–430
25. Minnikin, D. E., Minnikin, S. M., Goodfellow, M. & Stanford, J. R. (1982) *J. Gen. Microbiol.* 128, 817–822
26. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J. & Brennan, P. J. (1999) *Biochem. J.* 349, 1–6
27. Tsai, C. S. & Prusach, C. R. (1982) *Anal. Biochem.* 119, 115–119
28. Shaka A. J., Barker P. B. & Freeman R. (1985) *J. Mag. Reson.* 64, 547–552
29. Bax, A. D. & Davis, J. (1985) *J. Magn. Reson.* 63, 207–213
30. Kamerling, J. P., Gerwig, G. J., Vliegenthart, J. F. & Clamp, J. R. (1975) *Biochim. Biophys. Acta* 39, 1–10
31. Ciucanu, I. & Kerek, P. (1984) *Carbohydr. Res.* 131, 209–217
32. Gilleron, M., Ronet, C., Mempel, M., Monsarrat, B., Gachelin, G. & Puzo, G. (2002) *Biochim. Biophys. Acta* 151, 491–495
33. Chatterjee, D., Roberts, A. D., Lowell, K., Brennan, P. J. & Orme, I. M. (1992) *J. Gen. Microbiol.* 138, 340–349
34. Zhang, T., Bruser, M., Cohen, H., Bodkin, M., Law, K., Reinhart, J. & Rom, W. N. (1995) *J. Clin. Invest.* 95, 586–592
35. Chatterjee, D. & Khoo, K. H. (1998) *Glycobiology* 8, 113–120
36. Khoo, K. H., Tang, J. B. & Chatterjee, D. (2001) *J. Biol. Chem.* 276, 3868–3871
37. Initials of the authors are given in reverse order and are followed by the original year of publication.
Structural Study of Lipomannan and Lipoarabinomannan from *Mycobacterium chelonae* : PRESENCE OF UNUSUAL COMPONENTS WITH α 1,3-MANNOPYRANOSE SIDE CHAINS

Yann Guérardel, Emmanuel Maes, Elisabeth Elass, Yves Leroy, Philippe Timmerman, Gurdyal S. Besra, Camille Locht, Gérard Strecker and Laurent Kremer

*J. Biol. Chem. 2002, 277:30635-30648.*
doi: 10.1074/jbc.M204398200 originally published online June 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204398200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 26 of which can be accessed free at http://www.jbc.org/content/277/34/30635.full.html#ref-list-1