Isolation and Structural Characteristics of a Monoclonal Antibody-defined Cross-reactive Phospholipid Antigen from\textit{Mycobacterium tuberculosis} and \textit{Mycobacterium leprae}*

Jean-Jacques Fournié, Raymond J. Mullins, and Antony Basten

From the Centenary Institute of Cancer Medicine and Cell Biology, University of Sydney, Sydney, Australia

A low molecular weight antigen of \textit{Mycobacterium leprae} and other mycobacteria was previously defined in our laboratory by means of IgG₂ monoclonal antibody termed L₄. The antigen had an apparent molecular mass of 4.5-6 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was assumed to be a glycoprotein on the basis of its staining with periodic acid Schiff and sensitivity to periodate treatment. In the present work, the cross-reactive and phospholipidic nature of the antigen, present in \textit{Mycobacterium tuberculosis} as well as in \textit{M. leprae} sonicates, was demonstrated and this enabled us to undertake its purification from crude \textit{M. tuberculosis} phospholipidic extracts. The L₄-reactive antigen from \textit{M. tuberculosis} called L₄-PIM, was purified by means of silicic acid high pressure liquid chromatography. Its characterization by gas chromatography and FAB-MS showed the antigen to be the common mycobacterial dimannosylated phosphatidylinositol (PIM₂), the structure of which had been previously established by others (Lee, Y. C., and Ballou, C. E. (1964) \textit{J. Biol. Chem.} 239, 1316-1327; Lee, Y. C., and Ballou, C. E. (1965) \textit{J. Biochem. (Tokyo)} 4, 1395-1404). Delineation of the L₄ epitope on \textit{M. tuberculosis} L₄-PIM revealed the involvement of the axial 2-hydroxyl of the α-D-mannosyl residues, without any detectable contribution from the myo-inositol. Consequently, L₄ was shown to react with PIM₂, the structure of which contains twice the number of epitopes as does PIM₁.

By using both immunostained thin layer chromatography and indirect enzyme-linked immunosorbent assay, similar L₄-PIM epitopes were demonstrated in \textit{M. leprae} sonicate, thereby explaining the cross-reactive nature of the L₄-monoclonal antibody. Antibodies of IgG class directed against \textit{M. tuberculosis} L₄-PIM were detectable in sera from patients with leprosy, but no evidence of T cell reactivity to L₄-PIM was obtained. The demonstration of a correlation of anti-L₄-PIM IgG and anti-disaccharide-conjugated bovine serum albumin IgM antibody titers in the sera of leprosy patients indicates that measurement of antibodies directed against L₄-PIM may have the potential to be used as a complementary assay to the disaccharide-conjugated bovine serum albumin test for diagnosis and monitoring of patients undergoing leprosy therapy.

The identification and characterization of protein and non-protein antigens from \textit{Mycobacterium leprae} has been greatly facilitated by the development of monoclonal antibody (mAb) technology. During the last 5 years, seven protein antigens have been defined with mAbs (1) which were subsequently used as probes to screen a \textit{M. leprae} DNA (2). Sequence data are now available on most of them including three heat shock proteins of molecular mass 18, 65, and 70 kDa, all of which contain T cell reactive epitopes (3-4). Two glycolipids with B cell epitopes defined by specific mAbs have also been characterized viz. the phenolic glycolipid-I, PGL-I (5-8) and the phosphorylated lipoarabinomannan (9, 10). PGL-I and its synthetic analogue, d-BSA form the basis of standard tests for measurement of \textit{M. leprae}-specific antibodies. Among the components of \textit{M. leprae} previously detected in our laboratory by mAbs was a third type of antigen which reacted with an IgG₂ mAb designated L₄ and had an apparent \textit{M}_{r} of 4.5-6 kDa on immunoblotting. This antigen was assumed to contain carbohydrate as it stained with periodic acid-Schiff and its L₄-binding epitope was sensitive to periodic acid but not to periodate treatment (11). The L₄-reactive antigen elicited a potent antibody response in leprosy patients. Furthermore, it was not restricted to \textit{M. leprae} sonicates being present in many other mycobacteria including \textit{Mycobacterium tuberculosis}, \textit{Mycobacterium bovis BCG}, \textit{Mycobacterium smegmatis}, \textit{Mycobacterium gaesi}, \textit{Mycobacterium ulcerans}, \textit{Mycobacterium simiae}, \textit{Mycobacterium scrofulaceum}, \textit{Mycobacterium fortuitum}, \textit{Mycobacterium nonchromogenicum}, and \textit{Mycobacterium phlei} as well (12).

The aim of the current experiments was to isolate and characterize the L₄-defined antigen in more detail. The results indicate that it is not a glycoprotein but a dimannosylated glycolipid belonging to the previously described family

---

*This work was supported by the Leprosy Eradication and Education Program of Lion International, the Centre National de la Recherche Scientifique, France, (to J. J. F.) and the National Health and Medical Research Council of Australia (to R. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Centre de Recherches de Biochimie et Génétique Cellulaires, Centre National de la Recherche Scientifique, 118 Rte de Narbonne, 31062 Toulouse, France.

†The abbreviations used are: mAb, monoclonal antibody; d-BSA, disaccharide-conjugated bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high pressure liquid chromatography; ITLC, immunostained thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; PBM, peripheral blood mononuclear cells; PGL-I, major phenolic glycolipid from \textit{M. leprae}; PI, phosphatidylinositol; PIM₁, phosphatidylinositol mannoside, \(i\) is the number of mannose residues; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
of phosphatidylinositol mannosides (PIMs) synthesized by most mycobacterial species. Since both M. lepra and M. tuberculosis extracts were shown to cross-react with L4 and contain PIMs, it was possible to purify the L4-reactive PIM from M. tuberculosis and to characterize its structure as that of the well-known PIM2. The epitope recognized by L4 on this molecule lies on the axial 2-hydroxy group of the α-D-mannosyl residues, thereby explaining the cross-reactivity of the L4 mAb with other glycolipids containing the same structural motif, such as the higher mannosylated PIMs of which PIM5 is the commonest amongst mycobacterial species. IgG antibodies to L4-PIM were detectable in sera from patients with leprosy but no evidence for T cell reactivity with L4-PIM was obtained. The antigen has been designated L4-PIM and its serological usefulness is discussed.

MATERIAL AND METHODS

Source of Bacteria and Purified Phospholipids

Lyophilized M. leprae bacilli (batch CD69) were obtained from Dr. R. J. W. Rees through the IMM LEF component of the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. A sonicate was prepared as described previously (11, 12) and found to have a protein content of 1.3 mg/ml as measured by Folin’s reagent. M. tuberculosis var. hominis pooled strains P, AN, and DT bacilli were provided as freeze dried cultures from C.S.I.R.O., Animal Research Laboratories, Parkville, Australia. Purified glycolipid standards used in this study were kindly provided by Dr. F. Lakhdar-Ghazal, Centre de Recherches de Biochimie et Genetique Cellulaires. These were: Micrococcus luteus (Strain Institut Pasteur A 270) α-D-mannosyl-(1-3)α-D-mannosyl(1-3)-sn-glycero-diglyceride, whose fatty acyl chain composition is 98% iso and anteiso methyl branched pentadecanoic acid, and M. phlei (Strain ATCC 534) tri- and tetraacylated dimannosyl phosphatidyllectosides (and a small amount of PIM3), whose structural assignments were confirmed by their mobilities on silica gel TLC (respectively, 1.4 and 0.9 relative to P1 in solvent A) and mannose-to-inositol ratios of 1.8 as determined by gas chromatographic analysis, in accordance with already reported data (23, 24).

 soybean phosphatidylcholine (PC), phosphatidyl choline, and phosphatidylserine were purchased from Sigma. Phosphatidylcholine pentamannoside (PIM5) was purified by preparative TLC from a crude M. phlei phospholipid extract (23). Its mobility in silicic acid TLC (0.4 relative to P1 in solvent A) and mannose-to-inositol ratio of 5.2 were consistent with its structural assignment as PIM5.

Monoclonal Antibodies

The monoclonal antibody L4 (IgGα) was produced as described previously (11). It was purified from ascites fluid by passage through a protein A column, and stored as a 1 mg/ml stock solution. L7, an IgG, mAb directed against the M. lepra 70-kDa protein antigen (11) was used as a negative control.

Human Serum Samples

Sera were obtained from the following sources: 48 patients undergoing treatment for leprosy and tuberculosis, respectively, at Prince Henry and Royal Prince Alfred Hospitals, Sydney; 22 Nepalese patients of multicabilar bacillary leprosy (pooled, Dr. W. Britton, Kathmandu, Nepal); control sera were obtained from Mantoux negative healthy medical students with no history of exposure to tuberculosis nor of BCG vaccination (n = 28). The leprosy patients were classified according to the criteria of Ridley-Jopling (13).

Fractionation of M. tuberculosis Lipids

Lyophilized M. tuberculosis bacilli were repeatedly extracted by CHCl3/CH3OH, 2:1 (v/v) and the pooled extracts were dried to yield about 100 g of a “crude lipid” fraction. 500 ml of hot acetone (50 °C) was added to the crude lipids, and the acetone soluble fraction (nonreactive to molybdenum blue on TLC) was removed. This step was repeated twice and the acetone-insoluble pellet was dried and further partitioned by CHCl3 and H2O into an organic and water phase. The organic extract, reactive to molybdenum blue and orcinol stains on silica gel TLC is termed “crude phospholipidic fraction.” Column chromatography was performed on glass columns (1 x 70 cm) using silicic acid (Kieselgel G60, 70–230 mesh, Merck, Darmstadt, Germany), and CHCl3/CH3OH mixtures as eluant, as indicated in the text. HPLC separations were performed on a silica column (Spherisorb 10 5 μm, 250 x 10 mm, Phenomenex, Torrance, CA) protected by Sutton Guard-PAK RCSS precolumns (Waters Assoc, Milford, MA) using an isocratic mobile phase of solvent B (see below) at a flow rate of 2 ml/min (for 90 min) followed by a 20-min gradient from 0 to 100% of CH3OH in the previous solvent as a rinsing step. Absorbance was monitored at 280 nm on a UV spectrophotometer system. 2- butanol (30%) was added and were purified for TLC by L4 and reactivity against L4 monoclonal by ELISA (see below). The L4 monoclonal reactive HPLC-purified PIM component from M. tuberculosis was termed L4-PIM.

Analytical Methods

This layer chromatography was performed on silicic gel 10 x 20-cm aluminum backed foils (Kieselgel, G60, Merck, Darmstadt, Germany) with the following eluent mixtures as indicated in text: CHCl3/CH3OH/H2O 60:35:5 (v/v) (solvent A), CHCl3/CH3OH/H2O 60:35:5 (v/v) (solvent B); or CHCl3/CH3OH/hexane/2-butanol 40:10/10/10 (v/v) (solvent C). Phosphorus-containing lipids were located with molybdenum blue reagent and carbohydrates were detected by spraying the plate with ornicol reagent and heating it at 120 °C for 5 min. Mild alkaline hydrolysis was performed as described (23), and the lipidic phase was dried and methylated by diazomethane prior to GC analysis. PIMs (1 mg) were analyzed for carbohydrate content after acid hydrolysis with 1 mL of 2 N HCl for 2 h at 70 °C, followed by neutralization drying prior to trimethylsilylation and GC analysis. Gas chromatography was performed on a Girdel series 30 equipped with an OV1 coated 25-mm capillary column (inner diameter 0.3 mm) using N2 as vector gas at a flow rate of 2.5 ml/min and a flame ionization detector. The detector temperature was 250 °C and the oven temperature from 150 to 220 °C for trimethylsilyl derivatives of carbohydrates and from 150 to 290 °C for fatty acid methyl esters with a temperature increase of 3 °C/min.

SDS-PAGE and Immunoblotting with L4

SDS-PAGE of PIM fractions was performed on 5 x 7-cm slab gels as described (14). The lanes were loaded with 50 μg of PIM samples in SDS reducing buffer. After electrophoresis, the gels were fixed in 40% ethanola, 5% acetic acid overnight, then stained with either Coomassie Blue or silver nitrate. Silver staining was enhanced by a 5 min preincubation in 0.7% sodium periodate in fixing buffer as described (15). Immunoblotting of the SDS-PAGE with L4 mAb was performed as previously described (11, 12).

FAB Mass Spectrometry of L4-PIM

Both positive and negative mode FAB spectra were recorded on a VG 70-250 SEQ mass spectrometer operated with 8 kV maximal acceleration and a resolution of 1500. The calibration was based on CsI clusters. The scanning mode was a linear accelerating voltage scan from 1000 to 2000 a.m.u., with a multichannel accumulation of 10 scans. FAB ionization was performed with a cesium gun operated at 30 KeV and 2 μA emission current. The gun was directed against samples consisting of about 50 μg of phospholipids dissolved in solvent B and mixed 1:1 with matrix prior to deposition onto the FAB target.

Enzyme-linked Immunosorbent Assays

ELISA of HPLC-derived Fractions—After drying, the HPLC fractions were weighed, redissolved in solvent B, and adjusted to a final concentration of 10 μg/ml in absolute ethanol. 50 μl were added to the wells of polystyrene flat bottom 96-well microtiter plates (Linbro, Flow Laboratories, Australia Pty, Australia) and air dried. Control wells contained 50 μl of ethanol alone. ELISA plates were blocked overnight at 4 °C by 200 μl of blocking buffer consisting of 5% bovine serum albumin (Fraction V, Pentex, Miles Diagnostics, Kankeea, IL), 2% normal goat serum in PBS + NaN3 (0.1%). After one wash in PBS, the antibody solution (100 μl/well of 1/100 L4 mAb) was added and incubated for 27 h. After three washes in PBS containing 0.05% Tween 20, an alkaline-phosphatase-linked second antibody (sheep anti-mouse polyclonal antibody, Sigma) was added (100 μl/well) and incubated for 2 h at 37 °C. Finally the plate was washed again before addition of nitrophenylphosphate coloring re-
agent (100 μl/well). 20 min later the 405-nm color change was read with a Titertek Multiscan ELISA plate photometer. Positive reactions were designated as those with optical densities of three standard deviations above the mean of the negative control. Positive reactions were then pooled and rerun on the HPLC a further three times to yield “highly purified L4-PIM.”

Indel of ELISA of Glycolipid and Carbohydrate Fractions—Inhibition ELISAs were performed following a one-step procedure using inhibiting solutions comprised of either methyl glycoside solubilized in PBS (18) or glycolipid fraction solubilized in PBS containing 1 mg/ml sodium deoxycholate as previously described (44). These inhibitory solutions were mixed with L4 mAb present in a final dilution of 5-500, plus or minus their addition to ELISA wells containing 12% L. leprae sonicate or 50 ng of L4-PIM and blocked with 5% BSA in PBS. Two control reactions were performed. In the first, a similar procedure was followed except that the inhibitor was omitted (100% L4 binding) while in the second, L4 mAb 1:500 was reacted with solubilized inhibitor in wells without coated antigen (0% L4 binding). Each assay was performed in triplicate and the percentage of L4 specific binding was calculated as follows: (mean A 405 nm from test) – (mean A 405 nm from blank)/(mean A 405 nm from 100% L4 binding).

Human Serological Response to HPLC Purified L4-PIM—Flat bottom polystyrene 96-well microtiter plates (Linbro) were coated with 50 μl of 2.5 μg/ml PIM in ethanol. Negative control wells contained phosphatidylinositol (Soybean PI, Sigma) coated under the same conditions. The plates were dried, blocked with normal goat serum and substracted from the absorbance of the test wells.

Human Serological Response to d-BSA—The human serological response to HPLC purified L4-PIM was compared with the IgM response of 40 treated leprosy patients to d-BSA (the synthetic analogue of the L. leprae-specific phenolic glycolipid antigen). ELISA of human antibodies against the d-BSA synthetic antigen was performed in parallel according to the standardized protocol (19).

Immuno Thin Layer Chromatography

ITLC were performed on Silica Gel Whatman K6 plates 5 x 5 cm (Whatman, Maidstone, United Kingdom) (20) using the solvents indicated under “Results.” Samples consisting of 50 μg of crude M. tuberculosis PIMs, soybean PI, and M. leprae sonicate were applied from left to right on the bottom of each plate. The same chromatograms were performed in triplicate so that they could be stained by orcinol for carbohydrate as well as being immunoblotted with L4 and the irrelevant L7 mAb (11). After chromatography, the plates were blocked by incubation overnight at 4 °C in rocking chambers containing 40 ml of ELISA blocking buffer (see above). The plates were then transferred without rinsing into 40 ml of L4 mAb diluted 1:40 in PBS containing 0.1% BSA, 0.2% normal goat serum, 0.05% Tween 20, and 0.1% NaN3, incubated for 2 h at 37 °C with continuous rocking, and washed five times (to limit wash in) in Tris-buffered saline (TBS), pH 7.2. Subsequently anti-mouse IgG biotinylated second antibody (Vectorstain ABC Kits, Vector Labs Inc., Burlingame, CA) was added in TBS as recommended in the suppliers instructions for 2 h with rocking at 37 °C. After five washes in TBS, the plates were exposed to avidin-biotin-horseradish peroxidase complex (Vectorstain ABC Kits, according to suppliers instructions), rocked for 1 h at room temperature, and rinsed again five times. The immunostained spots were then revealed by the non-diffusing colored reagent produced by peroxidase and a freshly made dianisobenzidine solution with NiCl2 salts. For this purpose 20 ml of Tris-HCl 0.1 M, pH 7.2, containing 1 mg/ml dianisobenzidine was added to 13.5 ml of H2O2 at 30%, 0.4 ml of O2-methyl phosphenate, 5 ml of H2O, pH 7.2, and 0.2 ml of NiCl2 6% in 20 ml of distilled H2O. The reaction was stopped by washing in tap water, and after drying the plates were stored in the dark.

Proliferative Responses

Samples of blood were collected into preservative-free heparin (10 IU/ml, Weddel Pharmaceutical, Thornleigh, Sydney, Australia). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque density gradients, washed in RPMI-1640 (Flow Laboratories, Australia) containing 0.85 g/liter NaHCO3, 25 mm HEPES, penicillin (50 μg/ml), and streptomycin (100 μg/ml) and adjusted to a concentration of 107/ml in RPMI containing 10% heat-inactivated human A serum (Red Cross Blood Transfusion Service, Sydney). Flat bottom 96-well culture plates were coated with 50 μl of either 0.1 or 10 μg/ml of L4-PIM or sterile filtered ethanol alone (control) and dried. To other wells M. leprae sonicate (10 μg/ml) was added so that the proliferation response to L4-PIM could be compared. PBMC were then plated out in triplicate at a final cell density of 2 x 105 cells in 200 μl/well, and the trays were incubated for 5 days at 37 °C in a humidified atmosphere containing 5% CO2 in air. On day five each well was pulsed with 0.5 μCi of [3H]thymidine in sterile PBS. 24 h later, the cells were harvested onto glass fiber discs (Titertek, Flow Laboratories) with a cell harvester (Skatron, Liebyen, Norway). The discs were dried and transferred to vials containing 2 ml of 0.6% 2,6-diphenylxazole to toluene. The [3H] thymidine incorporation into newly synthesized DNA was measured in disintegrations/min by liquid scintillation spectrometry with a Rackbeta 1218 counter (LKB Wallac, Turku, Finland). Results were expressed as mean disintegrations/min of [3H]thymidine incorporation/culture, plus or minus one standard deviation.

RESULTS

The L4 Reactive Low Molecular Weight Antigen Is a Cross-reactive Glycosylated Phospholipid—Previous work (11, 12) had demonstrated that the monoclonal antibody L4 recognizes a cross-reactive carbohydrate epitope located in the bacterial cell wall of M. tuberculosis, M. bovis BCG, and other mycobacteria as well as M. leprae. In view of the limited supplies of M. leprae, the possibility of using M. tuberculosis as a source of material for characterizing the physicochemical nature of the L4 reactive antigen was examined in a series of inhibition experiments. As shown in Fig. 1A, not only did L4 mAb bind ELISA plates coated with M. leprae sonicate, but such binding was inhibited by both crude lipids (CH2OH/CHCl3 extract) and phospholipids (acetone insoluble pellet) derived from the M. tuberculosis cultures (Fig. 1B).

The phospholipid fraction was more inhibitory than the crude lipids (50% inhibition with 750 μg/ml crude lipids compared with 175 μg/ml with crude phospholipids), indicating this fraction to be enriched for the antigen recognized by L4 mAb. In order to confirm that L4 recognized a cross-reactive phospholipid antigen present in both M. tuberculosis and M. leprae, two additional experiments were performed. In the first, the migration of M. tuberculosis phospholipids was analyzed in silver-stained SDS-PAGE and immunoblots (Fig. 2). The M. tuberculosis-derived phospholipid fraction gave an obvious silver-stained band in SDS-PAGE, which corresponded in position to that of the L4-defined M. leprae low molecular weight antigen, being located at the bottom of the gel. No such reaction was observed in equivalent immunoblots performed with the unrelated L7 mAb. In the second experiment, 50 μg of M. tuberculosis phospholipids, M. leprae sonicate, and soybean phosphatidylinositol were run in parallel on analytical TLC eluted with solvents A and C (sprayed for sugars) and on ITLC to detect L4-reactive material. As shown in Fig. 3, L4 but not the control mAb L7 bound strongly to closely migrating polar phospholipids, present in both M. tuberculosis and M. leprae sonicates, the migration pattern of which matched that of the well known polar members of the mammophosphoinosidyl-PIM (21-25).

Taken together, these data clearly established that M. tuberculosis cultures could act as a source of the L4-defined antigen which appears to be a common member of the mycobacterial PIM glycolipid family rather than being a low mo-
bound and inhibition of deoxycholate and tested at the concentration indicated on the abscissa as lipids were diluted by sonication in PBS and 1 pg/ml subfraction. Problems have previously involved alkaline degradation of the crude phospholipid extract loaded onto ELISA plates (at 10 pg/ml crude lipids) and tested by direct ELISA, the binding of L4 to phospholipidic mixture prior to further separation (28, 29). How-ever, when both deacylated and untreated crude mycobacterial phospholipid extracts were coated onto ELISA plates (at 10 µg/ml) and tested by direct ELISA, the binding of L4 to phospholipids was abolished by alkaline treatment (data not shown). Conformational alterations of the L4-defined epitope and/or lack of binding of the deacylated antigen to the ELISA plate could explain this observation.

**FIG. 1.** Binding of L4 mAb to bound *M. leprae* sonicate (A) and inhibition of L4 binding to *M. leprae* sonicate by *M. tuberculosis* lipids (B). A, binding of L4 mAb diluted as indicated on abscissa to bound *M. leprae* sonicate, coated onto ELISA plates at: 50 µg/ml (●), 25 µg/ml (▲), 10 µg/ml (●), 5 µg/ml (●), 2.5 µg/ml (●), and 1 µg/ml (○) of protein antigen concentrations. B, *M. tuberculosis* lipids were diluted by sonication in PBS + 1% BSA + 0.1% sodium deoxycholate and tested at the concentration indicated on abscissa as inhibitors of the binding of L4 mAb diluted 1:300 (total volume) to bound *M. leprae* (2.5 µg/ml crude lipids (●) and phospholipidic subfraction (●) from crude lipids.

**FIG. 2.** Migration of *M. tuberculosis* phospholipids and L4-reactive material in SDS-PAGE. Silver nitrate stain (SN), L4 immunoblotting (L4), and L7 control (L7) antibody immunoblotting of SDS-PAGE loaded with 50 µg of *M. tuberculosis* phospholipids. Molecular weight glycoprotein as was previously thought (11, 12). Purification of the L4-reactive Antigen from *M. tuberculosis* Crude Phospholipids—The mycobacterial mannosphospholipidiosides have previously been shown to have serological reactivity, but in many cases, the precise nature of the B-cell epitopes remained unknown due to difficulties experienced in resolving such complex mixtures into purified molecular species (21, 23-26). One way of overcoming these separation problems has involved alkaline degradation of the crude phospholipid mixture prior to further separation (28, 29). However, when both deacylated and untreated crude mycobacterial phospholipid extracts were coated onto ELISA plates (at 10 µg/ml) and tested by direct ELISA, the binding of L4 to phospholipids was abolished by alkaline treatment (data not shown). Conformational alterations of the L4-defined epitope and/or lack of binding of the deacylated antigen to the ELISA plate could explain this observation.

Consequently, an alternative strategy for isolating the *M. tuberculosis* L4-reactive material was devised, based on its purification directly from the native phospholipidic extract by conventional liquid chromatography and HPLC on silicic acid.

*M. tuberculosis* crude phospholipids were separated on a silicic acid column eluted stepwise with CHCl₃, CHCl₃ + 10% CH₃OH, CHCl₃ + 50% CH₃OH, and CH₃OH only. Both the glycosylated phospholipids and L4-reactive material were recovered in the last two fractions (as measured by ITLC and ELISA, data not shown) which were pooled, dried, and solubilized in solvent B before separation on silicic acid HPLC. The HPLC mobile phase was an isocratic mixture of solvent B, with a flow of 2 ml/min; fractions were collected every 2 min, dried, weighed, and further resuspended in ethanol at a final concentration of 10 µg/ml for the coating of ELISA wells which were tested for reactivity to L4 mAb by ELISA. Fig. 4A is a representative HPLC chromatogram of *M. tuberculosis* phospholipids showing that almost all of the L4-reactive material was recovered in fractions 17–21 corresponding to those eluted with nearly 4.5 void volumes of solvent. These fractions gave a positive band in the low molecular weight region (bottom) of silver-stained SDS-PAGE and L4 immunoblots. They were therefore pooled and dried to yield 3 mg of a fraction designated L4-PIM.

On analytical TLC stained for carbohydrate (Fig. 4B), the *M. tuberculosis* L4-PIM appeared as two closely migrating spots whose chromatographic mobilities, relative to that of standard soybean phosphatidylinositol, indicated structures related to the well known phosphatidylinositol dimannosides (PIM₂) found in mycobacteria (21-23) and corynebacteria (27). Similar compounds were present in *M. leprae* sonicates (Fig. 4B, track A), in agreement with previous data (30) reporting the occurrence of polar PIMs in *M. leprae* harvested from infected armadillos.
mAbs-defined Mycobacterial Phospholipid Antigen

considered adequate for analysis of non-volatile and amphipathic biomolecules, but there is little information on its suitability for analysis of underivatized phospholipids (34-37). Initially, negative mode FAB was selected as it appeared to have the ability to detect stable and intense (M - H)^+ phosphatidyl diester anions (34, 36). To ensure that reproducible mass spectra from L4-PIM were obtained, particular care was taken over sample preparation in matrix prior to its addition to the FAB target (35), as phospholipids can form micelles which hamper their ejection from the target.

As indicated in Fig. 5A, successive scannings of the 1000-2000 a.m.u. zone of negative mode spectra obtained with L4-PIM in triethanolamine matrix consistently contained the intense m/z 1133 and 1175 signals with some minor m/z 1147 (+14) and m/z 1161 (+28) signals attributed to differences in chain length of fatty acid homologues. No stable and intense ions of higher mass were detected in these experiments, except a transient and much weaker signal at m/z 1217 suggesting that these signals could be legitimately attributed to the quasi molecular (M - H)^+ anions of dimannosylated phosphatidyl-inositol (PIM_2), the fatty acid composition of which consists of two palmitic acid (M = 1134 a.m.u.), one palmitic plus one tuberculostearic acid (M = 1176 a.m.u.), and two tuberculostearic acid (M = 1218, Fig. 5C). In agreement with the GC results, nearly equivalent distribution of the m/z 1133, 1175, and 1217 ions in these spectra would be expected as palmitate

identified by GC coinjections as methyl palmitate and methyl tuberculostearate, in a molar ratio of about 1:1 (for reviews, see Refs. 31–33). The hydrolysable fraction from the L4-PIM acid hydrolysis was analyzed by GC under its trimethylsilyl derivative, and only D-mannose and myo-inositol in a molar ratio of 2:1 were detected consistent with being a PIM_2, the dimannosylated phosphatidylinositol structure. The presence of one or two additional fatty acid (palmitate or tuberculostearate) esters on either the myo-inositol or a mannosyl residue however, is a well recognized feature of the PIM family of molecules (21-23, 30), and this structural feature could be expected in the case of L4-PIM in view of the sensitivity of the L4-reactive epitope to alkali treatment.

To determine whether the L4-PIM molecule contained one or more additional fatty acid esters in its carbohydrate portion, molecular mass measurement was undertaken on the underivatized L4-PIM. The FAB ionization technique is con-

FIG. 4. HPLC chromatogram (upper) of the L4-reactive material (lower) from M. tuberculosis phospholipids (A) and analytical TLC of the M. tuberculosis L4-PIM (B). A, M. tuberculosis phospholipids (1 mg) were loaded on a silicic acid (5μm, 300 x 8 mm) HPLC column and eluted by 2 ml/min solvent B with fractions being collected each 4 ml. UV detector (280 nm) profile (upper) with corresponding detection of the presence of L4-PIM in each fraction by direct ELISA measuring the binding of L4-mAb diluted 1:10 to wells coated with 10μg/ml of the collected fractions. B, orcinol stain on silica gel TLC eluted with solvent A of M. tuberculosis phospholipids (A), M. leprae sonicate (B), L4-PIM (C), and standard soybean P1 (D).

FIG. 5. FAB mass spectrometric molecular mass measurement of L4-PIM and example of PIM_2 structure. A, negative mode spectrum of L4-PIM in triethanolamine matrix. B, positive mode spectrum of L4-PIM in glycerol matrix. C, the PIM_2 structure shown above for L4-PIM, involving one palmitic and one tuberculostearic residue is a single isomer of PIM_2 since the respective fatty acid position on glycerol or positions of glycosidic linkages on inositol were not determined in the M. tuberculosis and M. phlei PIM_2 dimannosylated phosphatidylinositol structures published previously (22, 28, 29).
and tuberculostearate were found in similar amounts. However, the FAB ionization mode induces variable relative intensities for the signals obtained from glycolipid samples. This phenomenon is related to the time-dependent evolution of the amphipathic sample desorption process from FAB matrix. Therefore, the relative ion intensities in the present spectra should only be considered as indicative of the fatty acyl sample composition.

The experiment was repeated by scanning for ions in the positive mode. Although the results were less clear cut, they confirmed the molecular masses of 1134 and 1176 obtained in the negative mode. Interestingly, no protonated ion but only sodium and potassium clusters were detected, with a kinetic shift of dinatriated adducts in early scans toward dipotassium adducts in later scans (Fig. 5B). The signal m/z 1221 is the (M – H + 2Na)⁺ ion of M = 1176, m/z 1237 is (M – H + Na + K)⁺ heterologous mixed adduct of M = 1176; while m/z 1211 and m/z 1253 are the (M – H + 2K)⁺ dipotassium adducts of M = 1134 and 1176, respectively. It is worth noting that in both positive and negative mode spectra, ions 2 mass units lower than the molecular species and of nearly one-third their abundance were present. These represent H₂ losses which are known to occur in FAB spectra of many phospholipids (94) and particularly with the standard of phosphatidylinositol.

Taken together, the findings confirm that the M. tuberculosis L4-defined antigen, L4-PIM, is identical to PIM₂ dimannosylated phosphatidylinositol the structure of which was determined previously in M. tuberculosis and M. phlei (22, 28, 29). Since the respective positions of the fatty acid on the glycerol and location of the glycosidic linkages were not specified in the original studies an isomeric example of PIM₂ structure derived from published data is presented in Fig. 5C.

**Analysis of the L4-defined Epitope on the Mannophosphoinositides**—Two sets of experiments were performed in an attempt to define the B-cell epitope recognized by L4 per se. In the first, we compared the respective contribution of the carbohydrate residues in the L4-defined epitope since it had previously been shown to be degraded by sodium periodate treatment. For this purpose the capacity of a-D-methyl mannoside stereoisomers. Two comments can be made about these conclusions an isomeric example of PIM₂ structure derived from published data is presented in Fig. 5C.

**Fig. 6. Delineation of the L4 epitope on M. tuberculosis L4-PIM.** A, inhibition of binding of the reference L4 mAb (diluted 1:500) to 50 ng of L4-PIM (PIM₂) bound to ELISA plates by increasing concentrations of myo-inositol (Δ), α-D-methyl glucoside (▲), α-D-methyl galactoside (●), and α-D-methyl mannose solubilized in PBS with 1% BSA (□). Controls were performed in the same way except that the inhibitor was omitted (100% L4 binding) or antigen was omitted (0% L4 binding). Each experiment was performed in triplicate wells and the % of L4 mAb binding was calculated as follows: (mean A₄₀₅ nm from sample) – (mean A₄₀₅ nm from blank) / (mean A₄₀₅ nm from 100% L4 binding). B, direct ELISA of L4 mAb binding to 50 ng (pale shading) and 500 ng (dark shading) of the following antigens. Blank, (none): crude PIMs (crude M. tuberculosis phospholipid extract); PI (phosphatidylinositol); PE (phosphatidylethanolamine); PS (phosphatidylserine); Man₄GL (M. luteus) [Strain Institut Pasteur A 270] a-D-mannosyl(1-3) a-D-mannosyl(1-3)-sn-diglyceride; PIM₁ (the HPLC-purified M. tuberculosis L4-PIM₁); triacyl PIM₁ and tetracetyl PIM₁ (apolar M. phlei dimannosylated phosphatidylinositol) and PIM₂ (M. phlei phosphatidylinositol pentamannoside purified by preparative TLC).

Compared by direct ELISA. In order to ensure maximal glycolipid absorption to the wells, two antigen coatings of 50 and 500 ng (50 µl of 1 or 10 µg/ml of ethanol) were carried out. Both a positive control (crude M. tuberculosis phospholipids, A₄₀₅ > 1.00) and a negative control (dried ethanol alone, A₄₀₅ < 0.10) were included in the analysis. As shown in Fig. 6B, L4-PIM₁ (M. tuberculosis HPLC-purified L4-PIM₁) and M. phlei PIM₂ bound strongly at both concentrations (A₄₀₅ > 1.00), whereas the tri- and tetracylated derivatives of PIM₂ gave a weaker reaction (A₄₀₅ of 0.38 and 0.72, respectively) evident only at the high (saturating) antigen dose. Interestingly, an unrelated mannosylated glycerolipid from M. luteus was also recognized weakly at the highest antigen concentration (A₄₀₅ of 0.43), but no reactivity was demonstrated with phosphatidylinositol, phosphatidylcholine, or phosphatidylserine (A₄₀₅ of 0.06, 0.04, and 0.04, respectively) despite the fact that these phospholipid antigens can efficiently coat ELISA plates under these conditions (16-18). In other words, the epitope recognized by L4 on PIM is contained on α-D-mannoside residues but not on myo-inositol. The intense
cross-reactivity of L4 mAb with those PIMs expressing 2- and 5-mannoside residues was expected since these molecules bear two and four accessible mannoside epitopes, respectively (the 5th mannoside residue in PIMs, being glycosidically linked to position 2 of the 4th mannoside residue, (28, 29)). In addition, the results lead to the prediction that L4 should also bind to PIM3 and PIM5, although this was not tested in the current study.

Occurrence of the L4-defined Epitope in M. leprae—The results described thus far indicate that the L4-defined epitope present in most mycobacterial sonicates (11) is not a glycoprotein but carbohydrate in nature and is expressed on a group of cross-reactive mycobacterial mannophosphoinositides, which were detectable in L4-ITLC of M. tuberculosis extracts as well as M. leprae sonicates (Fig. 2).

In an attempt to confirm that L4-reactive PIMs are also present in M. leprae both L4-reactive PIM1 (M. tuberculosis HPLC-purified L4-PIM) and M. phlei PIMs were used to inhibit the specific binding of L4 mAb (diluted 1:500) to ELISA plates coated with 200 ng of M. leprae sonicate. As shown in Fig. 7 both molecules inhibited binding, and PIM1 was nearly 10-fold more potent than PIM3. The 50% inhibitory concentrations being about 10 and 100 μg/ml, respectively. This difference is not surprising since PIM3 expresses twice as many L4-epitopes as PIM1 and supports the conclusion that L4 binding to M. leprae sonicate is due to the presence of L4-epitopes on the PIMs within it.

Human Serological Response to M. tuberculosis L4-PIM—As it had been previously shown that the low molecular weight antigen of M. leprae recognized by L4 is a potent B-cell immunogen in man (11, 12), the question was asked whether the human serological response to M. tuberculosis HPLC-purified L4-PIM could be used as a serological marker for infection with M. leprae.

Antibody levels were measured by ELISA in sera from a range of human subjects undergoing treatment for tuberculosis or leprosy as well as healthy Mantoux negative controls (Table I). No difference in the IgG response to L4-PIM was observed between Mantoux negative controls and tuberculosis patients, whereas elevated levels were detected in the sera from treated and untreated leprosy patients across the clinical spectrum, being highest in newly diagnosed lepromatous cases. In patients with treated leprosy the IgG anti-L4-PIM response was correlated with the IgM response to d-BSA which is generally regarded as the most reliable diagnostic assay for leprosy infection (Fig. 8).

### Table I

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>No. tested</th>
<th>Anti-L4-PIM IgG response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Mantoux negative</td>
<td>28</td>
<td>0.280 ± 0.166</td>
</tr>
<tr>
<td>Treated tuberculosis patients</td>
<td>8</td>
<td>0.282 ± 0.262</td>
</tr>
<tr>
<td>Treated leprosy patients</td>
<td>7</td>
<td>0.513 ± 0.247</td>
</tr>
<tr>
<td>Healthy Mantoux positive</td>
<td>22</td>
<td>1.070 ± 0.070</td>
</tr>
</tbody>
</table>

**FIG. 7.** Cross-reactivity between M. leprae L4-defined antigen and L4-reactive PIMs. M. tuberculosis HPLC-purified L4-PIM (C) and M. phlei-derived PIMs (A) were solubilized in PBS containing 1 μg/ml sodium deoxycholate and tested as inhibitors of the specific binding of L4 mAb (diluted 1:500) to ELISA wells coated with 200 ng of M. leprae sonicate. Control reactions were performed in the same way except that the inhibitor was omitted (100% L4 binding) or antigen was omitted (0% L4 binding). Each experiment was performed in triplicate wells and the % of L4 mAb binding to M. leprae sonicate was calculated as follows: (mean A405 nm from sample) — (mean A405 nm from blank) / (mean A405 nm from 100% L4 binding to M. leprae sonicate alone).

**FIG. 8.** Correlation of anti-L4 IgG and anti-d-BSA IgM responses in leprosy patients. Antibody responses are expressed as optical densities at 405 nm with the vertical broken line representing the cut-off value for positive anti-d-BSA responses.

### Table II

<table>
<thead>
<tr>
<th>Subject</th>
<th>Background</th>
<th>L4-PIM</th>
<th>M. leprae sonicate (10 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) LL</td>
<td>NT</td>
<td>1.8 ± 0.4</td>
<td>13.8 ± 3.7</td>
</tr>
<tr>
<td>2) LL</td>
<td>NT</td>
<td>1.0 ± 0.1</td>
<td>34.8 ± 18.4</td>
</tr>
<tr>
<td>3) LL</td>
<td>NT</td>
<td>6.3 ± 3.3</td>
<td>39.0 ± 6.0</td>
</tr>
<tr>
<td>4) TT</td>
<td>NT</td>
<td>1.3 ± 0.3</td>
<td>182 ± 16.0</td>
</tr>
<tr>
<td>5) M</td>
<td>NT</td>
<td>2.6 ± 1.1</td>
<td>25.7 ± 11.5</td>
</tr>
<tr>
<td>6) M</td>
<td>NT</td>
<td>2.8 ± 1.0</td>
<td>38.9 ± 13.2</td>
</tr>
</tbody>
</table>

**Human T Cell Reactivity to M. tuberculosis L4-PIM—Mycobacterial glycolipids (although good B-cell immunogens) are not normally recognized by T cells. Possible T cell reactivity of M. tuberculosis highly purified L4-PIM was investi-
gated by comparing the in vitro proliferative responses to L4-PIM with those to M. leprae sonicate. PBM were obtained from three BCG-vaccinated Mantoux positive healthy subjects and three leprosy patients (two lepromatous and one tuberculoid) whose sera contained high levels of anti-L4-PIM and anti-d-BSA antibodies. These donors were selected on the basis that one or both groups should in theory possess T cell memory for specific or cross-reactive M. leprae antigens. A measurable proliferative response was observed in the cultures exposed to M. leprae sonicate particularly in the case of the tuberculoid patient (Table II) but in none of the cultures exposed to L4-PIM. On the contrary its presence either at 1 or 10 μg/ml led to a reduction in proliferation below background (medium only) similar to the inhibitory effect observed previously with lipoarabinomannan-B from M. tuberculosis (37, 38), the mycobacterial phenolic glycolipids (39), and peptidoglycolipids (40). It seems likely that such inhibitory properties are widespread among mycobacterial glycolipids, and we are currently investigating that phenomenon.

**DISCUSSION**

The results from this study indicate that the low molecular mass (4.5–6 kDa) cross-reactive antigen from M. leprae previously defined by the monoclonal antibody L4 (11) is a member of the phosphatidylinositol mannoside family of glycolipids. On the basis of immunoblotting from SDS gels, the antigen was initially thought to be a glycoprotein, the carbohydrate component of which constituted the epitope recognized by L4 (12). However, it could not be purified on an L4 affinity column, due in retrospect to the poor solubility of the PIMs in aqueous buffers. The presence of the antigen in crude extracts from other mycobacteria than M. leprae such as M. tuberculosis (Fig. 1) meant that the latter could be used as a source of large amounts of material for analysis rather than having to rely on scarce supplies of M. leprae per se. Since it was not possible to detect L4-reactive antigen by direct ELISA of the deacylated phospholipids, the antigen was purified from the silicic acid HPLC separated components obtained from crude M. tuberculosis PIMs (Fig. 4).

The evidence supporting the PIM nature of the L4-reactive M. tuberculosis antigen may be summarized as follows. Once purified by HPLC, the L4-PIM fraction gave a positive silver nitrate-stained band as did its immunoblot with L4 mAb in the low molecular weight region (bottom) of SDS-PAGE. Following analytical TLC L4-PIM appeared as two phosphorus and carbohydrate stained spots of chromatographic mobilities similar to that of PIM₁, the common mycobacterial dimannosyl phosphatidylinositol. GC analysis of the fatty acid and carbohydrate content of L4-PIM revealed the presence of characteristic palmitic and tuberculostearic acid residues together with myo-inositol and d-mannose in a molar ratio of 1:2 which is characteristic of PIM₁. Confirmation of the deacylated PIM₁ nature of the antigen was obtained from the molecular masses of 1134 and 1176 for L4-PIM as measured by both positive and negative mode FAB mass spectrometry (Fig. 5A and B). To our knowledge this is the first report of a molecular mass measurement for an underivatized mannosphosphoinositol, and the results are in fact consistent with the already published M. tuberculosis PIM₁ structure (28, 29); an isomer example of PIM₁ structure is presented in Fig. 5C.

Delineation of the L4 epitope on M. tuberculosis L4-PIM revealed that the immunodominant moiety does not lie in myo-inositol but in the α-d-mannoside residues, with an important contribution being made by the axial hydroxyl in position 2, as exemplified by the lack of reactivity of its glucos stereoisomer (Fig. 6A). The L4 mAb was shown to cross-react with other glycolipids bearing such epitopes, among which the most commonly encountered in mycobacteria in PIM₁ with a structure containing twice as many L4 epitopes as PIM₁. Furthermore, since the L4 epitope is located on the mannoside residue, the lack of L4 reactivity with deacylated PIMs in direct ELISA can in retrospect be attributed to inadequate binding of these molecules to the ELISA wells under the coating conditions used. Both L4-ITLC and indirect ELISA were used to show that M. leprae sonicate contains similar L4-binding PIMs, thereby explaining the cross-reactivity of the L4 monoclonal antibody. The occurrence of similar PIM₁ and PIM₂ in M. leprae, however, could not be formally demonstrated using similar purification techniques since sufficient supplies of M. leprae were not available.

The PIM family of glycolipids are known to be good B-cell immunogens (for review see Refs. 31–33). They have been shown to elicit significant antibody responses both in rabbits immunized with BCG and M. tuberculosis (23, 25), and in humans infected with M. leprae or M. tuberculosis (25, 26, 41–43). By coating ELISA plates with purified L4-PIM, high titer anti-L4-PIM IgG antibodies were detected in sera from patients with leprosy particularly at the lepromatous pole of the clinical spectrum. Interestingly, only low levels of antibody were detected in sera from tuberculosis patients despite the cross-reactivity of the L4-defined B-cell epitope. This could reflect the smaller antigenic loads in patients with tuberculosis as opposed to leprosy, or alternatively L4-PIM could be more abundant in cell walls from M. leprae than from M. tuberculosis. Despite its cross-reactivity, however, the serological response to L4-PIM appears to be relatively specific for infection with M. leprae and has the potential to be used as an alternative assay or as an adjunct to the anti-PGL-I assay for diagnosis or monitoring of the response to chemotherapy. In addition, the low molecular weight and stability of L4-PIM should permit the development of antigen detection assays in body fluids such as urine or cerebrospinal fluid.

No evidence was obtained to support the presence of T cell epitopes on L4-PIM when its capacity to stimulate a proliferative response by PBM was compared with that of crude M. leprae sonicate (Table II). This is consistent with current dogma that T cells only recognize peptides in association with MHC class II on antigen presenting cells. On the contrary L4-PIM appeared to exert a suppressive effect on T cell proliferation if used in sufficient concentration (Table II). Similar observations have been reported with other glycolipid B-cell antigens from mycobacteria (37–40), suggesting a potential role for them in modulating the immune response to mycobacteria, particularly at the site of infection.

_Acknowledgments—We wish to thank Dr. W. Britton, Anandaban Leprosy Hospital, Katmandu, Nepal, who developed the monoclonal antibody L4 and provided the leprosy patients’ sera. We also thank Dr. R. Garzia for the generous gift of tuberculosis patients’ sera and E. Adams for her assistance with lymphoproliferative assays. We are grateful to Dr. G. Puzo, Centre National de la Recherche Scientifique, Toulouse, France, for providing analytical facilities and continuous encouragement during the completion of this work._

**REFERENCES**