Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG

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Lipoarabinomannan (LAM), a major lipoglycan of the mycobacterial cell envelope, was previously recognized as existing in two major forms: LAM with arabinofuranosyl (Araf)-containing termini (AraLAM) and a mannose-capped version (ManLAM) in which the majority of these termini are modified by additional mannose residues. Since ManLAM was first recognized in the virulent (Erdman) strain of *Mycobacterium tuberculosis* and the non-capped version in a rapidly growing, attenuated, H37Ra strain, it was thought that mannose capping may be a key factor in virulence. In the present study, LAM from *M. bovis* BCG was isolated and the non-reducing termini sequenced through differential *O*-alkylation, partial depolymerization and gas chromatography-mass spectrometric analyses of fragments. LAM from *M. bovis* BCG contains a short mannan backbone, highly branched arabinofuranosyl-containing side chains and several mannosyl residues capping the non-reducing termini of these side chains. Thus, LAM from *M. bovis* BCG is of the ManLAM type, showing no major structural differences at the non-reducing ends from the *M. tuberculosis* Erdman product. This observation led us to examine the earlier strain and to conclude that it showed little resemblance to conventional strains of *M. tuberculosis*. Thus, the absence of mannose caps may be more a feature of rapid growth than of avirulence. These results demonstrate that the relationship between mannose capping and disease induction is not a simple one. However, use of a panel of LAM-specific monoclonal antibodies showed antigenic differences between the BCG and the Erdman products, suggesting the presence of features specific to the different strains and pointing to LAM as a molecule within which further species and strain variations reside.

Introduction

Among the carbohydrates of the mycobacterial cell wall, two polymers show strong antigenicity: arabinomannan and lipoarabinomannan (LAM) (Hunter *et al.*, 1986). LAM is widely distributed among *Mycobacterium* spp. (Misaki *et al.*, 1977), and its antigenic determinants are shared extensively among individual species (Gaylord *et al.*, 1987). Several investigations have focused on its wide biological activities. LAM from both *Mycobacterium tuberculosis* and *Mycobacteria leprae* is known to suppress T-cell proliferation (Kaplan *et al.*, 1987;

Moreno *et al.*, 1988; Molloy *et al.*, 1990), to inhibit γ -interferon-mediated activation of macrophages (Sibley *et al.*, 1988, 1990), to inhibit the synthesis of mRNA encoding IL-2, IL-5 and GM-CSF in human T cells (Chujor *et al.*, 1992), and to enhance the production of tumour necrosis factor (TNF) by mononuclear cells (Moreno *et al.*, 1989; Barnes *et al.*, 1990, 1992; Chatterjee *et al.*, 1992c). These and other results combined suggest that LAM may have broad immunosuppressive effects in mycobacterioses and contribute to the disease state (Levis *et al.*, 1987; Moreno *et al.*, 1988, 1989; Chan *et al.*, 1991).

Recently, LAM from what was believed to be a strain of *M. tuberculosis* H37Ra (Chatterjee *et al.*, 1991) and *M. tuberculosis* Erdman (Chatterjee *et al.*, 1992b) were described. Both products contain a phosphatidylinositol unit at the reducing end (Chatterjee *et al.*, 1992a), a mannan backbone with 6- and 2,6-linked mannopyranoses, and several arabinofuranosyl-containing chains marked by extensive branching. The non-reducing termini of the arabinan portion of LAM from the earlier H37Ra strain consist of either of two arrangements, β -D-

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Abbreviations: LAM, lipoarabinomannan; AraLAM, LAM with arabinofuranosyl-containing termini; ManLAM, mannose-capped LAM (see text); dLAM, deacylated LAM; TNF, tumour necrosis factor.

Araf-(1 → 2)- α -D-Araf-(1 → 5)- α -D-Araf → or [β -D-Araf-(1 → 2)- α -D-Araf-(1 →)]₂-(3 and 5)- α -D-Araf →; this product can be named AraLAM. In another strain (Erdman) of *M. tuberculosis*, the arabinan ends of LAM are extensively capped with single or di-, tri- or tetramannosyl units (Chatterjee *et al.*, 1992b). Consequently, we had termed this form of LAM as mannose-capped LAM or ManLAM and had speculated that these mannose residues may be implicated in the successful colonization or disease induction of strains of *M. tuberculosis*, a view reinforced by the observation that ManLAM is the dominant form present in *M. avium* (unpublished data) and, apparently, in *M. paratuberculosis* (Sugden *et al.*, 1987). It was thus of interest to investigate the structure of LAM from a vaccine strain of the *M. tuberculosis*-*M. bovis* complex whose LAM might *a priori* be expected to be of the type associated with non-virulence.

Methods

Isolation and purification of LAM from *M. bovis* BCG (Eli Lilly strain). The supernatant obtained after breakage of cells and centrifugation at 27 000 g was precipitated with 90% (v/v) acetone. The acetone precipitate was applied to a column (1.5 × 100 cm) of Sephacryl S-400 (Bio-Rad). Fractions enriched in carbohydrates were eluted with 6 M-guanidine·HCl in 10 mM-Tris/HCl, pH 7.4, dialysed, concentrated on an Amicon flowcell (Amicon 8200), and applied to a column (1.5 × 100 cm) of Sephacryl S-200 (Bio-Rad). Separation of LAM from lipomannan and phosphatidylinositolmannosides was achieved by elution with 10 mM-Tris/0.2 M-NaCl buffer containing 10 mM-EDTA, 0.02% NaN₃ and 0.25% deoxycholate. Fractions were monitored by SDS-PAGE. LAM-containing fractions were pooled, dialysed, concentrated and dried. In order to remove protein contaminants, phenol extraction was performed (Hunter *et al.*, 1986). Purified LAM thus obtained was subjected to chemical and immunochemical analyses.

The homogeneity of LAM from BCG was tested by HPLC. Gel filtration chromatography was conducted on a column (250 × 4.6 mm) of SynchroPack GPC 300 (Synchrom), with 0.1 M-potassium phosphate, pH 7.0, as eluting buffer. Hydrophobic interaction chromatography was performed on a column (100 × 4.6 mm) of Hypopore HIC 83 (Rainin). Different gradients of n-propyl alcohol in 0.1 M-triethylammonium acetate buffer, pH 7.0, were employed. Flow rates varied from 0.1 to 1 ml min⁻¹, and 200 μ l fractions were collected. The carbohydrate content of eluates was assayed with the phenol/H₂SO₄ reagent (Dubois *et al.*, 1966).

Analytical procedures. The relative amounts of mannose, arabinose and inositol were estimated by GC of the alditol acetate derivatives (Hunter *et al.*, 1986). In order to establish the absolute configuration of mannose and arabinose, trimethylsilyl derivatives of R-(–)- and S-(+)-2-butyl glycosides were analysed by GC/MS with appropriate standards (Gerwig *et al.*, 1978).

Deacylation of LAM (14 mg) was conducted with 0.1 M-NaOH in H₂O at 37 °C for 18 h (Hunter & Brennan, 1990). After neutralization with CH₃COOH, lipids were extracted with CHCl₃/CH₃OH (2:1, v/v). To remove salts, the aqueous fraction was applied to a column (1 × 100 cm) of Bio-Gel P-100 (Bio-Rad) as described by Hunter & Brennan (1990), to yield 6.3 mg of purified deacylated LAM (dLAM).

For analysis of glycosyl linkage composition, dLAM (6 mg) was per-O-methylated as mentioned by Daffé *et al.* (1990). A fraction (0.1 mg)

was further hydrolysed with 2 M-CF₃COOH at 120 °C for 2 h, reduced with NaB²H₄, acetylated and subjected to GC and GC/MS analyses (Daffé *et al.*, 1990; Chatterjee *et al.*, 1991). The remaining material (5.9 mg) was partially hydrolysed with 2 M-CF₃COOH at 75 °C for 1 h and reduced with NaB²H₄. Fragments of oligoglycosyl alditols thus obtained were O-ethylated and partially purified on a SEP-PAK (C-18) cartridge (Waters Associates), by eluting with 35 and 45% (v/v) CH₃CN in H₂O (Daffé *et al.*, 1990; Chatterjee *et al.*, 1991). Further separation was achieved by reverse-phase HPLC using a column (4 × 250 mm) of Lichrosorb RP18 (Merck). Fractions of 0.5 ml were collected and either analysed by GC/MS or completely hydrolysed, reduced with NaB²H₄, acetylated and the products identified by GC/MS (Daffé *et al.*, 1990; Chatterjee *et al.*, 1991).

¹³C-NMR was conducted in ²H₂O on a Bruker 300 instrument at Colorado State University, Department of Chemistry Central Instrument Facility (Chatterjee *et al.*, 1992b).

Electrophoresis and Western blotting. LAM preparations were routinely examined by analytical SDS-PAGE (Laemmli, 1970). Electrophoresis was conducted on 15% (w/v) acrylamide/0.8% bisacrylamide gels at 10 mA for 15 min and 15 mA for 45 min. Gels were silver stained (Tsai & Frasch, 1982) with a periodate oxidation step included. Two-dimensional electrophoresis was also performed on purified LAM (2 to 4 μ g) (O'Farrel, 1975). The first dimension involved isoelectric focusing in 15 M-urea with a 8% (v/v) ampholyte mixture (Bio-Lyte, Bio-Rad). The second dimension involved SDS-PAGE on a 15% polyacrylamide gel.

Immunoblotting was conducted on samples of the product from *M. bovis* BCG in parallel with purified LAM (1, 2, 4, 8 μ g) from *M. tuberculosis* H37Ra and *M. tuberculosis* Erdman. After electrophoresis, gels were transblotted to nitrocellulose (Bio-Rad) in Tris/glycine/methanol buffer under 40 V constant voltage (Gaylord *et al.*, 1987). The nitrocellulose sheets were blocked in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBST) and 5% (w/v) nonfat dry milk. Several antibodies diluted in TBST were used: an ascitic IgG anti-LAM monoclonal antibody CS-35 (ML9D3) (diluted 1:2000) (Chatterjee *et al.*, 1992b), a monoclonal antibody ML906 directed to LAM from *M. leprae* (diluted 1:250) (Gaylord *et al.*, 1987), and CS-40 raised to LAM from *M. tuberculosis* Erdman (undiluted) (Chatterjee *et al.*, 1992b). A pool of sera from tuberculous patients (diluted 1:1000, v/v), a serum sample from a nontuberculous patient (diluted 1:300, v/v), three sera from individual tuberculous patients (diluted 1:3000, v/v), three specimens from individual lepromatous leprosy patients (diluted 1:3000, v/v), four bovine serum samples from *M. bovis*-infected animals (diluted 1:1000, v/v), and one bovine serum sample from a non-infected animal (diluted 1:1000, v/v), were also tested. Antibodies were incubated overnight at room temperature. The nitrocellulose sheets were then washed with TBST and the appropriate antiserum conjugated with alkaline phosphatase (Sigma) was then applied. After a further 1 h of incubation at room temperature, bound antibodies were detected with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/p-nitro blue tetrazolium chloride (NBT) substrate (Sigma). Biotinylated concanavalin A (Boehringer Mannheim) was also used at a dilution of 1:3000 (v/v) in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBST). Bound concanavalin A was detected with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories) (Gaylord *et al.*, 1987).

Results

Purification of LAM from *M. bovis* BCG

After each purification step, the degree of purity of the LAM from *M. bovis* BCG was examined by SDS-PAGE, in which it appeared as a broad, diffuse band. HPLC or

Table 1. A comparison of the glycosyl composition of LAM from *M. tuberculosis* H37Ra, *M. bovis* BCG and *M. tuberculosis* Erdman

Product identified	Deduced form	Mol%		
		H37Ra*	BCG	Erdman†
1,4-di- <i>O</i> -Ac-2,3,5-tri- <i>O</i> -Me-arabinitol	t-Araf	7	3	2
1,2,4-tri- <i>O</i> -Ac-3,5-di- <i>O</i> -Me-arabinitol	2-Araf	9	11	7
1,4,5-tri- <i>O</i> -Ac-2,3-di- <i>O</i> -Me-arabinitol	5-Araf	43	37	41
1,3,4,5-tetra- <i>O</i> -Ac-3- <i>O</i> -Me-arabinitol	3,5-Araf	12	14	19
1,5-di- <i>O</i> -Ac-2,3,4,6-tetra- <i>O</i> -Me-mannitol	t-Manp	11	19	19
1,5,6-tri- <i>O</i> -Ac-2,3,4-tri- <i>O</i> -Me-mannitol	6-Manp	6	2	4
1,2,5,6-tetra- <i>O</i> -Ac-3,4,-di- <i>O</i> -Me-mannitol	2,6-Manp	9	5	9
1,2,5-tri- <i>O</i> -Ac-3,4,6-tri- <i>O</i> -Me-mannitol	2-Manp	0.6	9	8

* Chatterjee *et al.* (1990).† Chatterjee *et al.* (1992b).

two-dimensional electrophoresis were unable to further resolve the product, and it seems likely that LAM is not a single defined molecule, a view reinforced by evidence of multi-non-reducing termini (see below).

GC/MS of the fatty acyl methyl esters derived after alkali treatment of LAM showed the presence of methylhexadecanoate (42% of fatty acids), methyl-octadecanoate (17%), and methyltuberculostearate (41%) only. GC analysis of the polyol acetates or the alditol acetates from the purified product gave relative amounts of arabinose, mannose and inositol of about 67:32:1, respectively. The absolute configuration of arabinose and mannose was determined by GC/MS analysis of the trimethylsilyl derivatives of *R*-(-)- and *S*-(+)-2-butyl glycosides and by comparison with appropriate standards, which established that both arabinose and mannose were in the D-configuration.

Glycosyl linkage composition

In order to determine the linkage and the ring form of the arabinosyl and mannosyl residues, the dLAM was per-*O*-methylated, hydrolysed, reduced, acetylated and analysed by GC and GC/MS (Table 1). The product, 1,4,5-tri-*O*-Ac-2,3-di-*O*-Me-arabinitol, was attributed to 5-linked arabinofuranose units rather than 4-linked arabinopyranose based on the following evidence. When per-*O*-methylated dLAM was partially hydrolysed, reduced, *O*-ethylated and then completely hydrolysed, reduced and acetylated, 4-linked arabinopyranose would have given either 1,5-di-*O*-Ac-2,3-di-*O*-Me-4-*O*-C₂(²H)₅-arabinitol or 4-*O*-Ac-2,3-di-*O*-Me-1,5-di-*O*-C₂(²H)₅-arabinitol, whereas a 5-linked arabinofuranose would have yielded either 1,4-di-*O*-Ac-2,3-di-*O*-Me-5-*O*-C₂(²H)₅-arabinitol or 5-*O*-Ac-2,3-di-*O*-Me-1,4-di-*O*-C₂(²H)₅-arabinitol. Only the two latter compounds were found after hydrolysis of fragments 4, 11, 13, 16, 22 (Table 2). Likewise, 1,3,4,5-tetra-*O*-Ac-2-*O*-Me-arabinitol proved

to be indicative of 3,5-linked arabinofuranose rather than 3,4-linked arabinopyranose since the actual product identified upon hydrolysis of fragments 11 and 27 was 5-*O*-Ac-2-*O*-Me-1,3,4-tri-*O*-C₂(²H)₅-arabinitol; 3,4-linked arabinopyranose would have given rise to 4-*O*-Ac-2-*O*-Me-1,3,5-tri-*O*-C₂(²H)₅-arabinitol, which was never found in the hydrolysate from any of the fragments. Thus all of the arabinosyl residues are apparently furanoid and 2-, 5- or 3,5-linked. There were also some small amounts (approx. 3%) of terminal arabinofuranose. The mannosyl residues were clearly in the pyranose form, 2-, 6- or 2,6-linked. Terminal non-reducing mannopyranose was also present and accounted for about 19% of total sugars.

A comparison of the glycosyl composition of LAM from *M. tuberculosis* H37Ra, *M. tuberculosis* Erdman and *M. bovis* BCG is shown in Table 1. LAM from BCG and *M. tuberculosis* Erdman shared a similar sugar profile. However, the percentages of each form were noticeably different. The 6- and 2,6-linked mannopyranosyl constituents reflective of the mannan backbone (Chatterjee *et al.*, 1991) represented only 7% of the total sugar content of LAM from BCG, instead of the 15% and the 13% for LAM from H37Ra and Erdman, respectively. Moreover, the large amount of 3,5-linked arabinofuranose in the BCG product and the presence of only a few terminal arabinofuranose residues supported the existence of mannose-capped arabinosyl side chains which themselves must be highly branched. In summary, the data in Table 1 indicate that the mannan backbone of LAM from BCG is short, the arabinofuranosyl side chains are highly branched and mannosyl residues dominate the non-reducing ends of these side chains.

The regions of the anomeric carbons in the ¹³C-NMR spectrum of LAM from *M. bovis* BCG are shown in Fig. 1. The chemical shifts at δ 108.7, δ 108.3 and δ 106.7 are ascribed to C-1 of 5-, 3,5- and 2-linked α -arabinofuranose units, respectively (Daffé *et al.*, 1990; Chatterjee *et al.*,

Table 2. Proposed structures for the fragments arising from ManLAM of *M. bovis* BCG

Fragment no.	Proposed structure of the fragment as it appears in the original molecule	Structural proof
1*	t-Araf-(1 → 2)-Araf	($R_t = 7.649$), $m/z = 101, 143$ (aA ₂), 175 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 2-O-Ac-3,5-di-O-Me-1,4-di-O-C ₂ (² H) ₅ -arabinitol; 1,4-di-O-Ac-2,3,5-tri-O-Me-arabinitol; ratio 1:1
2	t-Araf-(1 → 5)-Araf	($R_t = 8.276$), $m/z = 101, 143$ (aA ₂), 175 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 312 (alditol cleavage)
3	→ 5-Araf-(1 → 3)-Araf 5 ↑	($R_t = 8.376$), $m/z = 101, 162$ (aA ₂), 194 (aA ₁), 249 (ald J ₂), 309 (ald J ₁), 395 (alditol cleavage)
4	→ 5-Araf-(→ 5)-Araf	($R_t = 9.001$), $m/z = 101, 162$ (aA ₂), 194 (aA ₁), 230 (ald J ₂), 290 (ald J ₁); 5-O-Ac-2,3-di-O-Me-1,4-di-O-C ₂ (² H) ₅ -arabinitol; 1,4-di-O-Ac-2,3-di-O-Me-5-O-C ₂ (² H) ₅ -arabinitol; ratio 1:1
5	→ 3-Araf-(1 → 5)-Araf 5 ↑	($R_t = 9.116$), $m/z = 181$ (aA ₂), 213 (aA ₁), 230 (ald J ₂), 276 (ald J ₀), 306, 350 (alditol cleavages)
6	→ 2-Manp-(1 → 5)-Araf	($R_t = 9.918$), $m/z = 88, 206$ (aA ₂), 238 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 331, 375 (alditol cleavages)
7	t-Manp-(1 → 5)-Araf	($R_t = 10.019$), $m/z = 88, 187$ (aA ₂), 219 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 312, 356 (alditol cleavages)
8	t-Manp-(1 → 2)-Manp	($R_t = 10.673$), $m/z = 88, 187$ (aA ₂), 219 (aA ₁), 274 (ald J ₂), 334 (ald J ₁), 401 (alditol cleavage)
9	→ 2-Manp-(1 → 2)-Manp	($R_t = 11.280$), $m/z = 88, 108$ (alditol cleavage) 206 (aA ₂), 238 (aA ₁), 274 (ald J ₂), 334 (ald J ₁)
10†	→ 5-Araf-(1 → x) Araf / \ t-Araf-(1 → x)	($R_t = 14.521$), $m/z = 120, 143$ (aA ₂), 162 (a'A ₂), 175 (aA ₁), 194 (a'A ₁), 390 (baA ₁), 409 (a'ald J ₂)
11	→ 5-Araf-(1 → 5)-Araf-(1 → 5)-Araf 3 ↑	($R_t = 15.158$), $m/z = 101, 162$ (aA ₂), 194 (aA ₁), 249 (ald J ₂), 309 (ald J ₁), 322 (baA ₂), 354 (baA ₁), 469 (bald J ₂), 510 (alditol cleavage); 5-O-Ac-2,3-di-O-Me-1,3,4-tri-O-C ₂ (² H) ₅ -arabinitol; 1,4-di-O-Ac-2,3-di-O-Me-5-O-C ₂ (² H) ₅ -arabinitol; 1,4,5-tri-O-Ac-2,3-di-O-Me-arabinitol; ratio 1:1:1
12	→ 5-Araf-(1 → 5)-Araf-(1 → 5)-Araf	($R_t = 15.341$), $m/z = 101, 162$ (aA ₂), 194 (aA ₁), 230 (ald J ₂), 276 (ald J ₀), 290 (ald J ₁), 322 (baA ₂), 354 (baA ₁), 450 (bald J ₁)
13	t-Araf-(1 → 5)-Araf-(1 → 5)-Araf	($R_t = 15.467$), $m/z = 101, 143$ (aA ₂), 175 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 303 (baA ₂), 335 (baA ₁), 428 (alditol cleavage), 450 (bald J ₁), 469 (bald J ₂), 472, 535 (alditol cleavages); 5-O-Ac-2,3-di-O-Me-1,4-di-O-C ₂ (² H) ₅ -arabinitol; 1,4-di-O-Ac-2,3,5-tri-O-Me-arabinitol; 1,4,5-tri-O-Ac-2,3-di-O-Me-arabinitol; ratio 1:1:1
14	→ 5-Araf-(1 → 5)-Araf-(1 → 5)-Araf 3 ↑	($R_t = 15.557$), $m/z = 101, 162$ (aA ₂), 194 (aA ₁), 230 (ald J ₂), 276 (ald J ₀), 290 (ald J ₁), 341 (baA ₂), 373 (baA ₁), 466 (alditol cleavage), 469 (bald J ₂), 510 (alditol cleavage)
15	t-Manp-(1 → 5)-Araf-(1 → 2)-Araf	($R_t = 16.012$), $m/z = 88, 101, 187$ (aA ₂), 219 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 347 (baA ₂), 379 (baA ₁), 390 (bald J ₂), 450 (bald J ₁); 2-O-Ac-3,5-di-O-Me-1,4-di-O-C ₂ (² H) ₅ -arabinitol; 1,4,5-tri-O-Ac-2,3-di-O-Me-arabinitol; 1,5-di-O-Ac-2,3,4,6-tetra-O-mannitol; ratio 1:1:1
16	t-Manp-(1 → 2)-Manp-(1 → 5)-Araf	($R_t = 16.632$), $m/z = 88, 101, 187$ (aA ₂), 219 (aA ₁), 230 (ald J ₂), 290 (ald J ₁), 391 (baA ₂), 423 (baA ₁), 434 (bald J ₂), 494 (bald J ₁); 5-O-Ac-2,3-di-O-Me-1,4-di-O-C ₂ (² H) ₅ -arabinitol; 1,5-di-O-Ac-2,3,4,6-tetra-O-mannitol; 1,2,5-tri-O-Ac-3,4,6-tri-O-Me-mannitol, ratio 1:1:1
17†	→ 5-Araf-(1 → 5)-Araf-(1 → x) Araf / \ t-Araf-(1 → x)	($R_t = 20.035$), $m/z = 101, 143$ (a'A ₂), 162 (aA ₂), 175 (a'A ₁), 194 (aA ₁), 322 (baA ₂), 354 (baA ₁), 436 (a'ald J ₀)

Fig. 1. ^{13}C -NMR of LAM from *M. bovis* BCG showing the anomeric regions only (between 95 and 110 p.p.m.).

nose from t- α -D-Manp-(1 $\rightarrow$ 2)- α -D-Manp (calculated value δ 103.4) and δ 99.4 to C-1 of 2,6-linked- α -D-mannopyranose from 2,6- α -D-Manp-(1 $\rightarrow$ 6)- α -D-Manp

(calculated value δ 99.4). The anomeric carbon of 2-linked mannopyranose can also resonate at δ 99.4 (Chatterjee *et al.*, 1992b). The ^{13}C -NMR spectra corroborate the results deduced from the methylation analysis, namely that all arabinosyl residues are in the furanosyl form and that the mannosyl residues are in the pyranosyl ring form.

Evidence for the presence of terminal mannosyl residues in LAM from *M. bovis* BCG

Per-*O*-methylated dLAM was partially hydrolysed. The fragments obtained were reduced, *O*-ethylated and isolated by reverse-phase HPLC. They were either further analysed by GC/MS or completely hydrolysed, reduced, acetylated and identified by GC/MS (Daffé *et al.*, 1990). With such a protocol, only one-third of the furanosyl residues were hydrolysed; the mannan backbone remained intact (Chatterjee *et al.*, 1991). A total of 28 fragments were characterized (Table 2); the evidence leading to structural elucidation is provided for fragments 20 and 28 in Figs 2 and 3. GC/MS showed that fragment 20 (Fig. 2) consisted of tetra-*O*-Me-Man $\rightarrow$ tri-*O*-Me-Man $\rightarrow$ di-*O*-Me-Ara $\rightarrow$ di-*O*-Me-1,5-di-*O*-C $_2$ (^2H) $_5$ -arabinitol. Hydrolysis, reduction and acetylation gave rise to 2-*O*-Ac-3,5-di-*O*-Me-1,4-di-*O*-C $_2$ (^2H) $_5$ -arabinitol, 1,4,5-tri-*O*-Ac-2,3-di-*O*-Me-arabinitol, 1,2,5-tri-*O*-Ac-

Fig. 2. Analysis of compound 20. (a) Total ion chromatography. (b) Mass spectrum and structural interpretation of the partially *O*-methylated, partially *O*-pentadeuterioethylated glycosyl alditol derivative leading to the structure t-Manp-(1 $\rightarrow$ 2)-Manp-(1 $\rightarrow$ 5)-Araf-(1 $\rightarrow$ 2)-Araf.

Fig. 3. Analysis of compound 28. (a) Total ion chromatography. (b) Mass spectrum and structural interpretation of the partially *O*-methylated, partially *O*-pentadeuterioethylated glycosyl alditols derivative leading to the structure t-Manp-(1 → 2)-Manp-(1 → 2)-Manp-(1 → 5)-Araf-(1 → 2)-Araf.

3,4,6-tri-*O*-Me-mannitol and 1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-Me-mannitol in the ratio of approximately 1:1:1:1. Thus, compound 20 is 2,3,4,6-tetra-*O*-Me-Manp-(1 → 2)-3,4,6-tri-*O*-Me-Manp-(1 → 5)-2,3-di-*O*-Me-Araf-(1 → 2)-3,5-di-*O*-Me-1,4-di-*O*-C₂(²H)₅-arabinitol, pointing to the presence of the fragment t-Manp-(1 → 2)-Manp-(1 → 5)-Araf-(1 → 2)-Araf in dLAM. Likewise the hydrolysis of fragment 28 (Fig. 3) produced 1,4,5-tri-*O*-Ac-2,3-di-*O*-Me-arabinitol, 2-*O*-Ac-3,5-di-*O*-Me-1,4-di-*O*-C₂(²H)₅-arabinitol, 1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-Me-mannitol and 1,2,5-tri-*O*-Ac-3,4,6-tri-*O*-Me-mannitol in the approximate ratio of 1:1:1:2. Thus the complete structure of fragment 28 is 2,3,4,6-tetra-*O*-Me-Manp-(1 → 2)-3,4,6-tri-*O*-Me-Manp-(1 → 2)-3,4,6-tri-*O*-Me-Manp-(1 → 5)-2,3-di-*O*-Me-Araf-(1 → 2)-3,5-di-*O*-Me-1,4-di-*O*-C₂(²H)₅-arabinitol, pointing to the presence of the unit t-Manp-(1 → 2)-Manp-(1 → 2)-Manp-(1 → 5)-Araf-(1 → 2)-Araf in LAM from *M. bovis* BCG.

Of the 28 fragments, 11 contained terminal mannoses, again emphasizing the extent of mannose capping of the putative terminal arabinofuranosyl units in this product. Thus, from fragments 7, 15, 21 and 24, it was evident that stretches of Araf are substituted with single mannose or dimannosyl units (evident from fragments 20, 25 and 26) or trimannosyl units (22 and 28). Mannosyl residues were all linked together by a 1 → 2 linkage (6, 8, 9, 16, 20, 22, 25, 26, 27). In agreement with the results obtained with LAM from *M. tuberculosis* Erdman, the mannosyl

side chain was often linked to an arabinose within the Ara-(1 → 5)-Ara-(1 → 2)-Ara motif (15, 20, 21, 24, 25, 26, 28). The arabinan side chain showed the frequent occurrence of the diarabinosyl unit, Ara-(1 → 5)-Ara, and 13 fragments in all contained 3,5-linked arabinose (3, 5, 10, 11, 14, 17, 18, 19, 21, 23, 25, 26, 28). The structural features of several of the tetrasaccharides and pentasaccharides demonstrated that the mannosyl residues were invariably bound to just two or three linear arabinofuranosyl units prior to the 3,5-linked branched arabinofuranose unit.

Western blotting (immunoblotting) of LAM from *M. bovis* BCG

Immunoblotting (Fig. 4), demonstrated that concanavalin A bound to LAM from BCG and *M. tuberculosis* Erdman with equal intensity but not to LAM of H37Ra, providing further evidence that LAM from BCG did contain mannosyl residues at the non-reducing end of the molecule. The antigenicity of LAM from BCG was examined by immunoblotting and by comparison with LAM from *M. tuberculosis* Erdman and *M. tuberculosis* H37Ra (Fig. 4). All products reacted strongly against pooled sera from tuberculosis patients, and against serum specimens from individual tuberculosis patients, lepromatous patients and *M. bovis*-infected animals. Thus the inherent antigenicity of ManLAM from *M.*

Fig. 4. SDS-PAGE and Western blot of LAM from *M. tuberculosis* H37Ra, *M. bovis* BCG and *M. tuberculosis* Erdman. Lanes: 1 (1 µg), 2 (2 µg), 7 (4 µg), 8 (8 µg) of *M. tuberculosis* H37Ra LAM; 3 (1 µg), 4 (2 µg), 9 (4 µg), 10 (8 µg) of *M. bovis* BCG LAM; 5 (1 µg), 6 (2 µg), 11 (4 µg), 12 (8 µg) of *M. tuberculosis* Erdman LAM. (a) Gel stained with silver nitrate. (b-f) Immunoblotting using (b) pooled tuberculous patients' sera (diluted 1:1000), (c) monoclonal antibody CS-35 (diluted 1:2000), (d) monoclonal antibody ML906 (1:250), (e) monoclonal antibody CS-40 (undiluted), (f) concanavalin A (diluted 1:3000). The positions of polypeptide markers (soybean trypsin inhibitor: 20.1 kDa, carbonic anhydrase: 30 kDa) are indicated in lane M.

bovis was demonstrated and so was its extensive cross-reactivity. Nevertheless, the use of monoclonal antibodies demonstrated the existence of elements of antigenic specificity in the various products. LAM from the three different sources reacted strongly against a monoclonal antibody (CS-35) originally raised to LAM from *M. leprae*. However, only the LAM from *M. tuberculosis* H37Ra reacted with another anti-*M. leprae* LAM antibody, ML906. In this case neither the product from Erdman nor that from BCG was recognized even when large amounts (8 µg) of LAM were loaded. The antibody CS-40, which had been raised against LAM from Erdman, surprisingly did not cross-react with the product from either H37Ra or BCG, even when large amounts (10 µg) were tested.

Discussion

To date, two major classes of lipoarabinomannan from various mycobacterial sources have been described: LAM (AraLAM) which possesses arabinofuranose-containing termini in either of two arrangements, β -D-Araf-(1 → 2)- α -D-Araf-(1 → 5)- α -D-Araf or [β -D-Araf-(1 → 2)- α -D-Araf-(1 → 3)- α -D-Araf], and which was found in an avirulent strain subcultured in laboratory media for many years and thought to be *M. tuberculosis* H37Ra (Chatterjee *et al.*, 1991); and ManLAM, in which the arabinan ends are extensively capped with single mannosyl, di-, tri- or tetramannosyl units and which was observed in virulent *M. tuberculosis* Erdman (Chatterjee *et al.*, 1992b). In the light of this dramatic difference between LAM from the two strains, we had speculated that the mannose adducts were of relevance to pathogenicity.

However, in view of the present evidence that LAM from *M. bovis* BCG is also of the ManLAM class, a direct relationship between mannose capping and patho-

genesis is hardly tenable. Indeed, arising directly from this study, we now question the authenticity of our *M. tuberculosis* H37Ra strain used in the initial work (Chatterjee *et al.*, 1991). It was always much more rapidly growing than other strains of *M. tuberculosis* H37Ra (Chatterjee *et al.*, 1992a, b). We have since shown that it is devoid of the majority of the key biochemical characteristics of H37Ra. In particular, the pattern of mycolic acids as their methyl esters on thin layer plates, conducted as described by Minnikin *et al.* (1975), showed the complete absence of methoxy mycolates, a hallmark of strains of *M. tuberculosis* and *M. bovis*. Thus, we conclude that the strain in the earlier study was a rapidly growing *Mycobacterium* sp. and that the absence of mannose capping is probably more a feature of rapid growth than of pathogenesis.

The relationship between structure and biological properties of LAM has been discussed. Several structural features are thought to play a key role in biological activity, such as the α -(1 → 5)-linked arabinosyl moieties, the *O*-acyl groups and the mannose capping. For example, it is known that α -(1 → 5)-linked arabinosyl residues located on the arabinan side chains are the main immunodeterminants, since the deacylated form of LAM, i.e. arabinomannan (and also arabinogalactan), after treatment with arabinases lost their immunological activity (Misaki *et al.*, 1977). Also, lipomannan has neither immunological activity (Gaylord *et al.*, 1987) nor capacity to induce secretion of TNF- α (Moreno *et al.*, 1989). Moreover, Moreno *et al.* (1988) found that LAM, as well as arabinomannan and D-arabino-D-galactan, but not L-arabino-L-galactan, were able to inhibit T-cell proliferation of human clones, leading to the hypothesis that the inhibition of major cellular immune functions by LAM is due to the polysaccharide region rather than the *O*-acyl groups. More recently (Chatterjee *et al.*, 1992c), it has been found that AraLAM was 100-fold more

Fig. 5. A hypothetical model representing the major structural features of LAM from *M. bovis* BCG.

effective in inducing TNF secretion than ManLAM, supporting the view that the masking of terminal arabinosyl residues by mannose directly influences biological activity. In addition, the *O*-acyl groups of LAM are thought to be involved in its biological properties. For example, de-*O*-acylation with alkali diminishes the release of TNF *in vitro* (Moreno *et al.*, 1989) and inhibits γ -interferon-mediated activation of mouse macrophages *in vivo* (Sibley *et al.*, 1988).

The results presented in the present paper demonstrate that the non-reducing termini of LAM from *M. bovis* BCG are mannose capped. Moreover, the data presented in Table 1 are consistent with the following structural features in BCG LAM: (i) a short mannan backbone with only 5 units of 2,6-linked mannose; (ii) an arabinofuranosyl side chain highly branched due to a plenitude of 3,5-linked D-Araf residues; and (iii) up to three 2-linked mannopyranosyl segments located at the non-reducing ends of the molecule. A model which accounts for most of the observed features of LAM from BCG is presented in Fig. 5.

Among fragments obtained after per-*O*-methylation and partial hydrolysis of BCG LAM, many correspond to motifs previously described for LAM from H37Ra (Chatterjee *et al.*, 1991) or from Erdman (Chatterjee *et al.*, 1992b). Although motifs containing terminal arabinofuranose (motif A) or long linear chains of arabinofuranose (motifs C and D) are not major components of LAM from BCG, fragments containing 3,5-linked Araf (motif B) are well represented. More outstanding are the similarities of motifs E, F, G from Erdman LAM with fragments 15, 20 and 28 from BCG LAM. However, there is no evidence for the presence of a tetramannosyl-

containing domain (motif H) in LAM from BCG. Thus, like that from H37Ra, LAM from BCG seems to be highly branched but, like that from Erdman, it possesses mannosyl domains at the non-reducing end of the molecule.

The antigenicity of LAM from BCG, and especially its reactivity with tuberculous patient sera and with some monoclonal antibodies, underlines again the well-known cross-reactivity of LAM. On the other hand, unlike LAM from H37Ra, that from BCG and *M. tuberculosis* Erdman did not react with yet another anti-*M. leprae* LAM antibody (ML906). The structural similarities between LAM from BCG and Erdman, more particularly the presence in both molecules of mannosyl domains on the arabinosyl side chain, may account for their similar antigenic behaviour. It was thus expected that an antibody raised against LAM from Erdman would recognize LAM from BCG. Unexpectedly, no reaction was observed between an anti-Erdman LAM antibody (CS-40) and BCG LAM, even at high concentration. In addition mouse serum raised against sonicated *M. avium* reacted with *M. avium* ManLAM but not with ManLAM from *M. tuberculosis* Erdman or *M. bovis* BCG (unpublished results). Thus, although LAM from BCG shows considerable structural analogies with Erdman LAM, it also shows antigenic differences, which are indicative of yet other niceties, such as: (i) some saccharide motifs (e.g. motif H) may exist only in Erdman LAM and not in BCG or H37Ra LAM; (ii) differences may lie in the involvement of acyl, acetyl or phosphoryl functions. It is noteworthy that the dominant structural features of LAM were established after deacylation and since deacylated LAM does not react with any antibodies, the nature of such functions and their roles in epitope recognition were not clearly investigated. The evidence for antigenic specificity within the ManLAMs from different mycobacteria further underlines the need for further immunochemical investigation of these molecules.

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