Structural Identification of a Major Mitogenic Lipid Derived from *Bacillus subtilis* as a Glycerophosphoglycolipid†

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ABSTRACT: Lipids extracted from *Bacillus subtilis* using a 2:1 mixture of chloroform and methanol have been found to be very mitogenic. These lipids were fractionated on a silica column and eluted with chloroform, acetone, and 60% methanol in chloroform, and the mitogenic activity was recovered in the last fraction. Further purification of the mitogenic components was achieved by HPLC on an amino−isopropyl bonded-phase column using a linear gradient of 5−20 mM ammonium acetate in a mobile phase consisting of hexane, 2-propanol, methanol, and water (5.5:8:1.5:1). Two major and several minor mitogenic peaks were observed. One major mitogenic lipid was isolated in pure form and structurally characterized by chemical degradation analysis, NMR spectroscopy, and mass spectrometry. Mild acid hydrolysis of the lipid released glycerol phosphate and a neutral glycolipid. Saponification of the lipid released a water-soluble head group and C_{14}−C_{17} branched fatty acids. Total acid hydrolysis of the head group revealed the presence of glycerol and glucose in a ratio of 1:1. Mild acid hydrolysis of the head group to remove the glycerol phosphate produced a neutral partial head group. The partial head group was methylated and then analyzed by GLC-CIMS and by the reductive-cleavage method, which revealed that it was composed of nonreducing terminal glucopyranosyl, 6-linked glucopyranosyl, and 3-linked glycerol residues in equimolar proportions. Finally, the molecular weight of the permethylated head group, obtained by fast atom bombardment mass spectrometry, was 724.3340, which is consistent with the composition of two glucose residues, one glycerol residue, and one glycerol phosphate residue. On the basis of all these results, the intact mitogenic lipid was identified as 1,2-di-*O*-acyl-3-*O*-[6-(*sn*-glycero-1-phospho)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]glycerol. The purified glycolipid possessed very potent mitogenic activity in a murine splenocyte proliferation assay at a concentration of 0.01−0.1 μg/mL.

Bacterial cell wall peptidoglycan possesses adjuvant and other immunological activities (Stewart-Tull, 1980). In order to identify the structural basis for the activity, peptidoglycan was digested by lysosomal enzymes and one of the fragments so obtained, *N*-acetyl-D-glucosaminyl-*N*-acetyl-D-muramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine, was found to be active as an adjuvant (Adam *et al*., 1972 & 1974). Compounds smaller than this fragment were synthesized and the minimal structure which still possessed the adjuvant activity was found to be *N*-acetyl-D-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, or MDP).[1]

MDP has been shown to enhance both cellular and humoral immune responses (Souvannavong *et al*., 1978, Boomford, 1980). Because MDP is structurally related to the bacterial peptidoglycan monomeric unit, it was hypothesized that MDP or structurally related small glycopeptides might be produced *in vivo* from degradation of cell walls by macrophages, which take up and destroy bacteria. The glycopeptides so produced might stimulate and amplify immune responses. To test this hypothesis, the processing of *Bacillus subtilis* peptidoglycan by macrophage-like RAW264 cells was investigated. Although MDP was not found, structurally related glycopeptides were indeed produced by macrophages and secreted into the culture medium (Vermeulen & Gray, 1984).

The enhancement of cell-mediated immune response by MDP can only be demonstrated *in vivo* when it is administered with mineral oil (Boomford, 1980; Carelli *et al*., 1981), or encapsulated in liposomes, or chemically derivatized with a lipid moiety (Azuma *et al*., 1978). To explain the cell-mediated immune response of peptidoglycan, the possibility of formation of lipophilic glycopeptides during the processing of peptidoglycan was examined. Macrophages were fed with *B. subtilis* cell walls radiolabeled in muramic acid, glucosamine, alanine, glutamine, and diaminopimelic acid residues. It was revealed that two lipophilic glycopeptides that contained glucosamine, muramic acid, and alanine of bacterial origin were present in the macrophage lipids after the feeding (Polanski & Gray, 1989). This result suggested an *in vivo* derivatization mechanism for the production of lipophilic glycopeptides capable of enhancing cell-mediated immune responses. Such a mechanism is supported by the direct observation of the immunological activities of the lipids derived from macrophages exposed

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[1] Abbreviations: Ala, alanine; CIMS, chemical ionization mass spectrometry; COSY, correlated spectroscopy; Dap, diaminopimelic acid; DMSO, dimethyl sulfoxide; DPM, disintegrations per minute; FABMS, fast atom bombardment mass spectrometry; GLC, gas−liquid chromatography; GlcNAc, *N*-acetyl-D-glucosamine; HPLC, high-performance liquid chromatography; isoGln, isoglutamine; LTPGLCRI, linear temperature programmed gas−liquid chromatography retention indices; MDP, *N*-acetyl-D-muramyl-L-alanyl-D-isoglutamine; MS, mass spectrometry; MurNAc, *N*-acetyl-D-muramic acid; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; TMSOMs, trimethylsilyl methanesulfonate.

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to bacterial peptidoglycan. Such lipids were shown to be strongly mitogenic to murine splenocytes and to stimulate the release of interleukin 1, superoxide (Wehner & Gray, 1991), and tumor necrosis factor α (M. Qi and G. R. Gray, unpublished data) by resident mouse peritoneal macrophages.

In the present work, lipids extracted directly from *B. subtilis* were examined and were also found to be very mitogenic. Therefore, an attempt was made to isolate and characterize these lipids. In this paper, we report the structural characterization of one of the major mitogenic lipids isolated from *B. subtilis*.

EXPERIMENTAL PROCEDURES

Bacillus subtilis Cell Culture. *B. subtilis* Porton strain was grown in a defined medium containing 0.65 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of ferric ammonium citrate, 0.5 g of $MgSO_4$, 20 g of glycerol, 4.0 g of L-glutamic acid, and 2.0 g of citric acid/L of water, adjusted to pH 7.4. Cells were cultured at 37 °C and harvested at late log phase by centrifugation at 17000*g* for 30 min, washed twice with 0.9% NaCl, and stored at −20 °C before use.

Lipid Extraction. Lipids were extracted from *B. subtilis* by Folch's method (Folch *et al*., 1957). Briefly, a suspension of bacteria was extracted with 20 volumes of chloroform:methanol (2:1) for 48 h. The extract was filtered and 0.2 volume of water was added to the filtrate. The mixture was allowed to separate into two phases, and lipids were recovered from the lower phase and stored at −20 °C.

Fractionation of Lipids by Silica Chromatography. Crude lipids (∼500 mg) extracted from the bacteria were loaded on a silica column (2.5 × 30 cm, Bio-Sil A, 200−400 mesh, Bio-Rad, Richmond, CA). The column was eluted by a step gradient of chloroform, acetone, and 60% methanol in chloroform (Kates, 1980). Fractions were collected and assayed for mitogenic activity.

High-Performance Liquid Chromatography. HPLC was performed on a Beckman System Gold chromatograph system with dual pumps. The lipids were separated on an amino−isopropyl bonded-phase column (Spherisorb amino 25.0 × 1.0 cm, Regis Chemical, Morton Grove, IL) with mobile phases modified from those originally developed for phospholipid separation (Hansons *et al*., 1981). Solvent **A** contained hexane:2-propanol:methanol:water (5.5:8:1.5:1) and solvent **B** consisted of 25 mM ammonium acetate in solvent **A**. The chromatographic program started at 20% **B** for 5 min, they linearly increased to 100% **B** over 5 min. After being held at 100% **B** for 30 min, the gradient was returned to 20% **B** over 2 min. The flow rate was 3 mL/min and fractions of 3 mL were collected. Aliquots of each fraction, diluted appropriately in methanol, were assayed for mitogenic activity. The active peaks were pooled and partitioned between chloroform, methanol, and water (Bligh & Dyer, 1959), and the mitogenic lipids were recovered from the chloroform phase.

Saponification of Lipids. The mitogenic lipid purified by HPLC was saponified with 0.2 M KOH in anhydrous methanol for 30 min at room temperature. Chloroform, methanol, and water were added to give a final ratio of chloroform:methanol:water of 2:2:1.8 and the mixture was allowed to separate into two phases. The lower phase was evaporated to dryness with a stream of nitrogen, and the upper phase was neutralized with a small amount of Dowex 50 (H^+) and then further purified by chromatography on a Sep-Pak C18 cartridge and by gel-permeation chromatography.

Separation by Sep-Pak C18 Cartridge. A Sep-Pak C18 cartridge (Vac RC, 500 mg, Waters, Milford, MA) was conditioned with 10 mL of methanol followed by a wash with 10 mL of water. The aqueous phase from saponification (previous step) was passed through the cartridge, which was then washed with 10 mL of water. All the effluent was collected and lyophilized.

Gel Permeation Chromatography. The head group released by saponification and isolated by chromatography on a Sep-Pak C18 cartridge was further purified by gel-permeation chromatography on a column (1 × 60 cm) of Bio-Gel P2 (Bio-Rad, Richmond, CA). The column was eluted with distilled water at a flow rate of 3.2 mL/h. Fractions of 0.8 mL were collected an an aliquot was removed and assayed for the presence of sugar by the periodate oxidation method (Dixon & Lipkin, 1954).

Mild Acid Hydrolysis of Lipids. Lipids were hydrolyzed by treatment with 98% acetic acid at 100 °C for 45 min as previously described (Fisher & Landgraf, 1975). The acetic acid was then evaporated under a stream of nitrogen and the residue was partitioned between chloroform and water (1:1). Both the upper and the lower phases were collected.

Alditol Acetate Analysis. Samples were hydrolyzed in 1 mL of 2 N TFA at 120 °C for 1 h. TFA was removed by evaporation under a stream of nitrogen and the hydrolyzed products were reduced with $NaBH_4$ (20 mg/mL in 1 M NH_4-OH) for 90 min at 40 °C. The reduction was stopped by the addition of 10 μL of acetic acid. The borate formed was removed by repeated evaporation with a mixture of methanol and acetic acid (9:1). Alditols were acetylated for 30 min with 100 μL of acetic anhydride and 10 μL of *N*-methylimidazole. The alditol acetates were recovered by extraction with chloroform and analyzed by GLC (Blakeney *et al*., 1983).

Methylations. The head group released by saponification and the partial head group obtained by mild acid hydrolysis of the head group were methylated by the method of Ciucanu and Kerek (1984) as modified for microscale work. The lyophilized head group (or partial head group), dissolved in 100 μL of DMSO, was mixed with 100 μL of NaOH/DMSO (120 mg/mL) and stirred for 30 min and then 10 μL of CH_3I was added and stirring was continued for 2 h. The reaction was stopped by the addition of 100 μL of water and the methylated product was extracted twice with 100-μL portions of dichloromethane. The dichloromethane extract was then back-extracted three times with 100-μL portions of water. The permethylated product was recovered from the dichloromethane solution.

Reductive Cleavage Analysis. The permethylated compound was analyzed by the reductive cleavage method (Rolf & Gray, 1982) modified for microscale analysis (J. B. Zheng and G. R. Gray, unpublished). The methylated sample in 20 μL of CH_2Cl_2 was mixed with 30 μL of a mixture of CH_2Cl_2:$(CH_3CH_2)_3SiH$:TMSOMs:$BF_3 \cdot OEt_2$ (300:13:13:2 v/v/v/v) and stirred for 24 h under argon. Acetic anhydride (2 μL) was then added for *in situ* acetylation and stirring was continued for another 24 h. The reaction was quenched with 200 μL of water and the products were recovered by extraction into 100 μL of CH_2Cl_2. The dichloromethane

solution was then back-extracted twice with 100-μL portions of water. The reductive cleavage products in the dichloromethane solution were then analyzed by gas−liquid chromatography.

Gas−Liquid Chromatography. Gas−liquid chromatography was performed on a Hewlett-Packard gas−liquid chromatograph (HP5890) equipped with DB-5 (J&W Scientific, Folsom, CA) and RTx 200 (Restek, Bellefonte, PA) capillary columns. The temperature was programmed from 80 to 250 °C at 2 °C/min with no initial hold. Compounds were identified by comparing their linear temperature programmed gas−liquid chromatography retention indices (LTPGLCRI), obtained on both columns, to those of authentic standards.

Instrumental Methods. NMR spectra were obtained on a Varian 500-MHz spectrometer. Mass spectra were recorded on a Finnigan Mat 95 mass spectrometer. Fast atom bombardment mass spectra were recorded on a VG Analytical, Ltd., 7070E-HF high-resolution double-focusing mass spectrometer; a *m*-nitrobenzyl alcohol (MNBA) matrix was used.

Mouse Splenocyte Proliferation Assay. The mitogenic activity was assayed as previously described (Wehner & Gray, 1991) except for a modification of sample preparation. Mouse spleen cells from endotoxin-resistant C3H/HeJ mice were prepared in minimal Eagle's medium (MEM) plus 2% fetal calf serum and cultured in a 96-well microtiter plate at 37 °C, 5% CO_2 and 85% humidity. Aliquots (5 μL) of lipid samples dissolved in methanol were added to each well, and then 0.5 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) was added after 24 h and the culture was incubated for another 24 h. Cells were harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA). The incorporation of radioactivity was measured by liquid scintillation counting. The assay was carried out in triplicate, and methanol or Hanks' balanced salt solution was used as a control.

Endotoxin Assay. Endotoxin levels in the lipid samples were assayed by the limulus amebocyte lysate assay method using a kit obtained from Biowhittaker (Walkersville, MD). The lipid sample (5 μL) in methanol was incubated with 50 μL of limulus amebocyte lysate (LAL) solution and 50 μL of water for 30 min at 37 °C. The substrate (100 μL) was then added and the reaction was stopped after 6 min with 100 μL of 25% acetic acid (v/v in water). The absorbance was measured at 410 nm and the level of endotoxin was calculated from a standard curve determined from the standard solution provided in the kit. Methanol was used as a control and included in the determination of the standard curve.

RESULTS

Isolation of the Mitogenic Lipid. Lipids extracted from *B. subtilis* were first fractionated on a silica column and then eluted sequentially with chloroform, acetone, and 60% methanol in chloroform. Neutral lipids were eluted in the chloroform fraction, which contained no mitogenic activity. The acetone fraction, which contained glycolipids, was only slightly active. The major mitogenic activity was eluted in the last fraction, which contained phospholipids (data not shown). This fraction was further purified by HPLC. The assay results of HPLC fractions revealed that there were two

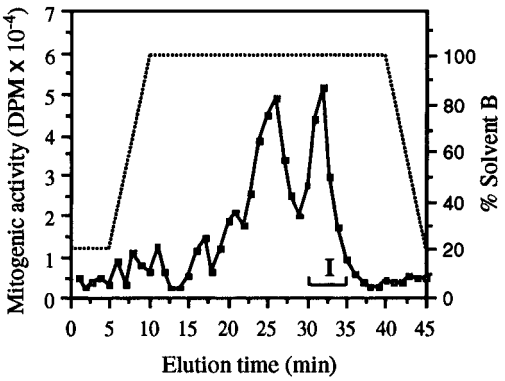


FIGURE 1: Fractionation of the phospholipid fraction derived from *B. subtilis* by HPLC on a Spherisorb amino column. The column was equilibrated with 20% solvent **B** in solvent **A** (see Experimental Procedures) and then eluted over 5 min to solvent **B**. Fractions were assayed for mitogenic activity as described in the text.

major and several minor mitogenic components (Figure 1). A pure lipid was recovered from the second major peak (**I**) and its structure was elucidated as 1,2-diacyl-3-*O*-[6-(*sn*-glycero-1-phospho)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]glycerol (**I** in Scheme 1). The experimental data to support this structure are presented in detail herein (refer to Scheme 1 for the identity of all compounds).

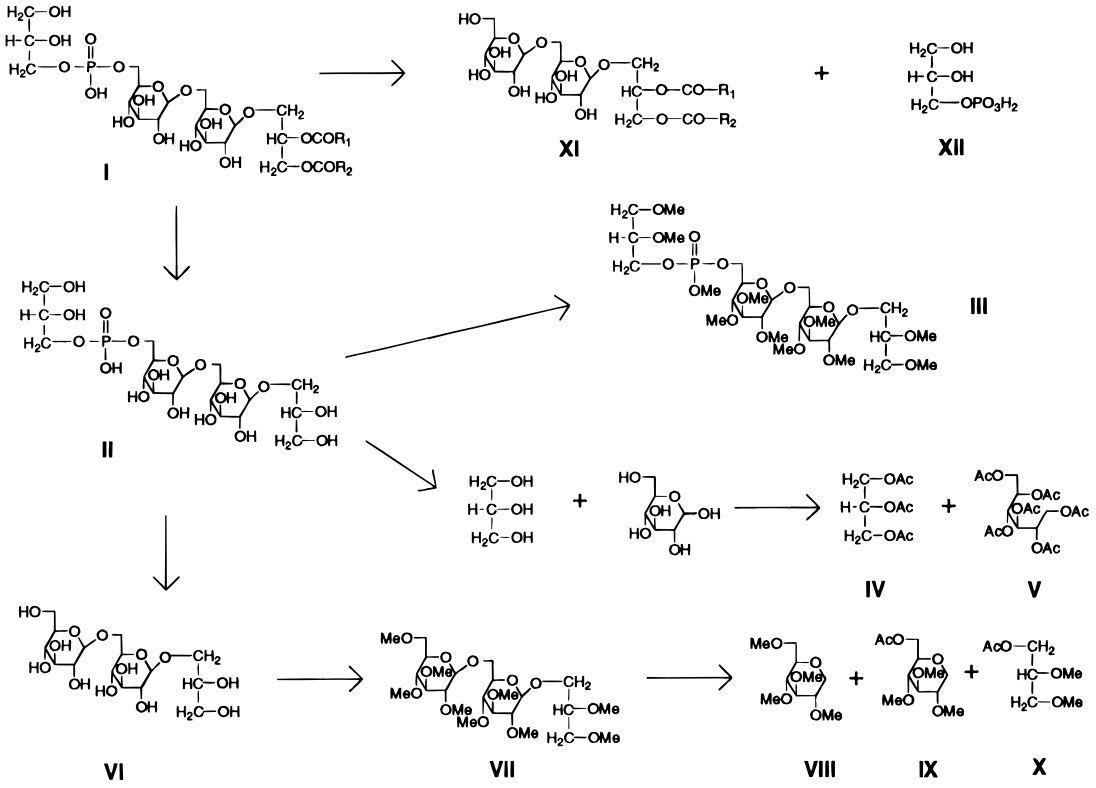
Chemical Degradation Studies. Saponification of the lipid (**I**) removed the acyl groups and released a water-soluble component (head group, **II**). The molecular weight of the permethylated head group (**III**), obtained by fast atom bombardment mass spectrometry (FABMS spectrum not shown), was 724.3340, which is in very good agreement with the calculated value (724.3282).

Hydrolysis of the head group (**II**) by TFA followed by alditol acetate analysis produced glycerol triacetate (**IV**) and glucitol hexaacetate (**V**), which were identified by their linear temperature programmed gas−liquid chromatography retention indices (LTPGLCRI) and confirmed by GLC−MS. The molar ratio of glucitol to glycerol, corrected for molar response by the effective carbon response method (Ackman, 1964; Sweet *et al*., 1975), was 1:0.87, which is consistent with the proposed structure.

When the head group was subjected to mild acid hydrolysis (98% acetic acid), the glycerol phosphate group was removed and a neutral partial head group (**VI**) was obtained. This partial head group was methylated and analyzed by GLC−CIMS. A single peak was eluted and its molecular weight was measured as 542 ($M + NH_4^+ = 560.3$), corresponding to the per-*O*-methylated partial head group (**VII**).

The structure of this permethylated partial head group (**VII**) was further analyzed by the reductive-cleavage method (Rolf & Gray, 1982). After reductive cleavage of the glycosidic bonds followed by acetylation, three products were separated by gas−liquid chromatography and identified by their LTPGLCRI values on two columns (J&W DB-5 and Restek RTx-200) and by GLC−MS. These products were 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**VIII**), 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**IX**), and 3-*O*-acetyl-1,2-di-*O*-methylglycerol (**X**), which were derived from nonreducing terminal glucopyranosyl, 6-linked glucopyranosyl, and 3-linked glycerol residues, respectively. This result established the structure of the partial head group

Scheme 1



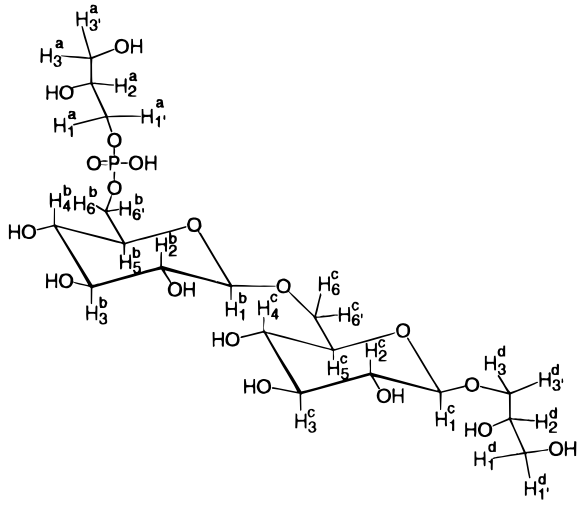
(**VI**) as 3-*O*-[D-glucopyranosyl-(1→6)-D-glucopyranosyl]-glycerol.

That glycerol phosphate was cleaved from the head group by mild acid hydrolysis was confirmed by similar experiments with the intact mitogenic lipid (**I**). Lipid **I** was subjected to hydrolysis with 98% HOAc, which cleaved the phosphodiester bond to the glucose residue. The products were partitioned between chloroform and water and, as expected, a glycolipid (**XI**) was extracted into the chloroform phase. The major compound released into the aqueous phase was identified as α-glycerophosphate (**XII**) by NMR spectroscopy. This result also established that, in the intact lipid, the phosphoglycerol group was not acylated. Otherwise, a diacylglycerol phosphate would have been produced and extracted into the chloroform phase.

NMR Studies. The presence of a diacylated glycerol moiety was clearly indicated in the NMR spectrum of the mitogenic lipid (**I**) (spectrum not shown; see Chart 1 for atom labels). In the intact lipid, H_2^d was observed at δ 5.22 and was coupled to H_1^d and $H_{1'}^d$ at 4.20 and 4.35 ppm, respectively. The downfield shift of these resonances established that both the 1 and 2 positions were acylated. H_2^d was also coupled to H_3^d and $H_{3'}^d$ at 3.94 and 3.71 ppm, respectively, indicating no acylation at the 3-position. Upon saponification, H_2^d was shifted upfield to 3.76 ppm, and H_1^d and $H_{1'}^d$ were shifted upfield to 3.5 ppm (Figure 2). Therefore, the head group (**II**) was acylated at both the 1 and 2 positions of the glycerol residue in the intact lipid (**I**).

The head group (**II**) was also studied in detail by NMR spectroscopy, which established the anomeric configuration of the two glucopyranosyl residues and the position of attachment of the glycerol phosphate to the glucose. The two anomeric proton resonances were observed at 4.33 and 4.38 ppm, respectively, and both had a coupling constant of 8.0 Hz. This unambiguously established that both glucopyranosyl residues had a β configuration. H_6^b and $H_{6'}^b$, which were assigned at 4.02 and 3.91 ppm, respectively, exhibited unusual multiplet patterns, suggesting coupling to phosphorus. Therefore, the glycerol phosphate was 6-linked to the terminal glucopyranosyl residue.

Chart 1



The existence of phosphorus in the compound was further confirmed by ^{31}P NMR spectroscopy. A broad, poorly resolved quintet was observed at 3.33 ppm (triphenyl phosphate as an external standard, spectrum not shown), consistent with the phosphodiester linkage.

Fatty Acid Analysis. The composition of fatty acids in the lipid was analyzed by GLC−MS of their methyl esters, which were formed by saponification with potassium hydroxide in methanol. The NMR spectrum of the mixture of methyl esters established that they were branched fatty acids. Analysis by GLC−MS identified nine different saturated

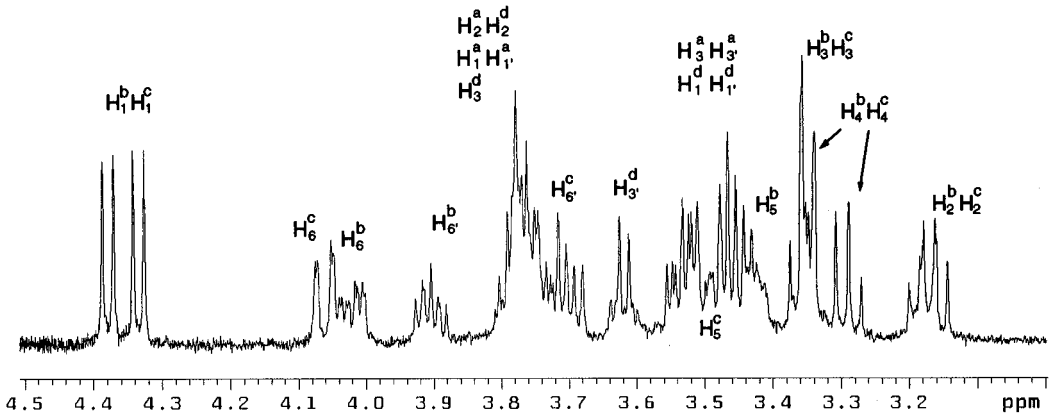


FIGURE 2: ^{1}H NMR spectrum (500 MHz) in D_2O of the head group (**II**) derived by saponification of the mitogenic lipid **I**. The indicated resonances were assigned by COSY.

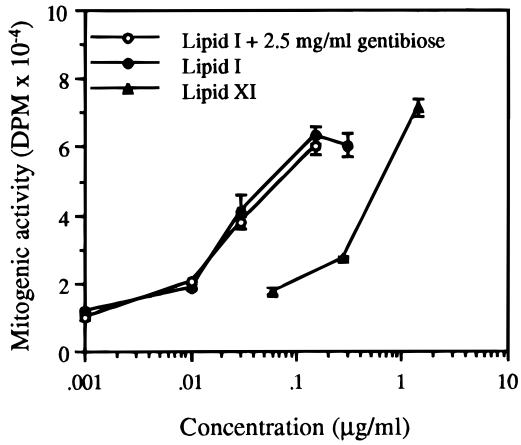


FIGURE 3: Stimulation of proliferation of splenocytes derived from C3H/HeJ mice by lipid **I** and the glycolipid (**XI**) derived from **I** by mild acid hydrolysis.

$C_{14}-C_{17}$ fatty acids. The major components were branched but the detailed structures of these fatty acids were not determined.

Mitogenic Activity. The purified lipid (**I**) showed strong dose-dependent stimulation of the proliferation of splenocytes from C3H/HeJ mice. The activity could be detected at a concentration as low as 10 ng/mL and a maximal activity was obtained at 0.1 μg/mL (Figure 3). This lipid also stimulated proliferation of human blood mononuclear cells in the same range of concentration (data not shown). Because the disaccharide moiety of the lipid is gentibiose, its effect on the activity of **I** was also studied. Gentibiose did not show any mitogenicity itself, nor did it inhibit activity of the intact lipid in the range of concentration of (4 μg−2.5 mg)/mL (Figure 3; only one concentration of gentibiose at 2.5 mg/mL is shown).

The effect of mild acid hydrolysis of the lipid on the mitogenic activity was also evaluated. After hydrolysis of **I**, the lipid was extracted and rechromatographed by the same HPLC procedure (see Experimental Procedures). As expected, mitogenic activity could be detected at the position of elution of **I**, due to incomplete hydrolysis, but a glycolipid (**XI**) eluting near the void volume was recovered as the major product. This glycolipid (**XI**) was also mitogenic. It was able to stimulate mouse splenocyte proliferation to the same level as the intact lipid, but at a 10-fold higher concentration (Figure 3). This result not only demonstrated that the glycerophosphate group was not absolutely required for the activity but also established that the lipid (**I**) as originally isolated was responsible for the mitogenic activity.

DISCUSSION

Many bacterial products have long been known to possess a variety of immunological activities. Among those that are mitogenic are peptidoglycan, lipoteichoic acids (LTA), and lipopolysaccharides from Gram-negative bacteria. However, all of these are high molecular weight biopolymers, and there have never been any reports on the mitogenic activity of small molecules in the lipids extractable from bacteria by a mixture of chloroform and methanol. Herein it has been shown that a glycerophosphoglycolipid is highly mitogenic in both mouse splenocytes and human blood mononuclear cells. The mitogenic activity observed for this lipid derivative could not be due to contamination by endotoxin, as the endotoxin level in the lipid sample was undetectable by the limulus amebocyte lysate assay.

As the structure of the mitogenically active component (**I**) was emerging, we realized that it was very similar to one of the phosphoglycolipids isolated by Fisher *et al.* (1978). Further structural work confirmed that the mitogenic component we isolated was indeed the same. To our knowledge, however, this lipid has never been reported to be mitogenic.

This lipid is actually a part of the structure of lipoteichoic acid (LTA) of *B. subtilis*, and it has been suggested as the precursor of lipoteichoic acid biosynthesis (Fischer, 1988). Lipoteichoic acids have been known to possess many immunological activities (Levy *et al.*, 1990; Usami *et al.*, 1988; Takada *et al.*, 1995). The lipid isolated herein might be the active core of the lipoteichoic acid. A lipoteichoic acid fraction from *Enterococcus hirae* ATCC9790 was found to be very potent in inducing TNF-α, α/β interferon, and interferon-γ in *Propionibacterium acnes*-primed ICR mice. A phosphatidylglycolipid fraction from the acid-hydrolyzed LTA was also active in all these bioassays (Tsutsui *et al.*, 1991), suggesting that the glycolipid anchor of the LTA was the active core. Later examination by Takada *et al.* (1995) using synthetic glycolipids argued against such a conclusion. However, since the synthetic glycolipid prepared by Takada *et al.* was not exactly the same structurally as the glycolipids derived from the LTA, it has not been conclusively established that the natural glycolipid is inactive. Therefore, the structural basis for the immunological activities of LTAs is still not well defined. It seems that both the hydrophilic and hydrophobic regions play important roles, as suggested from

the structure of nontoxic *Rhodobacter capsulatus* lipid A, which is very similar to the toxic *Salmonella* lipid A in the hydrophilic head but significantly different from the latter in the hydrophobic region (Krauss *et al*., 1989). We have shown that lipid **I** is highly mitogenic and that its corresponding glycolipid is also active, although much less potent. It will be interesting to determine whether lipid **I** also possesses any other activities, such as TNF-inducing and antitumor activities (Usami *et al*., 1988; Takada *et al*., 1995).

Structural studies were also conducted on the first major mitogenic peak (Figure 1) eluting from the HPLC column. This peak was contaminated by other phospholipids, but NMR and mass spectral data indicated that the mitogenic lipid in that peak has the same head group as that of lipid **I**. The difference in chromatographic behavior may arise as a result of the presence of different fatty acids esterified to the head group.

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