Structural features of the exocellular polysaccharides of *Mycobacterium tuberculosis*

Anne LEMASSU and Mamadou DAFFÉ*

Département des Glycoconjugués et Biomembranes, LPTF du CNRS, 118 route de Narbonne, 31062 Toulouse Cédex, France

The cell envelope which surrounds pathogenic mycobacteria is postulated to be a defence barrier against phagocytic cells and its outermost constituents have a tendency to accumulate in the culture medium. The present work demonstrates that the exocellular material of *Mycobacterium tuberculosis* contains large amounts of polysaccharides with only traces, if any at all, of lipids. Three types of polysaccharides were purified by anion-exchange and gel-filtration chromatography; all were found to be neutral compounds devoid of acyl substituents. They consisted of D-glucan, D-arabino-D-mannan and D-mannan, which were eluted from gel-filtration columns in positions corresponding to molecular masses of 123, 13 and 4 kDa respectively. Their predominant structural features were determined by the characterization of the per-O-methyl derivatives of enzymic, acetolysis and Smith-degradation products and by ^1H - and ^{13}C -n.m.r.

spectroscopy of the purified polysaccharides, using mono- and two-dimensional homonuclear chemical-shift correlated spectroscopy and two-dimensional heteronuclear ($^1\text{H}/^{13}\text{C}$) spectroscopy. The glucan which represented up to 90% of the polysaccharides was composed of repeating units of five or six $\rightarrow 4\text{-}\alpha\text{-D-Glcp-1}\rightarrow$ residues and a $\rightarrow 4\text{-}\alpha\text{-D-Glcp}$ substituted at position 6 with an $\alpha\text{-D-Glcp}$, indicating a glycogen-like highly branched structure not related to the so-called polysaccharide-II previously identified in tuberculin. The arabinomannan consisted of a mannan segment composed of a $\rightarrow 6\text{-}\alpha\text{-D-Man-1}\rightarrow$ core substituted at some positions 2 with an $\alpha\text{-D-Manp}$. The arabinan termini of the arabinomannan were found to be extensively capped with mannosyl residues. The possibility that these polysaccharides contribute to the persistence of the tubercle bacillus in the macrophage by molecular mimicry is discussed.

INTRODUCTION

Mycobacterium tuberculosis, the etiological agent of tuberculosis, is the most serious mycobacterial pathogen. It is responsible for the deaths of 3 million individuals annually arising from the large pool of infected persons currently estimated at 1 billion [1]. Consequently, interest in the study of the tubercle bacillus stems not only from its importance as a pathogen, but also from the hope that such work would reveal the factor(s) responsible for its ability to multiply within human tissues and, at the same time, for its capacity to withstand host defence mechanisms.

In spite of the considerable amount of work devoted to the chemical characterization and biological activities of individual mycobacterial somatic or wall-associated components (for a review see [2]), the general conclusion drawn from the published work is that the substances identified so far cannot, alone or together, account for the virulence of certain strains or the lack of virulence of others. Interestingly, it has been observed that virulent mycobacteria growing intracellularly are surrounded by an electron-transparent zone, also called capsule, which may be part of a defence mechanism permitting all mycobacterial pathogens to resist being killed by phagocytic cells [3]. This protective capsule not only controls access from the medium to the inside of the mycobacterial cell but also determines what components come into contact with host cells and tissues. Furthermore, the outermost constituents of the bacilli, confined around the bacterial cells when the environment happens to be a macrophage, may be released by actively growing mycobacteria in artificial culture media [4].

In the past few years numerous studies have been devoted to the isolation, characterization and biological activities of the

proteins derived from early-logarithmic-phase culture filtrates [5]. Despite the recent demonstration of the immunosuppressive effects of mycobacterial carbohydrate-containing molecules, notably those attached to lipoarabinomannan (see [2]), few efforts have been made to characterize the polysaccharides released in mycobacterial culture filtrates (under non-autolytic conditions).

The goal of the present study was the identification, purification and chemical characterization of the major polysaccharides present in the culture filtrates of actively growing cells of *M. tuberculosis*.

EXPERIMENTAL

Strains and growth conditions

Strains CIPT 1400100-001 (H37Rv), -59, -59R and -60 of *M. tuberculosis* [6] were grown on Sauton's medium as surface pellicles at 37 °C.

Isolation and fractionation of the crude exocellular material

The bacteria were harvested by filtration through a Durieux filter no. 111 (Durieux, Paris, France) and the culture filtrates were re-filtered through 0.2 μm -pore-diam. sterile filterware (Nalge Co, New York, NY, U.S.A.). The filtrates were pooled and concentrated under vacuum to one tenth of the original volume; chloroform and methanol were then added to the filtrate to obtain a partition mixture composed of chloroform/methanol/water (3:4:3, by vol.). The aqueous phase contained Ara and Man residues (molar ratio 1:1) and traces of Glc, whereas Glc was the only sugar constituent detected in the interphase. Thus, the glucan was recovered as an opalescent solution by

Abbreviations used: CIPT, Collection Institut Pasteur Tuberculose; tms, trimethylsilyl; f, furanosyl; p, pyranosyl; COSY, chemical-shift correlated spectroscopy; f.a.b.-m.s., fast-atom bombardment m.s.

* To whom correspondence should be addressed.

extracting the interphase several times with water. The Ara/Man- and Glc-rich fractions were concentrated and the polymers were precipitated overnight at 4 °C with 6 vol. of cold ethanol. The precipitates were collected after centrifugation at 14000 g for 1 h, dissolved in distilled water, the polymers precipitated again with ethanol, re-centrifuged and the precipitates dialysed for 3 days against water to eliminate traces of salts, before being lyophilized and weighed.

Purification of the different polysaccharides

The Ara/Man-containing polysaccharides were chromatographed on a column (55 cm × 1.8 cm) of DEAE-Trisacryl (IBF, Villeneuve-La-Garenne, France) gel and the neutral fraction was re-chromatographed on a column (90 cm × 1.8 cm) of Bio-Gel P-10 (Bio-Rad) using water or 1% (v/v) acetic acid as eluent. The Glc-rich polysaccharide was similarly purified by chromatography on DEAE-Trisacryl, followed by chromatography on a Sephadex G-200 column (90 cm × 1.8 cm; Pharmacia, Uppsala, Sweden) using 0.1 M NaCl in 1% acetic acid as eluent. The elution profiles of both types of gel-filtration chromatography were monitored by refractive index detection and the collected fractions were assessed for their carbohydrate content. The gel-filtration columns were calibrated using dextrans (Dextran Blue 2000, T110, T40, T20 and T10) purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

α -Mannosidase digestion of the arabinomannan

Arabinomannan (10 mg) was dissolved in 3 ml of sodium acetate buffer (0.05 M) and incubated at room temperature with 200 μ l of α -mannosidase (extracted from almonds; Sigma Chemical Co., St. Louis, MO, U.S.A.); subsequent treatments of the reaction mixture were performed as previously described [7].

Amyloglucosidase digestion of the glucan

A 2 mg sample of glucan was incubated with 0.1 ml of HClO₄ (0.6 M), 0.1 ml of potassium bicarbonate (1 M) and 2 ml of amyloglucosidase from *Aspergillus niger* (Sigma Chemical Co.) for 2 h at 40 °C. The glucose content of the mixture was determined as described previously [8].

Smith degradation

A 10 mg sample of purified polysaccharides was treated with 10 ml of 0.1 M sodium iodate in 50 mM sodium acetate buffer (pH 5.0) and allowed to react for 4 days in the dark at room temperature [9]. Ethylene glycol (3 mg) was then added to the mixture to stop the reaction and the reaction products were reduced by stirring with 40 mg of sodium borohydride for 5 h. The reaction mixture was then treated as described previously [9].

Acetolysis

Dry polysaccharide samples (10 mg) were acetolysed [10]. A 1 ml sample of a mixture of acetic acid/acetic anhydride/sulphuric acid (10:10:1, by vol.) was added to the samples and the mixtures were left at 40 °C for 30 min, 1 h or 3 h. Saturated sodium carbonate (3 ml) and methylene chloride (3 ml) were added, followed by thorough mixing. The organic phase was removed and washed three times with 3 ml of water, dried and analysed by t.l.c. on silica gel 60-coated plates (0.25 mm thickness; Merck, Darmstadt, Germany) developed with benzene/

methanol (92:8, v/v) as solvent, using peracetylated mono- and oligo-saccharides as standards. The acetylated oligosaccharides obtained from acetolysis were deacetylated [7] and the oligo-saccharide products were fractionated on Bio-Gel P-4 columns in water.

Methylation of polysaccharides

Pure polysaccharides (2 mg) were O-methylated four times according to Ciucanu and Kerek [11]. Portions of the per-O-methylated products were hydrolysed with 2 M trifluoroacetic acid at 110 °C for 2 h, reduced with sodium borohydride and acetylated. The different partially O-methylated and partially acetylated alditols were identified by g.c.-m.s. and by co-chromatography using a capillary column with authentic standards of Man and Glc derivatives (kindly supplied by Dr. M. McNeil, Fort Collins, CO, U.S.A.) and of Ara derivatives prepared from arabinogalactan [12].

Miscellaneous analytical techniques

The dialysed culture filtrates and the various chromatographic fractions were assessed for the presence of protein [13] and carbohydrate [14]. The percentage of carbohydrate was also determined by g.l.c. using erythritol as the internal standard. To determine the nucleic acid content of the materials, u.v. spectra were recorded.

The absolute configuration (D or L) of Ara, Man and Glc residues were determined by g.l.c. analysis of the trimethylsilyl (tms) derivatives of R-(−)- and S-(+)-2-butyl glycosides as described [15].

G.l.c. was performed on alditol acetates and tms derivatives of monosaccharides using a Girdel chromatograph (model G 30) equipped with a fused-silica capillary column (25 m length × 0.22 mm internal diam.) containing WCOT OV-1 (0.3 μ m film thickness, Spiral, Dijon, France). A temperature gradient of 100–280 °C at 2 °C/min was used.

G.c.-m.s. was performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5989A mass selective detector. The column was a 12 m HP-1 (Hewlett-Packard, Avondale, PA, U.S.A.). Samples were injected in the Splitless mode. The oven temperature was programmed to hold at 80 °C for 1 min followed by a 15 °C/min rise to 290 °C. The mass spectrometer was set to scan from 50 to 600 atomic mass units.

Fast-atom bombardment m.s. (f.a.b.-m.s.) was performed on a ZAB-HS reverse-geometry mass spectrometer (VG Analytical, Manchester, U.K.). Spectra were generated by an 8 eV xenon-atom beam. Samples were dissolved in chloroform/methanol (1:1, v/v). A 1 μ l sample of this solution was mixed on the probe tip with 1 μ l of *m*-nitrobenzyl alcohol and 1 μ l of a 10% (w/v) NaI solution. Ten scans of 10 s/decade were accumulated to obtain a spectrum.

N.m.r. spectra were obtained in ²H₂O with a Bruker AM 300 WB instrument equipped with an Aspect 3000 computer. One-dimensional spectra were obtained with 40° pulses, and acquisition times of 1.56 s for ¹³C n.m.r. (spectral width: 5263 Hz) and 2.73 s for ¹H n.m.r. (spectral width: 2994 Hz) were used. The chemical-shift reference used was that of tetramethylsilane. The homo-nuclear two-dimensional chemical-shift correlated spectroscopy (COSY) spectra were obtained using a previously described pulse sequence [16] with 256 × 1024 matrix data points over a spectral range of 2092 Hz; the matrix data points were expanded to 1024 × 2048 by zero-filling. A sine-bell window function was used. A total of 256 experiments with 24 accumulations for each one were performed. The heteronuclear two-dimensional COSY

$^{13}\text{C}/^1\text{H}$ -n.m.r. spectra were recorded using carbon-proton phase-sensitive chemical-shift correlation software supplied by Bruker over a spectral width of 6250 Hz for the ^{13}C dimension and 310 Hz for the ^1H dimension. A total of 34 experiments with 256 accumulations for each one were performed. ^{13}C -n.m.r. spectra were obtained using a Bruker J-modulated sequence.

U.v. spectra were recorded using a Perkin-Elmer (Lambda 5) spectrophotometer. Optical rotation (at a wavelength of 589 nm) was determined in water with a Perkin-Elmer spectropolarimeter (model 241) at 25 °C.

The lipid content of the culture filtrates was determined by analysing the chloroform/methanol phase by t.l.c. on silica gel 60-coated plates (0.25 mm thickness; Merck) developed either with chloroform/methanol (99:1, or 90:10, v/v), or with chloroform/methanol/water (30:8:1 or 65:25:4, by vol.). Glycolipids were visualized by spraying plates with 0.2 % anthrone in concentrated sulphuric acid, followed by heating.

RESULTS

Chemical nature of the exocellular material

The polymers derived from the ethanol precipitation of the unfractionated culture filtrates represented 20 % of the dried bacterial mass of both early- and late-growth-phase cultures. When a chloroform/methanol/water partition was applied before the ethanol precipitation, the organic phase derived from the extraction of the culture filtrates of *M. tuberculosis* contained

only traces, if any, of lipids. The precipitates were found to be devoid of DNA and were composed of up to 85 % carbohydrate, depending on the growth phase of the examined strains. The monosaccharide composition of the exocellular material determined by acid hydrolysis of the polymers was found to be qualitatively constant during the logarithmic growth phase. Glc, Man and Ara were detected in the acid hydrolysis products of all the precipitates. Glc was always found to be the major constituent of the sugar mixture (60–90 %). However, Gal and 6-*O*-methylglucose were detected in the exocellular material derived from the stationary-growth-phase cultures, suggesting that these two sugars are probably derived from autolysis compounds of the cells, namely wax-D and 6-*O*-methylglucose-containing lipopolysaccharides respectively [17].

Purification and characterization of the major polysaccharides

The glucans were not retained on the anion-exchange column; when analysed by gel-filtration chromatography over a Sephadex G-200 column (Figure 1a) the polysaccharides were eluted at a position corresponding to an apparent molecular mass of 123 000 Da.

Most (90 % in weight) of the Ara/Man-containing polysaccharides were eluted from the DEAE-Trisacryl column with the buffer containing no NaCl. Chromatography of these neutral polysaccharides over a Bio-Gel P-10 column using 1 % acetic acid in water as eluent led to the isolation of two major fractions (Figure 1b). The first major peak, exhibiting an apparent molecular mass of approx. 13 000 Da, contained Ara and Man in a molar ratio Ara:Man of 2:1. The second major fraction consisted of mannan with an apparent molecular mass of 4000 Da. The minor peak, which eluted in a position corresponding to a molecular mass of 20 000 Da, corresponded to an incomplete dissociation of the two polysaccharides.

Absolute configuration of the Glc, Ara and Man residues

G.c.-m.s. analysis of the tms derivatives of both *R*(-)- and *S*(+)-2-butyl glycosides and appropriate standards [15] showed the Glc, Ara and Man residues to be more than 98 % in the *D* configuration, thus confirming that both Ara and Man in the arabinomannans of *M. tuberculosis* are *D* [18].

Optical rotations of the purified polysaccharides

The optical rotation ($[\alpha]_{589}^{25}\text{D}$) of the pure glucans isolated from the different strains were found to be equal to +167–168° (c 1 in water).

The optical rotations measured for the purified arabinomannans and mannans were: $[\alpha]_{589}^{25}\text{D} = +76\text{--}77^\circ$ and $+37\text{--}40^\circ$ (c 1 in water) respectively. These values were significantly lower than those previously reported for alkali-treated arabinomannans and mannans [18].

Structure of the glucan

Examination of Table 1 reveals that the vast majority of the glycosyl residues are either 4-linked Glcp or 5-linked Glcf; some branched 4,6- or 5,6-glucosyl residues were also detected. The average chain length of the repeated unit (linear versus branched residues) was estimated to be five to six residues, indicating a highly branched structure. In order to address the question of the anomeric configuration and of the ring form of the glucosyl units, the purified glucans were subjected to ^{13}C -n.m.r. analysis. The anomeric resonance signal was seen at 101 p.p.m. which can

Figure 1 Gel filtration of the major neutral glucan fraction derived from the culture filtrate over a Sephadex G-200 column (a) and of the major neutral Ara/Man-rich polysaccharide fraction over a Bio-Gel P-10 column (b)

The eluent of the gel-filtration columns consisted of 1 % acetic acid. Abbreviations: V_0 , void volume; AM, arabinomannan; M, mannan. Fractions (4 ml) were collected, and the carbohydrate content of each fraction was determined as described in the Experimental section.

Table 1 Glycosyl-linkage analysis of the exocellular polysaccharides of *M. tuberculosis*

Conditions for O-methylation of the polysaccharides, hydrolysis, reduction, acetylation and g.c.-m.s. analysis are described in the Experimental section. Proof of linkage and ring form are given in the text. Abbreviation: t, terminal.

Polysaccharides	Glycosyl residue	O-Ac positions	O-Me positions	Deduced linkage and ring form	Mole (%) [*]	
					Native	Treated [†]
Arabinomannan	Ara	1,4	2,3,5	t-Araf	4.5	10.0
	Ara	1,2,4	3,5	2-Araf	11.0	16.5
	Ara	1,4,5	2,3	5-Ara//4-Arap	40.0	41.0
	Ara	1,3,4,5	2	3,5-Ara//3,4-Arap	18.0	19.0
	Man	1,5	2,3,4,6	t-Manp	19.0	12.0
	Man	1,5,6	2,3,4	6-Manp	1.6	1.0
	Man	1,2,5,6	3,4	2,6-Manp	1.8	0.3
	Man	1,2,5	3,4,6	2-Manp	4.0	0.3
Glucan	Glc	1,5	2,3,4,6	t-Glcp	13.2	
	Glc	1,4,5	2,3,6	4-Glcp/5-Glcf	73.5	
	Glc	1,4,5,6	2,3	4,6-Glcp/5,6-Glcf	13.2	

^{*} The mole (%) is given for strain 140010060. Similar results were obtained for the other examined strains.

[†] Treatment with α -mannosidase.

only result from the resonances of C-1s of α -Glcp, the C-1 resonances of β -Glcp and of α - and β -Glcf being expected at lower field (104–110 p.p.m. [19]). No acyl substituent was detected by either ¹H- or ¹³C-n.m.r. in the glucans.

Acetolysis applied to the glucans for 30 min, 1 h or 3 h resulted in the visualization on t.l.c. plates of two major spots, the R_f values of which corresponded to those of the two anomers of peracetylated glucosides; in addition, minor spots which exhibited the same chromatographic mobility as peracetylated di-, tri- and tetra-glucosides were also detected. Analysis of the f.a.b. mass spectrum of the peracetylated oligoglucosides confirmed the t.l.c. data: two prominent peaks at m/z 413 and 331, corresponding respectively to the pseudomolecular ion peak ($M+Na$) of peracetylated glucoside and to the loss of acetate from the entire molecule ($M-59$), were observed. Besides, ($M+Na$) and ($M-59$) ion peaks corresponding to peracetylated di-, tri- and tetra-glucoside molecular species were also observed but in less abundance. Deacetylation of the oligosaccharide mixture, followed by fractionation on a Bio-Gel P-4 column in water, demonstrated that the almost exclusive compound (> 90%) was eluted from the gel at a position corresponding to that of glucose. Per-O-methylation of the acetolysis products followed by hydrolysis, reduction, acetylation and analysis by g.l.c. allowed the identification of terminal Glcp and 4-Glcp. These results support a structure based on a linear $\rightarrow 4\text{-}\alpha\text{-D-Glcp-1}$ substituted mainly by a glucosyl residue at some 6 positions.

The glucans were readily digested by amyloglucosidase which hydrolyses the $\rightarrow 4\text{-}\alpha\text{-D-1}$ and $\rightarrow 6\text{-}\alpha\text{-D-1}$ linkages of glycogen and structurally related polysaccharides [8].

As expected from the proposed structure, periodate oxidation of the glucans resulted in the identification of glycerol and erythritol, but not of Glc.

Structural features of the arabinomannans

Glycosyl-linkage analysis

Aliquots of the purified arabinomannans were O-methylated, hydrolysed, reduced, acetylated and analysed by g.c.-m.s. (Table 1). The arabinan segments consisted of terminal, 2-, 3,5- and 5-linked Araf residues; the presence of substantial amounts of

pyranosyl residues was ruled out by n.m.r. analyses (see below). The mannan segments contained terminal 2-, 6- and 2,6-linked pyranosides as all the mannosyl residues, after O-methylation, contained a methoxyl group located on carbon 4, in agreement with the published data [18,20].

Determination of the ring forms and of the anomeric configurations of the glycosyl residues by n.m.r.

In order to address the question of the ring form of the majority of the arabinosyl residues, the purified arabinomannans were analysed by ¹³C n.m.r. (Figure 2). All the signals were observed between 60 and 110 p.p.m., indicating that the polysaccharide was not acylated, a conclusion confirmed by ¹H n.m.r. (results not shown). In the anomeric carbon resonance region (90–110 p.p.m.) several groups of signals were observed. The most deshielded resonances (108–109 p.p.m., $J_{C-1-H-1} = 170$ Hz) were assigned to those of 5-linked $\alpha\text{-D-Araf}$ residues [12,19,21]. Because O-substitution of $\alpha\text{-Araf}$ at position 2, but not at position 3 or 5, is known to cause the corresponding C-1 chemical shift to move upfield by 1–2 p.p.m. [12,19], the signal at 107 p.p.m. ($J_{C-1-H-1} = 170$ Hz) was assigned to the resonance of the anomeric carbons of the 2-linked $\alpha\text{-D-Araf}$. The assignment was confirmed using two-dimensional ¹³C/¹H correlation n.m.r. (Figure 2) and homonuclear two-dimensional COSY (not shown). These experiments demonstrated that the C-1 signal at 107 p.p.m. has its anomeric proton resonance at 5.25 p.p.m., allowing the identification of the resonances of the corresponding H-2s at 4.20 p.p.m., which in turn showed connectivities with carbons having their resonances at 88–89 p.p.m. (Figure 2). The signal at 102 p.p.m. was assigned to the resonance of the C-1s of the recently revealed $\beta\text{-D-Araf}$ [12,20]. The occurrence of $\beta\text{-D-Araf}$ in the arabinomannans was also suggested by the presence of a signal at 64 p.p.m. assignable only to the resonance of unsubstituted C-5 of this residue [19]. The remaining anomeric signals at 100 and 104 p.p.m. were assigned to the resonances of the C-1s of the D-Manp residues. From the literature data, the most shifted resonance was assignable to that of the 6-linked Manp , whereas the terminal and the 2-linked Manp have their resonances at 103–104 p.p.m. [22]. These assignments were confirmed by the analysis of the homo- and hetero-nuclear (Figure 2) two-

Figure 2 Two-dimensional heteronuclear ($^{13}\text{C}/^1\text{H}$)-n.m.r. spectrum of the arabinomannan of *M. tuberculosis*

The assignments of the main cross-peaks are indicated.

dimensional spectra. The resonances of the C-1s at 104 p.p.m. correlated with those of the H-1s at 5.10 p.p.m, which in turn were connected to two groups of H-2 at 4.05 and 4.10 p.p.m. (results not shown). The corresponding C-2 resonances were observed at 79 and 70 p.p.m. respectively (Figure 2) and were assignable to those of a 2-linked and of a terminal Manp [18]. Similarly, the resonances of C-1s at 100 p.p.m. were assigned to those of 2,6- and 6-linked Manp. All the Manp residues were shown to occur in the α configuration as the $J_{\text{C-1-H-1}}$ values were found to be 170–172 Hz, instead of the 160 Hz characteristic of β -Manp [23]. The enzymic degradation of the arabinomannans by α -mannosidase (Table 1) supports this conclusion.

The question of the occurrence of substantial amounts of Arap residues in the polysaccharides was also addressed by n.m.r. By showing that all of the coupling constants ($J_{\text{C-1-H-1}}$) of the anomeric carbon atoms were ≥ 170 Hz, it was obvious that the α -Arap residue ($J_{\text{C-1-H-1}} = 160$ Hz [21]) was not present in significant amounts in the arabinomannans. Because the resonances of C-2, C-3 and C-4 of β -Arap appeared at 69.5 p.p.m. [19], it was clear that the majority of the polysaccharide was devoid of β -Arap residues as the spectra showed only substituted primary alcohol carbon signals in this region. Based on the n.m.r. data

(Figure 2), which were very similar to those of the previously described lipoarabinomannans [20,24,25], it can be concluded that the native exocellular arabinomannans of *M. tuberculosis* share some predominant structural features with the latter related compounds. They mainly consist of an arabinan composed of at least three major structural domains [12,20,24] and of an α -D-mannan core (see below). The predominant structural features of the arabinan involved 5-linked α -D-Araf residues, representing more than 50 % of the arabinosyl units (Table 1) and occurring as linear oligosaccharides which may be interrupted by branched 3,5-linked α -D-Araf residues.

Evidence of the presence of mannosyl residues on the non-reducing termini of the arabinan segment

In mycobacterial arabinogalactan [12] and uncapped lipoarabinomannan [20], the non-reducing end region was always characterized by a disaccharide composed of terminal β -D-Araf and 2- α -D-Araf, which has been shown to represent the antigenic motif of the polysaccharides [18,26]. The occurrence of such a conserved structure implies a molar ratio for the two glycosyl residues of 1. Examination of Table 1 showed that the arabino-

Figure 3 One of the possible structures of the capped arabinomannan and an explanation of the effects of acetolysis and of α -mannosidase treatment

From the apparent molecular mass of the arabinomannan (13000 Da) and the Ara/Man molar ratio (2:1), it was estimated that the polysaccharide contains roughly 68 Ara and 25 Man. The O-methylation data (Table 1) showed that 2 mol of 2,6-linked- and 4 mol ($m+n$) of 2-linked-Man were present per molecule. Acetolysis demonstrated that terminal Man was linked to position 2 of 2,6-linked Man. It follows that most of the terminal Man residues are directly linked to Ara residues.

mannan described herein did not fulfil this criterion, suggesting some differences in the terminal region of its arabinan segment. The combined evidence of small amounts of terminal Ara_f (as compared with 3,5-linked Ara_f) and the excess of terminal Man_p (as compared with branched Man residues) strongly suggests the substitution of the non-reducing end of the arabinan segments of the arabinomannan by either a terminal Man residue or an oligomannoside. As expected with this hypothesis, treatment of the native polysaccharide by α -mannosidase (Figure 3) resulted in a significant increase of the percentage of terminal Ara_f (Table 1).

Structure of the mannan segment of the arabinomannan

The mannan segments of the D-arabino-D-mannans examined herein consisted of terminal, 2-, 6- and 2,6-Man_p (Table 1). From these structural data, two possible arrangements exist: a 1 $\rightarrow$ 6 backbone of mannosyl residues substituted with 1 $\rightarrow$ 2 mannosyl segments and vice versa. Based on the results of α -mannosidase digestion (Table 1) showing the decrease of the percentage of both 2-linked and 2,6-linked Man_p, it is clear that the first arrangement is the valid one. To determine the length of the oligosaccharides branched on the mannan backbone, arabinomannans were submitted to acetolysis (Figure 3) and the resulting peracetylated products were analysed by t.l.c. and f.a.b.-m.s. Both techniques demonstrated that peracetylated mono- and disaccharides were the acetolysis products: a major spot, whose R_F value corresponded to that of peracetylated mannoside, and a minor spot, which exhibited a chromatographic mobility similar to that of peracetylated diglycoside, were observed on t.l.c. plates. Analysis of the f.a.b.-mass spectrum of the acetolysis products showed two pseudomolecular ion peaks ($M+Na$) at m/z 413 and 701 and two peaks corresponding to the loss of acetate ($M-59$) from peracetylated mono- and di-glycoside species at m/z 331 and 619 respectively. Per-O-methylation of the acetolysis products, followed by acid hydrolysis, sodium borohydride reduction, acetylation and g.c.-m.s. analysis, allowed the identification of terminal Man_p and 2-Man_p.

Smith degradation of the polysaccharides, followed by the analysis of the degradation products, allowed the identification

of glycerol and Ara but not of Man, in agreement with the proposed structure.

DISCUSSION

The presence of mycobacterial products in culture media has been known from the earliest work on the tubercle bacillus and notably that of Seibert and collaborators [27–29]. They demonstrated the high complexity of the material derived from the culture filtrate and also used for skin testing, namely tuberculin. Analysis of the Seibert fractions established the presence of several proteins and two types of polysaccharides (for a review see [30]): polysaccharide-I consisted of Ara and Man whereas polysaccharide-II contained mainly Glc. However, the subsequent demonstration of the presence of arabinogalactan, a true cell wall constituent [12,26], as the major antigenic component of polysaccharide-I [30], questioned the origin of the tuberculin products; accordingly, the impression prevails that these products are derived from the autolysis of bacterial cells. Consequently, the important question of the possible origin of the substances isolated was addressed in the present study. By demonstrating the absence of several somatic and wall-associated compounds in the culture filtrates during the growth phase, the isolated polysaccharides appeared to be true exocellular material. Neither the cell-wall-associated compounds (lipids and wax D), somatic DNA, 6-O-methyl-containing lipopolysaccharides or glycogen were detected before reaching the stationary growth phase. In addition, polysaccharides are present in the early-growth-phase culture filtrates. Although the glucan was found to be structurally related to glycogen (highly branched structure), the length of the branched segments differs in the two polysaccharides: a glucosyl residue substitutes some six positions of the glucan core whereas seven to nine glucosyl units are present in the branched segments of the glycogen isolated from *M. tuberculosis* [31]. Furthermore, while the glucan exhibited an apparent molecular mass of 123000 Da, mycobacterial glycogen was found with an average molecular mass of 100×10^6 Da (1000-fold greater than that determined for the glucan) [31].

The exocellular polysaccharides described herein should be compared with those previously isolated from tuberculin. Despite

a similar glycosyl composition, the glucan characterized in the present study is different from polysaccharide-II. This latter polysaccharide was found to consist of a linear $\rightarrow 2\text{-}\alpha\text{-D-Glc-1}\rightarrow$ chain, as revealed by its glycosyl-linkage analysis [32]. As expected from the proposed structure, polysaccharide-II was not sensitive to β -amylase which hydrolyses $\rightarrow 4\text{-}\alpha\text{-D-Glc-1}\rightarrow$ glycosyl bonds and did not give the reddish brown colour with iodine typifying glycogens [29]. In contrast, the glucan herein described was readily hydrolysed by amyloglucosidase, as expected from its glycogen-like structure.

The second class of exocellular polysaccharides which we characterized shares with polysaccharide-I, derived from tuberculin, the same glycosyl composition. To our knowledge, no detailed structural information is available on polysaccharide-I. Nevertheless, the capacity of this polysaccharide to be adsorbed on to the erythrocyte surface, thus sensitizing the cells, which in turn agglutinate in the presence of the corresponding antiserum (haemagglutination or Middlebrook-Dubos reaction) implies the presence of a lipid moiety [33]. The arabinomannan characterized in the present study was devoid of lipid, as deduced from the analysis of the n.m.r. spectra. Furthermore, the acylated and non-acylated arabinomannans isolated by various vigorous treatments of the bacterial cells were shown to exhibit acidic groups [7,25,34,35] whereas the major polysaccharides obtained from the culture filtrates analysed were neutral. These results reinforce the concept that the outermost cell envelope polysaccharides are not derived from autolysis of the cells.

In an *in vivo* context, the major outermost polysaccharide (i.e. glucan) may play a role in the defence mechanisms of mycobacteria. For instance, because of its highly branched structure, related to that of glycogen, the glucan may be mistaken for the latter compound, so the bacteria endowed with this non-immunogenic polysaccharide may be left unrecognized by the host defence mechanisms, as previously reported for other bacterial pathogens using molecular mimicry [36,37]. Likewise, based on the occurrence of $\alpha\text{-D-Manp}(1\rightarrow2)\text{-}\alpha\text{-D-Manp}$ residues in the oligosaccharide chains of mammalian cell surface glycoconjugates [38], the mannan as well as the Man-capped arabinomannan may play a similar role in the non-recognition of the tubercle bacillus by host cells. Furthermore, the immuno-suppressive action of the arabinomannan purified from culture filtrates of *M. tuberculosis* towards human lymphocytes [39] has potential relevance to the pathogenesis of human tuberculosis and the nature and limitation of the resulting host response. Further studies, involving the characterization of the exocellular constituents of both pathogenic and non-pathogenic mycobacterial species, are in progress in order to substantiate the possible role(s) of these outermost components of grown cells *in vivo*.

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