Acylation State of the Phosphatidylinositol Mannosides from Mycobacterium bovis Bacillus Calmette Guérin and Ability to Induce Granuloma and Recruit Natural Killer T Cells*

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Previous studies have found that, when injected into mice, glycolipidic fractions of mycobacterial cell walls containing phosphatidylinositol mannosides (PIM) induced a granuloma and recruitment of Natural Killer T cells in the lesions. The dimannoside (PIM2) and the hexamannoside (PIM6) PIM were isolated from Mycobacterium bovis bacillus Calmette Guérin and shown to act alike, but the activity was found to be dependent on the presence of the lipidic part. The chemical structure of PIM was then re-evaluated, focusing on the characterization of their lipidic part defining mono- to tetra-acylated PIM. The structure of these acyl forms was elucidated using a sophisticated combination of chemical degradations and analytical tools including electrospray ionization/mass spectrometry, electrospray ionization/mass spectrometry/mass spectrometry, and twodimensional NMR. Finally, the acyl forms were purified by hydrophobic interaction chromatography and tested for their capacity to induce the granuloma and Natural Killer T cell recruitment. We found that there is an absolute requirement for the molecules to possess at least one fatty acyl chain, but the number, location, and size of the acyl chains was without effect. Moreover, increasing the complexity of the carbohydrate moiety did not lead to significant differences in the biological responses.

Phosphatidyl-myoinositol mannosides (PIM)1,2 constitute a group of phospholipids found in the cell wall and the cytoplasmic membrane of mycobacteria along with cardiolipid, phosphatidylethanolamine, and phosphatidylinositol. Known from the 1940s and fully described by Ballou and colleagues (1) in the 1960s, they were shown to consist of phosphatidyl-myoinositol di-, tri-, tetra-, and pentamannosides (PIM2 to PIM5). These authors showed unequivocally that in PIM6 the mannosyl units were glycosidically attached at positions 2 and 6 of the myo-Ins ring (2) and that for the more glycosylated forms, chain elongation occurred on the mannosyl present at position 6 (3). Recently, PIM from Mycobacterium smegmatis were reanalyzed in their deacylated form (4). They were shown to have a structure based on that defined by Ballou et al. (1) but containing only six mannosyl residues (PIM4). The structure of the glycosidic part of PIM6 was unambiguously established and corresponds to Man-α1→2-Man-α1→6-Man-α1→4Glc-α1→6mannosyl residue present at position 2. This structure was confirmed by an NMR spectroscopy strategy applied to deacylated PIM4 (5).

Concerning the lipidic part of the PIM, the situation is still unclear, although the presence of multiacylated forms of PIM was already reported in 1960s by several authors (6–9) working on different mycobacterial strains. More recently, in a chromatographic and fatty acid quantitation study of mycobacterial lipoglycans, Leopold and Fisher (10) also inferred the existence of multiacylated forms of PIM. However, the sites of attachment of the acyl groups were not established. Re-evaluating the multiacylated nature of Mycobacterium tuberculosis and Mycobacterium leprae PIM using FAB-MS analysis, Brennan’s group (11) presented evidence for tri-acylated PIM, assuming an extra fatty acyl group on the C-6 of one of the Manp residue beside the fatty acids already localized on the C-1 and C-2 of the glycerol. Our data derived from an NMR study of a tetra-acylated form of PIM2 (Ac4PIM2) purified from Mycobacterium bovis BCG clearly inferred the previously described positions and demonstrated the fourth acylation site as the C-3 of the myo-Ins (12).

Recently, PIM received a renewed interest due to the fact that PIM2 constitute the anchor motif of two important constituents of the mycobacterial cell walls, namely lipomannans (LM) and lipoarabinomannans (LAM). LAM, ubiquitous in the Mycobacterium genus, exhibit a wide spectrum of immunoregulatory effects (for reviews see Refs. 13–15). It is now clearly established that most of these effects are abolished by alkaline hydrolysis, highlighting the importance of the lipidic part of the anchor. Moreover, PIM6 as well as ManLAM from M. leprae and M. tuberculosis Erdman strain are recognized by human CD4+ CD8+ αβ T cells in the context of a presentation by CD1b-expressing antigen-presenting cells (16). The high affin-
ity interaction of CD1b molecules with the PIM acyl side chains was then established (17). The phosphatidylinositol moiety plays a central role in the process of PIM and ManLAM binding to CD1b proteins. In the mouse, CD1a, -b, and -c genes are missing. The murine CD1d gene is homologous to the CD1d gene in man (18). This CD1d molecule restricts CD4+ or DN T cells (NK T cells) using a distinctive TCR characterized by an invariant α chain (19–21). The crystal structure of the mouse CD1d molecule showed a hydrophobic binding site (22), and cellular glycosylphosphatidylinositol was proposed as a major natural ligand (23). Due to the homology between CD1a, -b, -c, and -d molecules, it has been hypothesized that mouse CD1d may bind mycobacterial glycolipids. In this respect, a recent study reported that granuloma formation in response to a crude fraction containing PIM from H37Rv M. tuberculosis was dependent upon CD1d-restricted T cells (20). However, no reports have mentioned PIM presentation by CD1d molecules, and the respective roles of the lipidic and carbohydrate moieties in the PIM-induced response was not established.

In this study, the chemical structure of PIM isolated from M. bovis BCG was evaluated, focusing on the characterization of the lipidic part of the PIM, thus defining the notion of “acyl forms.” The structure of these acyl forms was elucidated using sophisticated analytical tools such as ESI-MS, ESI-MS/MS, and two-dimensional NMR. Finally, these acyl forms were purified and used in a bioassay aimed at determining the extent of inflammation they caused and the recruitment of NK T cells into the granulomatous lesions they induced.

**EXPERIMENTAL PROCEDURES**

Mice—C57BL/6 mice were 6–8-week-old and obtained from IFFA-Credo (L’Arbresle, France). Mice were injected subcutaneously with 50 μg of each molecule and adsorbed onto 12.5 μl of a neutral carrier (Alu-Gel-S, Serva) in a total volume of 0.1 ml. The cellular infiltrate at the site of the injection was excised 7 days after the injection and either fixed for histological analysis or used for mRNA characterization.

**RNA Extraction and cDNA Synthesis**—Total RNA was isolated by using the Trizol technique (Life Technologies, Inc.). cDNA was synthesized from 10 μg of total RNA, using a dT20 primer, 25 units of RNasin (Promega Corp., Madison, WI), and 10 units of avian myeloblastosis virus-reverse transcriptase (Roche Molecular Biochemicals) in the provided buffer.

**mRNA Quantification, PCR Procedure, and Immunoscope Analysis**—HPGRT-standardized amounts of cDNA solution were PCR-amplified using Vα14- and Co-specific primers (20). PCR products were subjected to primer extension using an internal, fluorescently labeled Co-specific primer (Alu-Gel-S, Serva) in a total volume of 0.1 ml. The cellular infiltrate at the site of the injection was excised 7 days after the injection and either fixed for histological analysis or used for mRNA characterization.

**DNA Extraction and cDNA Synthesis**—Total DNA was isolated by using the Trizol technique (Life Technologies, Inc.). cDNA was synthesized from 10 μg of total DNA, using a dT20 primer, 25 units of RNasin (Promega Corp., Madison, WI), and 10 units of avian myeloblastosis virus-reverse transcriptase (Roche Molecular Biochemicals) in the provided buffer.

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**Experimental Mouse Procedures**—The mice were handled in accordance with the European Community guidelines for the care and use of laboratory animals. All procedures were approved by the University of California Laboratory Animal Care Committee.

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relation spectra (HMQC) were obtained according Bax’s pulse sequence (27). The GARP sequence (28) at the carbon or phosphorus frequency was used as a composite pulse decoupling during acquisition. Multiple-bond \(^{1}H^{13}C\) correlation spectra (HMBC) (29) were processed in the magnitude mode. The pulse sequence used for \(^{1}H\)-detected heteronuclear relayed spectra (HMQC-HOHAHA) was that of Lerner and Bax (30).

RESULTS

Isolation of PIM Acyl Forms Inducing Granuloma—From previous data (31), phosphatidyl-myoinositol mannosides (PIM) are known to be found in the acetone-insoluble fraction of the chloroform/methanol (1:1, \(v/v\)) mycobacterial extract. The contaminating neutral compounds were eliminated by QMA anion exchange chromatography, irrigated with neutral eluents (chloroform, followed by chloroform/methanol, 1:1, \(v/v\) and then by methanol). Then, phospholipids were eluted with ammonium acetate-containing organic solvents resulting in three fractions, namely A-C. Each fraction was adsorbed onto alum and injected subcutaneously to C57BL/6 mice. Seven days after the injection, the cell infiltrates were excised, sectioned, and stained. The three fractions generated a highly organized and large granuloma with a central core of neutrophils surrounded by a densely organized rim of macrophages, lymphocytes, and fibroblasts (Fig. 1, \(E\) and \(F\)), mimicking the granuloma observed during murine tuberculosis. By contrast, a minor inflammatory reaction was evidenced in PBS-injected (control mice) (Fig. 1, \(A\) and \(B\)) or deacylated PIM-injected mice (Fig. 1, \(C\) and \(D\)). In this case, the lesions were found to predominantly contain a disordered array of macrophages and neutrophils with few lymphocytes. This points to the absolute requirement for the lipidic moiety of PIM to cause a granulomatous lesion.

In order to characterize the PIM composition of the different fractions, the fractions A–C were analyzed by ESI-MS in negative mode. The attribution of the deprotonated molecular ions (M – H\(^{-}\)) (Fig. 2) was based on the predominant fatty acids as deduced from GC/MS analysis (not shown), i.e. palmitic (C\(_{16}\)), tuberculostearic (C\(_{19}\)), and stearic (C\(_{18}\)) acids. ESI-MS mass spectrum of fraction A predominantly showed ions corresponding to Ac\(_{3}\)PIM\(_{2}\) (m/z 1415.0) and Ac\(_{4}\)PIM\(_{2}\) (m/z 1653.5, 1681.5 and 1695.5) PIM\(_{2}\) in addition to the diacyl form of phosphatidylinositol (Ac\(_{2}\)PI at m/z 852.3) (Fig. 2a). Fraction B mass spectrum (Fig. 2b) appeared more complex showing predominantly the previously described Ac\(_{3}\)PIM\(_{2}\) and also Ac\(_{2}\)PIM\(_{2}\) (m/z 2063.7) and Ac\(_{3}\)PIM\(_{2}\) (m/z 2302.1, 2330.2, and 2344.2) PIM\(_{2}\). Finally, the mass spectrum of fraction C (Fig. 2c) was dominated by one peak at m/z 2063.7 assigned to (M – H\(^{-}\)) of Ac\(_{3}\)PIM\(_{6}\), beside the other acyl forms of PIM\(_{6}\). In summary, tri- and tetra-acylated forms of PIM\(_{2}\) and PIM\(_{6}\) appeared to be the most abundant PIM acyl forms of \(M.\) bovis BCG, and they may be responsible for the granuloma formation.

Purification of the PIM Acyl Forms—The first attempt to go further in the separation of the different PIM acyl forms consisted of using silicic phase flash-chromatography with chloroform/methanol/water in different proportions as eluents. By using this strategy, Ac\(_{3}\)PIM\(_{2}\) was purified from fraction A with a chloroform/methanol/water, 70:30:2, \(v/v/v\) eluent (12). The fractionation of the more polar PIM\(_{1}\) and PIM\(_{6}\) was unsuccessful using successive isocratic flash chromatographies. An alter-
A native approach was developed, using hydrophobic interaction chromatography on an octyl-Sepharose column, with propanol-1 as eluent. Fig. 3 shows the purification of PIM$_2$ acyl forms issued from fraction A. The different acyl forms were eluted at propanol-1 concentrations ranging from 25 to 50% and separated into 5 sub-fractions according to the mannose content profile. Each sub-fraction (I–V) was collected and analyzed by ESI-MS and NMR.

**Strategy for the Structural Elucidation of the Acyl Forms, the Example of Ac$_3$PIM$_2$—** The negative ESI mass spectrum of fraction IV is dominated by the signal at $m/z$ 1414.2 (Fig. 4c), beside peaks at $m/z$ 1442.2 and $m/z$ 1456.2 assigned to deprotonated molecular ions of tri-acylated forms of PIM$_2$ (Ac$_3$PIM$_2$). Indeed, the most abundant (M – H$^-$) ions at $m/z$ 1414.2 (77%) characterized Ac$_3$PIM$_2$ esterified by 2C$_{16}$,1C$_{19}$, whereas the ions at $m/z$ 1442.2 (14%) and 1456.2 (9%) corresponded to PIM$_2$ esterified by 1C$_{16}$,1C$_{18}$,1C$_{19}$ and 1C$_{16}$,2C$_{19}$, respectively.

The acylation sites on PIM$_2$ were determined previously to be at both positions of the Gro, the C-3 of the myo-Ins unit and the C-6 of the Man$_2$ unit linked to C-2 of the myo-Ins thanks to an NMR strategy based on two-dimensional $^1$H-$^3$P and $^1$H-$^13$C experiments applied on the tetra-acylated form of PIM$_2$ (Ac$_4$PIM$_2$) (12). The NMR strategy we applied on each acyl form is presented briefly below. From the $^1$H-$^3$P-HMQC experiment, the prochiral H-3 and H-3' Gro protons and the myo-Ins H-1 were easily assigned. Then, the remaining myo-Ins and Gro protons were observed on the two-dimensional $^1$H-$^3$P HMQC-HOHAHA spectrum (Fig. 5) and were assigned from their multiplicity and chemical shifts (32) and with the help of the $^1$H-$^1$H HOHAHA spectrum. The different chemical shifts typified the presence or absence of an acyl appendage. For example, a di-acyl-Gro could be evidenced by an H-2 around 5.10 ppm (and H-1 around 4.00/4.25 ppm) (32) and a lyso-Gro should then be assigned by an H-2 around 3.75 ppm and H-1 around 3.95 ppm. These attributions were then supported by the downfield resonance of the lyso-Gro $^1$H resonance compared with that of di-acyl-Gro $^3$P. Likewise, the downfield shift of the myo-Ins H-3 of about 1 ppm signed a C-3 acyl appendage.

The fourth position of acylation, the C-6 of the Man$_2$, was checked with the $^1$H-$^13$C HMBC spectrum, which implied the complete determination of $^1$H and $^13$C chemical shifts achieved by $^1$H-$^1$H HOHAHA and $^1$H-$^13$C HMQC analysis. When this position is acylated, the H6/H6' was deshielded from 3.47/3.64 to 3.95/4.07 ppm.

This analytical approach applied to Ac$_3$PIM$_2$ (Fig. 5c) revealed that the major acyl form corresponded to PIM with two fatty acids on the Gro and one fatty acid on the Man$_2$ unit. This was definitively demonstrated from the $^1$H-$^13$C HMQC spectrum, by the chemical shifts of the H-2 (4.98 ppm) and C-2 (7.06 ppm) of Gro and of the H-6/H-6' (4.07/3.95 ppm) and C-6 (63.6 ppm) of Man$_2$. Beside this species, another one was observed in less quantity. Indeed, $^1$H-$^13$C HMQC and $^1$H-$^1$H HOHAHA highlighted the presence of an acylated (δ$_{H}$a, 5.55) myo-Ins and of a mono-acyl Gro with the acyl appendage present in position 2 as evidenced by the H-2 at δ 4.80.

The nature of the fatty acids esterifying the different sites was investigated by mass spectrometry using two different approaches. The first consisted in obtaining fragment ions produced by ESI-MS/MS from each pseudo-molecular ion of the native molecule (Fig. 6a). Concerning Ac$_3$PIM$_2$, Fig. 6b shows the MS/MS spectrum obtained from the precursor ions at $m/z$ 1414.2 (2C$_{16}$/C$_{19}$ PIM$_2$). This spectrum is dominated by peaks at $m/z$ 297.7 and 255.6 assigned to C$_{19}$ and C$_{16}$ carboxylic fatty acids, respectively. In addition, in the high mass range loss of C$_{16}$ gives the fragment ions at $m/z$ 1158.5 which in turn led to the fragment ions $m/z$ 860.0 by loss of C$_{19}$. More interesting are the fragment ions C at $m/z$ 803.9 highlighting a C$_{16}$ acyl appendage on the C-6 of the Man$_2$ residue. Moreover, this spectrum shows fragment ions at $m/z$ 433.4 corresponding to mono-C$_{19}$ Gro and at $m/z$ 153.2 (not shown) corresponding to deacylated Gro. So, ESI-MS/MS fragmentations did not appear suitable in order to establish accurately the structure of the fatty acid located on the glycerol moiety. Moreover, in the case of Ac$_3$ forms, C ions were unable to discriminate between acyl appendages located on the Man$_2$ and the myo-Ins.

A second approach was then developed, consisting in ESI-MS analysis of the acetylation reaction products of each PIM$_2$ species. Acetylation cleaves the phosphoglycerol linkage without altering the fatty acid esters, leading to two entities as follows: the dimannosyl-inositol phosphate moiety (Man$_2$-Ins-P) (I, II) and the acyl-glycerol residue (III) (Fig. 7a). The dimannosyl-inositol phosphate moiety was observed in negative mode as [M – H$^-$] ions I and II, and the acyl-glycerol residue was analyzed in positive mode in the presence of ammonium acetate as [M + NH$_4$]$^+$ ions III. The acylation reaction produces two populations of dimannosyl-inositol phosphate moieties, one with one acetate on the phosphate group (I) and the other without (II). So to simplify the MS spectra, the acetylated products were subsequently peracetylated to obtain predominantly the di-ester species (ions I). The number of acetate groups was verified by deutero-acetylation followed by perdeutero-acetylation.

Ac$_3$PIM$_2$ was treated by acetylation, and the reaction products were analyzed by ESI-MS in positive and negative modes. As expected, the positive ESI-MS spectrum (Fig. 7c) showed an intense peak at $m/z$ 670.9 assigned to ammonium adduct of the di-acylated C$_{16}$/C$_{19}$ Gro. The negative mass spectrum (Fig. 7d) mainly showed one peak at $m/z$ 1284.2 corresponding to the Man$_2$-Ins-P moiety acylated with one C$_{19}$ arising from the C$_{16}$/C$_{19}$PIM$_2$. Besides this peak, other peaks in lower intensity were observed at $m/z$ 1312.3 and 1326.4 corresponding to Man$_2$-Ins-P moiety acylated with one C$_{16}$ and one C$_{19}$, arising from 2C$_{16}$/C$_{19}$- and 2C$_{19}$/2C$_{19}$-PIM$_2$, respectively. Heterogeneity in terms of the nature of the fatty acyl appendage was then observed on the Man$_2$ unit, but the major population corresponded to C$_{16}$-Man$_2$, as deduced from the intensity of the $m/z$ 1414.2 ions in the ESI source spectrum.

Surprisingly, the parent ion at $m/z$ 1456.2 (C$_{16}$/2C$_{19}$PIM$_2$) gave rise by MS/MS to $m/z$ 745.7 fragment ion identifying the 2C$_{16}$-Gro and to the corresponding $m/z$ 803.9 fragment ion identifying the C$_{16}$-Man$_2$-Ins-P. This is proof that a Gro bearing 2C$_{19}$ exists in minor amounts. Moreover, another ion at $m/z$ 1042.0 was observed corresponding to 2C$_{16}$-Man$_2$-Ins-P.
and confirming the presence of the lyso-Gro population evidenced by NMR.

Taken together, these results indicate that Ac3PIM2 predominantly exists with 2C16 and 1C19, the glycerol being diacylated by C16/C19 and the mannose bearing a C16.

Ac4PIM2 Acyl Forms—The negative ESI mass spectrum of fraction V (Fig. 4d) showed more peaks than that of the fraction IV (Fig. 4c) revealing a higher number of acyl forms. However, all these peaks were in agreement with tetra-acyl forms of PIM₄ (Ac₄PIM₂) and arose from the presence of three types of fatty acids, C₁₆, C₁₈, and C₁₉. Indeed, the most abundant [M-H]⁻ ions at m/z 1652.4 (33%), 1680.5 (12%), and 1694.5 (36%) characterized PIM₂ acylated by (3C₁₆, 1C₁₉), (2C₁₆, 2C₁₉), and (2C₁₆, 1C₁₈, 1C₁₉), respectively. Astonishingly, the signal at m/z 1678.5 (13%) highlighted a PIM₂ tetra-acylated with 2C₁₆, 1C₁₆:1, and 1C₁₉. Besides these major compounds, another rather abundant species (5%) was observed at m/z 1666.4 corresponding to PIM₂ acylated with 2C₁₆ and 2C₁₈.

The acylation positions were determined previously in an NMR study of Ac₄PIM₂ (12). Three of the fatty acyl residues were localized on both positions of Gro and on the C-3 of the myo-Ins unit by two-dimensional ¹H-³¹P HMQC and HMQC-HOHAHA analysis (Fig. 5d). The localization of the fourth fatty acid, on the C-6 of the Manp, was deduced from the ¹H-¹³C HMBC spectrum. As for the Ac₃PIM₂, the acetylation experi-

FIG. 4. Negative ESI mass spectra of the fractions I (a), III (b), IV (c), and V (d) of the octyl-Sepharose chromatography. PIM fatty acyl compositions were based on the most abundant fatty acyl chains found by GC/MS analysis, i.e. palmitate (C₁₆), tuberculostearate (C₁₉), and for a lesser extent stearate (C₁₈). Species <2% were not considered. a, fraction I corresponds to Ac₃PIM₂ (895.5). b, fraction III corresponds to Ac₂PIM₂ acylated by C₁₆/C₁₆ (1133.8) (38%) and C₁₆/C₁₉ (1175.9) (62%). c, fraction IV corresponds to Ac₃PIM₂ acylated predominantly by 2C₁₆,C₁₉ (1414.2) (77%) but also by C₁₆,C₁₆,C₁₉ (1442.2) (14%), and C₁₆,C₂₁ (1456.2) (9%). d, fraction V corresponds to Ac₄PIM₂ acylated predominantly by 3C₁₆,C₁₉ (1652.4) (33%), 2C₁₆,2C₁₉ (1694.5) (36%), 2C₁₆,C₁₆,C₁₉ (1678.5) (14%), and 2C₁₆,C₁₆,C₁₉ (1680.5) (12%) but also by 2C₁₆,2C₁₈ (1666.4) (5%). C₁₆,2C₁₆,C₁₉ (1708.5) and C₁₆,C₁₆,2C₁₉ (1722.5) also exist as minor components.

FIG. 5. ¹H-³¹P HMQC-HOHAHA spectra of Ac₁PIM₂ (a), Ac₂PIM₂ (b), Ac₃PIM₂ (c), and Ac₄PIM₂ (d) dissolved in CDCl₃/CD₃OD/D₂O 60:35:8 at 303 (a and b), 305 (c), and 293 K (d). Numerals correspond to the proton number of the myo-Ins unit, and numerals with letter G correspond to the proton number of the glycerol unit.

FIG. 6. Fragmentation scheme (a) and negative ESI-MS/MS spectrum of Ac₃PIM₂ (2C₁₆,C₁₉) (1414.2) (b), m/z 255.6, C₁₆ carboxylate; m/z 297.7, C₁₉ carboxylate; m/z 433.4, opening of the phosphate linkage with retention of the glycerol with R₃,R₄ = H, C₁₆; m/z 803.9, ion C with R₃,R₄ = H, C₁₆; m/z 821.9, ion D with R₃,R₄ = H, C₁₆; m/z 860.0, loss of C₁₆ and C₁₉ in the carboxylic form; m/z 878.4, loss of C₁₆ in the acidic form and C₁₉ as a ketene; m/z 1042.0, ion C with R₃,R₄ = C₁₆,C₁₆; m/z 1158.5, loss of C₁₆.
were never observed. The negative mode mass spectrum of PIM₂ (Ac₃PIM₂) containing C₁₆ and C₁₉ for fractions I and II, respectively. The ³¹P HMQC experiment led to the definition of H-1 at δ 3.90/3.94 and H-3 at δ 3.70/3.75. These chemical shifts were in agreement with an acylation of position 1 by comparison with Gro chemical shifts of the standard 1-α-lysophosphatidylinositol in Me₃SO-d₆ (1-acyl-2-lyso-sn-glycero-3-phospho-(1-α-my o-Ins); δ_H/δ_31P: 3.99/3.97; δ_H/δ_31P: 3.75; δ_H/δ_31P: 3.78/3.76) and the standard 1-oleyl-2-lysophosphatidic acid in chloroform/methanol/water (δ_H/δ_31P: 4.05/4.11; δ_H/δ_31P: 3.95; δ_H/δ_31P: 3.83/3.89). Moreover, the δ_H of Man at 62.7 ppm and the δ_H of Gro between 3.47 and 3.64 ppm excluded acylation on this position. Thus, it can be proposed that mono-acyl forms of PIM₂ are acylated on the Gro at the C-1 position whatever the nature of the fatty acid (C₁₆ or C₁₉). This conclusion is in agreement with ESI-MS/MS experiments and ESI-MS analysis of the acetylation products of Ac₃PIM₂. Indeed, the Man₂-Ins-P moiety of Ac₃PIM₂ was found devoid of fatty acids (ions at m/z 565.4/583.4 and 1087.8, respectively). Concerning the Gro part, fragment ions corresponding to C₁₀-Gro at (m/z 433.5/451.6) were obtained from the m/z 937.5 parent ion (C₁₀PIM₂). The analogous fragments corresponding to C₁₂-Gro at (m/z 391.5/409.5) expected from the m/z 895.5 parent ions (C₁₂PIM₂) were not observed. However, the acylation experiment unambiguously evidenced C₁₆-Gro ((M + NH₄)⁺ at m/z 432.6) and C₁₉-Gro ((M + NH₄)⁺ at m/z 474.7).

The ESI mass spectrum corresponding to fraction III showed two peaks at m/z 1133.8 and 1175.9 (Fig. 4b) assigned to deprotonated molecular ions of di-acyl forms of PIM₂ (Ac₃PIM₂). They characterized (M – H)⁻ ions of PIM₂ acylated by 2C₁₆ (m/z 1133.8 (38%)), 1C₁₆, and 1C₁₉ (m/z 1175.9 (62%)). The acylation positions were investigated by NMR analysis. The ⁳¹P HMQC-HOHAHA spectra (Fig. 5b) clearly showed two lines of correlations, highlighting two distinct populations of Ac₃PIM₂. The most representative one (80%, determined by ²⁷Na signal height) corresponded to a population bearing fatty acids on both positions of Gro since the H-2 was deshielded at 5.06 ppm (Fig. 5b). The Gro proton chemical shifts of the other population were similar to those described for Ac₃PIM₂ since all the Gro protons resonated between 3.7 and 4.0 ppm. These chemical shifts were in agreement with acylation of Gro at position 1. Moreover, the m/z 937.5 fragment ions were also similar to those described in the case of Ac₃PIM₂, we assumed that myo-Ins was not acylated and, by deduction, that Man was acylated in position 6. The existence of these two populations is perhaps more obvious from the exchange correlations, where the two sets of correlations, i.e., those corresponding to the di-acyl Gro and those corresponding to the lyso-Gro, were clearly observed as two different phosphorus resonances (not shown). Two populations of Ac₃PIM₂ evidenced by NMR, were confirmed by MS/MS data. Indeed, beside the ions corresponding to a non-acylated Man₂-Ins-P moiety (m/z 565.4), ions arising from C₁₀-Man₂-Ins-P (m/z 803.9/821.9) were observed from the parent ions at m/z 1133.8 (2C₁₆) and m/z 1175.9 (C₁₀/C₁₉) parent ions. Similarly, in addition to the non-acylated Man₂-Ins-P (m/z 1087.8), the
infiltration by NK T cells was nearly exclusive since no other granulomatous response, also induced the local infiltration of NK T cells identified by the presence of the invariant V\textsubscript{14-J281} TCR \alpha chain. PBS-Alum, used as a negative control and which did not support recruitment of CD1\text{d}-restricted NK T cells (Fig. 8, left panel) and that of V\text{a}14-J281\textsuperscript{+} (i.e. CD1\text{d}-restricted T cells) (Fig. 8, right panel). The fully deacylated PIM\textsubscript{2} generated identical signals thus extending to the recruitment of NK T cells, the absolute dependence of the response upon the presence of fatty acid chains. By contrast, PI with two acyl chains as well as PIM\textsubscript{2} with one to four acyl chains, in addition to generating a granulomatous response, also induced the local infiltration of the lesions by NK T cells (Fig. 8, right panels). Moreover, the infiltration by NK T cells was nearly exclusive since no other V\text{a}14\textsuperscript{+} T cells were recovered in the lesions, excluding random infiltration of lymphocytes that should have mimicked the infiltrate of total V\text{a}14\textsuperscript{+} T cells (Fig. 8, bottom panels). The data obtained by using PCR at saturation were only semi-quantitative but, for an identical HPRT mRNA content, suggested an equivalent number of V\text{a}14-J281 mRNA chains in all positive samples. Thus, the number of fatty acid chains does not interfere with the recruitment of NK T cells, and the recruitment of the latter cells by PI of mycobacterial origin strengthened the hypothesis according to which the carbohydrate moiety played only a minor role in the biological response.

**DISCUSSION**

The results presented herein solve several questions left in abeyance for many years concerning the structural definition of the phosphatidylinositol-mannosides. Although their mannoligosaccharidic part is perfectly defined (5), a blur existed concerning the acylated state of these molecules. This point is plainly of great importance as the metabolic control of the acylation state may be involved in the fine-tuning of their biological activities. For a structural elucidation of the different populations existing, purification is a crucial step. We developed a rapid and effective method allowing the retrieval of purified acyl form in three steps as follows: (i) lipidic extraction and phospholipidic precipitation, (ii) QMA column to discriminate neutral from charged molecules and PIM\textsubscript{2} from PIM\textsubscript{6}, and (iii) octyl-Sepharose column on PIM\textsubscript{2} or PIM\textsubscript{6} mixtures to separate acyl forms. PIM were predominantly found in the chloroform/methanol extract. Despite our efforts to purify them from the ethanol/water extract, very little was retrieved. The heterogeneous nature of these closely related molecules defies their purification to homogeneity using isocratic silicic acid chromatography. Ac\textsubscript{2}PIM\textsubscript{2} could be easily purified in this way (12) as it is very apolar, but this strategy was unsuccessfully applied to purify the more polar PIM\textsubscript{6}, as they co-migrate with the more acylated forms of PIM\textsubscript{6}. The strategy described here allowed us first to separate PIM\textsubscript{2} from PIM\textsubscript{6} and second to

**TABLE I**

| Ac\textsubscript{p}PI | C\textsubscript{16}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | Manp, 6 | m\textsubscript{y}O-Ins, 3 | Abundance |
|----------------------|----------|----------------|----------------|--------|----------------|-----------|
| Ac\textsubscript{2}PIM\textsubscript{2} | C\textsubscript{16} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 62% |
| Ac\textsubscript{2}PIM\textsubscript{2} | C\textsubscript{16}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | 38% |
| Ac\textsubscript{3}PIM\textsubscript{2} | 2C\textsubscript{16}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 77% |
| Ac\textsubscript{3}PIM\textsubscript{2} | C\textsubscript{16}, C\textsubscript{19}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 14% |
| Ac\textsubscript{3}PIM\textsubscript{2} | Ac\textsubscript{2}PIM\textsubscript{2} C\textsubscript{16}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 9% |
| Ac\textsubscript{3}PIM\textsubscript{2} | 3C\textsubscript{16}, C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 42% |
| Ac\textsubscript{3}PIM\textsubscript{2} | 2C\textsubscript{16}, 2C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 38% |
| Ac\textsubscript{3}PIM\textsubscript{2} | 2C\textsubscript{16}, 2C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | C\textsubscript{19} | C\textsubscript{16} | C\textsubscript{19} | 14% |

**Fig. 8.** **Immunoscope profiles of the V\text{a}14 rearranged TCR \alpha chains in cell infiltrates.** Cell infiltrates were collected on day 7 following the injection. The molecules injected are shown on the left of the figure. Left panels, V\text{a}14-C\textsubscript{a} rearrangements, and right panels, V\text{a}14-J281 rearrangements. **Ordinate**, relative scale of fluorescence intensity. **Abscissa**, size of the CDR3 region in amino acids.
fractionate them according to their acylation degree. 

_M. bovis_ BCG and _M. smegmatis_ strain 607*3 were found to mainly contain two PIM families, the dimannoside (PIM₂) and the hexamannoside (PIM₆). PIM₁, PIM₃, PIM₄, and PIM₅ were observed in very small quantities, suggesting that they are biosynthetic intermediates. For each glyco form, tri- and tetraacylated molecules were the most abundant.

The structural strategy used to characterize acylation sites combines the potential of two complementary analytical techniques, NMR and mass spectrometry. NMR has the advantage of taking into account the globality of the fraction but the sensitivity is less than that of mass spectrometry. Electrospray ionization mode was chosen rather than FAB as the molecules could be analyzed without derivatization preventing the loss of any labile substituents. Classical chemical procedures as protection of free hydroxyls by base-stable O-(1-methoxy)ethylated groups after reaction with methyl vinyl ether, where only acylation sites are revealed as O-methylated sugars by GC/MS analysis (35) or Prehm methylolation followed by FAB-MS analysis as described by Khoo et al. (11), could help answering this question. They were discarded as they imply several chemical steps and as they require higher amounts of sample than ESI mass spectrometry. Moreover, in our strategy, NMR was used to characterize the acylation positions, and mass spectrometry gave information concerning the nature of the fatty acids present at each position. The results obtained are summarized in Table I. Ac₁PIM₂ is identified with C₁₆ or C₁₉ in position 1 of the Gro. Ac₂PIM₂ appears as two populations equally represented, one with 2 C₁₆ and one with 1 C₁₆ and 1 C₁₉, both fatty acids being on Gro. These data contrast with those of Brennan and co-workers (11) who demonstrated that the Ac₂PIM₂ exists predominantly as lyso-PIM₂. Concerning Ac₃PIM₂, a major acyl form was observed, corresponding to PIM₂ with 2 C₁₆ and 1 C₁₉, the glycerol being di-acylated by C₁₀/C₁₉ and the mannose bearing a C₁₆. Interestingly, the acyl form corresponding to 3C₁₆-PIM₂ was not observed, indicating that Ac₃PIM₂ arose from 3C₁₀/C₁₉-PIM₂. Ac₄PIM₂ was present as three populations: 3C₁₀/C₁₉, 2C₁₀/2C₁₉, and 2C₁₀/C₁₉-C₁₉-PIM₂. The acylation positions were clearly elucidated in an earlier NMR study (12) as being both positions of Gro, the C-3 of the myo-Ins unit and the C-6 of the Manp linked to the C-2 of the myo-Ins unit, excluding the presence of a fatty acid to the C-6 of the Manp linked on the C-6 of the myo-Ins unit envisaged by Khoo et al. (11). Taken together, the results indicate that the glycerol is preferentially acylated by C₁₀/C₁₉, the mannose by one C₁₆, even if a weak variation exits. However, the nature of the fatty acid present on the inositol appears highly variable being C₁₆, C₁₈, or C₁₉.

These results have led to advances in answering the question whether PIM are biosynthetic precursors of LM and LAM or not. The definition of the acyl forms of LM and LAM was approached by an NMR strategy applied on the native molecules (12, 36, 37). Indeed, the one-dimensional ³¹P NMR spectra of LM and LAM from _M. bovis_ BCG typify the anchor heterogeneity and lead to the characterization of at least five populations of differently acylated molecules. Then two-dimensional ¹H-³¹P NMR spectroscopy allowed the definition of mono- to at least tri-acylated molecules. In the case of LM and LAM, NMR was unable to confirm the highly suspected presence of fatty acid on the C-6 of the Manp due to the larger number of mannose units. Nevertheless, the purification of each population followed by matrix-assisted laser desorption ionization/time of flight-mass spectrometric analyses allowed the unambiguous characterization of the tetra-acyl form, in addition to the mono- to tri-acyl forms.³ Taken together, the results highlight the same major fatty acids and acylation sites for all these molecules strongly suggesting a biosynthetic filiation between them.

PIM, independently of their glyco forms and acyl forms, have the capacity to recruit NK T cells in the granulomatous region. From our results, two main conclusions can be reached concerning the structural requirement of such activity. First, to generate a granulomatous response and to recruit NK T cells, there is an absolute requirement for the molecules to possess at least one fatty acyl chain, with no evidence for differences related to their number, location, or size. Second, the increasing complexity of the carbohydrate moiety is not reflected in significant differences in the biological responses. In this respect, it is worth noting that mycobacterial PI behaved primarily like PIM₂ and PIM₆, a finding indicative of the minor role of the carbohydrates in NK T cell recruitment as well as a lack of specificity of the TCR of NK T cells toward sugars as suggested independently by analysis of the TCR repertoire (21, 38) and the use of CD1d1 tetramers (39).

The Vo₁₄⁺ invariant TCR α chain typifies CD1d-restricted NK T cells that are activated by exogenous antigens such as α-GalCer. This TCR of CD1d-restricted NK T cells is highly discriminating for α-GalCer analogs. Indeed, discrete structural modification of the α-GalCer carbohydrate part either by adding an extra α-Gal or by changing the α-anomer into a β-anomer or by epimerization of the hydroxyl group at position 2 of α-GalCer led to non-activation of CD1d-restricted NK T cells (40–42). In contrast to these results, our data reveal that PI, which does not contain carbohydrate, presents the same ability as PIM₂ or PIM₆ to recruit Vo₁₄⁺-invariant NK T cells preferentially to other rearranged Vo14⁺ T cells, which are undetectable in the granuloma. This suggested the absence of a TCR-specific recognition by the TCR of NK T cells and consequently the absence of role of the TCR-CD1d axis in NK T cell recruitment.

In addition, Ac₁⁻ and Ac₂PIM₂ show the same activity as Ac₃PIM₂ and Ac₄PIM₂. To date, the glycolipids loaded by CD1b molecules have two aliphatic chains (43). This is also the case for the exogenous antigen α-GalCer presented by CD1d. This is consistent with the fact that CD1d is composed of a hydrophobic antigen-presenting groove with two large pockets that probably accommodate two acyl appendages (44). In the case of PIM, the extra appendages located on Manp and/or on myo-Ins could prevent tri- and tetra-acylated PIM recognition by the TCR of NK T cells again supporting the idea that recruitment of Vo₁₄⁺ invariant NK T cells is not controlled by the TCR-CD1d axis. This hypothesis is in agreement with adoptive transfer experiments of wild type NK T cells into CD1d⁻/⁻ mice showing that CD1d₁ expression is not required for the early recruitment of NK T cells to the injection site.⁴ Taken together, these data strongly suggest that the TCR-CD1d axis is not important for the NK T cell activation and recruitment to the injection site. These data rather fit with NK T cells being attracted to the site of inflammation caused by glycolipids, irrespective of their carbohydrate and fatty acyl moieties.

It should be kept in mind that the present bioassay does not reflect only early events in the recognition of glycolipids but rather the result of complex and sequential cell-to-cell interactions due to the occurrence of foreign glycolipids. The rationale for the use of the present bioassay is based on the inability to activate NK T cells in vitro using reagents other than α-galac-

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³ M. Gilleron and G. Puzo, unpublished results.

⁴ M. Mempel, C. Ronet, F. Suasez, M. Gilleron, G. Puzo, Y. Koezuko, L. van Kaes, A. Lehuen, P. Kourilsky, and G. Gachelin, manuscript submitted for publication.
tosylceramide (41) or anti-CD3 monoclonal antibodies (45). This precludes the monitoring of the early release of interferon-γ and interleukin-4 (46) that could occur following encounters with foreign ligands. The need for such an assay aimed at deciphering early events following glycolipid recognition is stressed by the finding that PIM recruit NK T cells without inducing their apoptosis (data not shown), whereas NK T cells are involved in a variety of immune processes ranging from the keeping of pregnancy to anti-cancer responses (47).

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Acylation State of the Phosphatidylinositol Mannosides from *Mycobacterium bovis* Bacillus Calmette Guérin and Ability to Induce Granuloma and Recruit Natural Killer T Cells

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