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Revisiting the Structure of the Anti-neoplastic Glucans of *Mycobacterium bovis* Bacille Calmette-Guérin

STRUCTURAL ANALYSIS OF THE EXTRACELLULAR AND BOILING WATER EXTRACT-DERIVED GLUCANS OF THE VACCINE SUBSTRAINS*

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The attenuated strain of *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), used worldwide to prevent tuberculosis and leprosy, is also clinically used as an immunotherapeutic agent against superficial bladder cancer. An anti-tumor polysaccharide has been isolated from the boiling water extract of the Tice substrain of BCG and tentatively characterized as consisting primarily of repeating units of 6-linked-glucosyl residues. *Mycobacterium tuberculosis* and other mycobacterial species produce a glycogen-like α-glucan composed of repeating units of 4-linked glucosyl residues substituted at some 6 positions by short oligoglucosyl units that also exhibits an anti-tumor activity. Therefore, the impression prevails that mycobacteria synthesize different types of anti-neoplastic glucans or, alternatively, the BCG substrains are singular in producing a unique type of glucan that may confer to them their immunotherapeutic property. The present study addresses this question through the comparative analysis of α-glucans purified from the extracellular materials and boiling water extracts of three vaccine substrains. The polysaccharides were purified, and their structural features were established by mono- and two-dimensional NMR spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of the enzymatic and chemical degradation products of the purified compounds. The glucans isolated by the two methods from the three substrains of BCG were shown to exhibit identical structural features shared with the glycogen-like α-glucan of *M. tuberculosis* and other mycobacteria. Incidentally, we observed an occasional release of dextrans from Sephadex columns that may explain the reported occurrence of 6-substituted α-glucans in mycobacteria.

Tuberculosis, an infectious disease that has plagued man over the last several centuries, remains a leading cause of mortality worldwide, with 2–3 million people dying of the disease annually. It is estimated that almost 2 billion people, one-third of the world's population, are or have been infected by *Mycobacterium tuberculosis*, the causative agent of tuberculosis. To date the only effective vaccine strain against the disease is the so-called Bacille Calmette-Guérin (BCG).[1] This attenuated strain derived from 230 passages of *Mycobacterium bovis in vitro* between 1908 and 1921 (1). The original attenuated live vaccine has subsequently been sent to various countries and is conserved through continued passages that led to a profusion of phenotypically different substrains, as recently established by comparative genomics (2–5). BCG has also shown efficacy against tumors, notably as an immunotherapeutic agent for the treatment of superficial bladder cancer, the fourth most common cancer among men (6). Because the use of the whole BCG is sometimes associated with significant morbidity, the search for immunologically active BCG subcomponents is an attractive alternative. With the underlying idea of identifying defined components responsible for the anti-neoplastic properties of the BCG vaccine, an α-glucan with the expected activity was isolated from a boiling water extract of the Tice substrain of *M. bovis* BCG (7). The polysaccharide exhibited a molecular mass estimated between 65 and 87 kDa by gel filtration and consisted primarily of repeating units of →6-α-D-glucosyl residues; it also contained a few →4-α-D-glucosyl residues substituted at position 6 with a monoglucosyl residues (Fig. 1*A*). In other mycobacterial species, however, no such polysaccharide has been described to date. Rather, an α-D-glucan, a D-arabino-D-mannan, and a D-mannan were isolated from culture filtrates of surface-exposed materials of *M. tuberculosis* and some other pathogenic mycobacterial species (8–10). Structural studies demonstrate that these α-glucans are glycogen-like polysaccharides (Fig. 1*B*), with an apparent molecular mass of 100 kDa, and are composed of repeating units of 5–6 →4-α-D-glucosyl residues substituted at position 6 with short oligoglucosyl residues (9–10). These polysaccharides are also produced by tubercle bacilli inside macrophages (11) and are involved in the nonopsonic binding of *M. tuberculosis* to mammalian cells through the complement receptor 3 (12). More importantly, polysaccharides with similar chromatographic behavior were purified from the Pasteur substrain of BCG and were shown to exhibit an anti-tumor activity against superficial bladder cancer (13). Finally, glycogen-like α-glucans have been character-

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[1] The abbreviations used are: BCG, Bacille Calmette-Guérin; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; BWG, boiling-water glucan; CFG, culture filtrate glucan; COSY, homonuclear (^{1}H-^{1}H) COSY; DEPT, distortionless enhancement by polarization transfer; GC, gas chromatography; HMQC, heteronuclear (^{1}H-^{13}C) multiple quantum correlation; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; RD, Region of deletion; TOCSY, two-dimensional total correlation spectroscopy.

ized from the cell wall preparations of an unspecified substrain of *M. bovis* BCG (14). Consequently, the impression prevails that mycobacteria or at least *M. bovis* BCG contain two types of glucans with different cell locations and/or extractabilities, (i) glycogen-like α-glucans loosely attached to the bacterium and partly released in the culture fluid (9–10) and (ii) →6-α-D-glucans present in deeper compartments of the cell envelope and extractable only with boiling water (7) but not with mild mechanical extraction (10, 15). Alternatively, the latter polysaccharides may typify the BCG substrains, thereby explaining their greater ability to protect against both tuberculosis and cancer than other mycobacterial strains. The present study was undertaken to address this question. For this purpose, the α-glucans from the culture filtrates (CFG) and boiling water extracts (BWG) of three substrains of *M. bovis* BCG were purified and comparatively analyzed by mono-and two-dimensional NMR spectroscopy, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), and conventional chemical techniques.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The Tice, Pasteur, and Connaught substrains of *M. bovis* BCG examined in this study were grown on the synthetic Sauton's medium (16) (100 ml/flask) either as surface pellicles or on an orbital shaker at 37 °C.

Primers and RT-PCR Analysis—Chromosomal DNA was extracted from BCG Connaught, Pasteur, and Tice substrains as previously described by Torrea *et al.* (17) with some modifications. Briefly, 10 ml of log phase cultures of *M. bovis* BCG were centrifuged, and the bacterial cells were resuspended in 500 μl of lysis solution containing sucrose (25% w/v), 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 500 μg of lysozyme/ml. The suspension was incubated overnight at 37 °C, and 500 μl of proteinase K (400 μg/ml in 100 mM Tris, pH 8, 1% SDS) were added. After a 4-h incubation at 55 °C, 200 μl of 5 M NaCl were added, and DNA was extracted with phenol and chloroform and precipitated with ethanol. Pellets were then resuspended in water and stored at −20 °C before use.

PCR were performed in a final 50-μl volume and contained per reaction 10–50 ng of DNA template, 100 nM concentrations of each primer, 4 μl of nucleotide mix (10 mM), 5 μl of buffer 10× (AmpliTaq Gold buffer, Applied Biosystems), 4 μl of 25 mM $MgCl_2$, 5 μl of Me_2SO, and 0.25 units of *Taq* polymerase (AmpliTaq Gold Applied Biosystems). An initial denaturation step of 1 min at 95 °C was followed by 30 cycles of 1 min at 58 °C, 1 min at 72 °C and terminated by 1 elongation step of 10 min at 72 °C. Primers sequences are listed in Table I. Those corresponding to internal RD14 and RD15 internal regions were generous gifts of Dr. R. Brosch (Institut Pasteur, Paris, France). Primer sequences from RD15 flanking sequences were designed by using the Primer3 web site www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi and should amplify ~500-bp fragments.

Isolation of the Culture Filtrate Macromolecules and Boiling Water Extracts—Bacterial cells were removed from the growth medium by filtration through a Nalgene 0.22-μm filter (Nalge, NY), and the culture filtrates were concentrated (20 times) by rotary evaporation under vacuum. High molecular weight carbohydrates and proteins were precipitated overnight at 4 °C with 6 volumes of cold ethanol. The precipitates were collected after centrifugation at 14,000 × *g* for 1 h, dissolved in distilled water, dialyzed for 1 day against water to eliminate traces of salts, lyophilized, and weighed. The polymers were dissolved in distilled water, and the α-glucans were purified as previously described (9). BCG cells were suspended in 100 ml of phosphate-buffered saline and centrifuged twice at 14,000 × *g* for 10 min. The washed cells were extracted in a boiling water flask by refluxing with 100 ml of distilled water for 2 h as described (7). The extracts were filtered through a sterile Nalgene 0.22-μm filter (Nalge, NY). The filtrates were concentrated by rotary evaporation under vacuum, and the concentrated filtrates were precipitated overnight at 4 °C with 6 volumes of cold ethanol. The precipitates were collected after centrifugation at 14,000 × *g* for 1 h, dissolved in distilled water, and dialyzed overnight against 5 liters of distilled water, lyophilized, and weighed.

Purification and Chemical Characterization of Glucans—The ethanol precipitates originated from the culture filtrates, and the boiling water extracts were separately chromatographed first on a column (55 × 1.8 cm) of DEAE-Trisacryl (IBF, Villeneuve-La-Garenne, France) gel, and the neutral fractions were re-chromatographed either on a Bio-Gel P-100 (Bio-Rad) or a Sephadex G-200 (Amersham Biosciences) column (90 × 1.8 cm) using 0.5% acetic acid or 0.1 M NaCl in 1% acetic acid as eluent. The elution profiles of the gel filtration chromatography were monitored by refractive index detection, and the collected fractions were assessed for their carbohydrate content (18). Samples of purified polysaccharides were *O*-methylated (19); portions of the per-*O*-methylated products were hydrolyzed with 2 M CF_3COOH at 110 °C for 2 h, reduced with $NaBH_4$, and acetylated. The different partially *O*-methylated and partially *O*-acetylated glucitols were identified by gas chromatography (GC) and GC-MS.

Lipid Analysis—Wet cells were first extracted with chloroform-methanol (1:2, v/v) then five times with chloroform-methanol (1:1, v/v) and finally once with chloroform-methanol (2:1, v/v). These extracts were pooled, dried under vacuum, and partitioned between water and chloroform (1:1, v/v); the organic phase was extensively washed with distilled water, evaporated to dryness to yield the crude lipid extract, and weighed. The washed lipid extract was redissolved in chloroform and analyzed by TLC on silica gel Durasil 25-precoated plates (0.25 mm thickness; Macheray-Nagel). Lipid spots were resolved by TLC run in the solvent mixtures petroleum ether-diethyl ether (9:1, v/v) for analyzing phthiocerol dimycocerosates and triacylglycerol (15, 20), chloroform-methanol (9:1, v/v) for phenolic glycolipids and trehalose dimycolates (15, 20), and chloroform-methanol-water (60:30:8, by volume) for trehalose monomycolates and phospholipids (15). Sugar-containing compounds were visualized by spraying the plates with 0.2% anthrone in concentrated sulfuric acid followed by heating at 110 °C. The Dittmer-Lester reagent was used to detect phosphorus-containing substances. The ninhydrin reagent was used to reveal the presence of free amino groups; a spray with 10% molybdophosphoric acid in ethanol solution followed by heating at 110 °C was used to detect all of the lipid spots. Mycolates were obtained by saponification of delipidated cells with 5% KOH in methanol/benzene (8:2, v/v) for 16 h at 80 °C (21); after acidification with sulfuric acid, fatty acids were extracted with diethyl ether, extensively washed with water, and then methylated with diazomethane.

Pullulanase Digestion of Glucans—Samples (2 mg) of purified mycobacterial glucans, pullulan (positive control, Pfanstiehl, Laboratories Inc., Waukegan, IL), and maltoheptaose (negative control, Sigma) were dissolved in 200 μl of CH_3COONa (0.5 M, pH 5.6). Twenty μl of pullulanase (4.2 mg/ml, EC 3.2.1.41, Sigma) from *Klebsiella pneumoniae* were added to the reaction mixtures and incubated for 24 h at 40 °C. The enzyme was inactivated by heating at 100 °C for 10 min, and the reaction mixtures were dried and analyzed by ^1H NMR for the completeness of the digestion (the absence of signal resonances at 4.99 ppm assigned to Glc *p*-(1→6)- residues, see below, under "Results"). Per-*O*-acetylation of the oligosides released by enzyme digestion were performed by adding a mixture of acetic anhydride/anhydrous pyridine (1:1, v/v) to the samples; the reaction mixtures were left at 37 °C for 90 min and then dried. Dichloromethane (2 ml) and distilled water (2 ml) were added followed by thorough mixing. The organic phases were removed and washed 4 times, dried, and analyzed by MALDI-TOF MS.

Acetolysis—Dry polysaccharide samples (1 mg) were acetolysed as previously described (22). Briefly, a 1-ml sample of a mixture of acetic acid/acetic anhydride/sulfuric acid (10:10:1, by volume) was added to the samples, and the mixtures were left at 40 °C for 3 h. Anhydrous pyridine (1 ml) and methylene chloride (1 ml) were added followed by thorough mixing. The organic phases were removed and washed three times with water (1 ml), dried, and analyzed.

Sugar Analysis—Samples (200 μg) were hydrolyzed with 2 M CF_3COOH at 100 °C for 1 h. The resulting materials were dried and dissolved in 200 μl of anhydrous pyridine (Riedel-de Haën, RdH Laborchemikalien GmbH Co., Seelze, Germany) followed by the addition of 100 μl of hexamethyldisilazane (Aldrich) and 50 μl of trimethylchlorosilane (Aldrich) as described (23). The reaction was incubated at room temperature for 15 min. The mixture was dried under nitrogen, and the trimethylsilyl derivatives were solubilized in diethyl ether and analyzed by GC and GC-MS.

Aminopyrene Trisulfonic Acid Derivatization of Polysaccharides—Samples (250 μg) of glucans were derivatized with 8-aminopyrene 1,3,6-trisulfonic acid (APTS) by adding a large excess (0.55 μl of a 15% acetic acid solution (0.12 M)) of APTS trisodium salts (Fluka) and 30 μl of 1 M $NaBH_3CN$ (Aldrich) in an Eppendorf tube. The reaction mixture was heated at 55 °C under a ventilated hood for 24 h. The APTS derivatives of polysaccharides were purified by gel filtration chromatography on a PD10 (Amersham Biosciences) column using distilled water as eluent. The elution profile was monitored by spectrophotometry (λ 450 nm), and the monosaccharide composition of the fractions was determined by acid hydrolysis, trimethylsilylation, and GC.

Miscellaneous Analytical Techniques—^{1}H NMR spectra of purified glucans and dextran (Amersham Biosciences) were obtained in D_2O (99.9% D) on a Bruker AMX-500 spectrometer in 5-mm tubes at 500.13 MHz for ^{1}H and at 125.77 MHz for ^{13}C using a 5-mm BBI probe. The temperature was maintained at 343 K to obtain a reasonable line width. The double quantum-filtered COSY experiment was performed using the standard Bruker® pulse-field gradient program cosydfprtp, with presaturation during relaxation delay on HOD signal, 1.5-s recycle delay, 0.41-s acquisition time, 4096 data points in the F2 dimension, and 512 increments in the F1 dimension. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 4096 points, and a shifted sine-bell apodization function was interactively optimized and applied before Fourier transformation.

The two-dimensional TOCSY spectra (clmlevprtp) were recorded with an effective spin lock time of 112 and 160 ms using MLEV17 sequence for mixing, a 1.5-s recycle delay, and a 1.46-s acquisition time. The parameters for the two-dimensional heteronuclear (^{1}H-^{13}C) multiple quantum correlation (HMQC) spectra were as follows: inv4gptp standard Bruker® program, 1.5-s recycle delay, 0.68-s acquisition time, GARP ^{13}C decoupling during acquisition. ^{13}C-NMR spectra were obtained using the Bruker® DEPT (distortionless enhancement by polarization transfer) sequence.

MALDI-TOF mass spectra (in the positive mode) were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in the reflector mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive ion mode of 20 kV. To improve the signal-to-noise ratio, 150 single shots were averaged for each mass spectrum, and typically, four individual spectra were accumulated to generate a summed spectrum. Mass spectrum calibration was performed using the calibration mixture 1 of Sequazyme™ peptide mass standards kit (Perseptive Biosystems), including known peptide standards in a mass range from 900 to 1600 Da. Internal mass calibration was performed as previously described (24). The stock solutions of peracetylated oligosides were prepared in chloroform at a concentration of 1 mM and were directly applied on the sample plate as 1-μl droplets followed by the addition of 0.5 μl of matrix solution (2,5-dihydroxybenzoic acid (10 mg/ml) in chloroform/methanol (1:1, v/v)). Samples were allowed to crystallize at room temperature.

The diffusion parameters of the purified polysaccharide solutions (1 mg/ml in distilled water) were determined by dynamic light scattering. The apparatus consisted of a DynaPro-MS/X dynamic light scattering instrument (ProteinSolutions, Charlottesville, VA), providing a 830 nm light beam. Samples were analyzed in a 12-μl quartz sample cell at 20 °C. Using the Dynamic V6 software, dynamic light scattering autocorrelation function data were collected at a scattering angle of 90° with a data acquisition time of 200 s.

GC of monosaccharide derivatives was performed on a Hewlett-Packard 5890 series II apparatus equipped with an OV1 capillary column (0.30 × 25m) using helium gas. The temperature separation program involved an increase from 60 to 100 °C at the rate of 20 °C/min followed by an increase from 100 to 300 °C at the rate of 5 °C/min.

GC-MS analyses were performed on a HP 5889X mass spectrometer (electron energy, 70 eV) coupled to a HP 5890 series II gas chromatograph fitted with a column identical to that used for GC; GC-MS analyses were realized in the electron impact mode.

RESULTS

The structural differences between the glucans obtained from the CFGs and surface-exposed materials of mycobacteria, including an unspecified BCG substrain (14) and those derived from the BWG of the Tice substrain of *M. bovis* BCG (Fig. 1), raised the question of the relatedness of the Tice substrain to other BCG daughter substrains. It was, thus, important to re-examine some of the well defined characters of this strain to clarify its taxonomic status before the structural analysis of its polysaccharide content.

Molecular Characterization of BCG Substrains—The Tice substrain of BCG is believed to have derived from the original vaccine strain after 1931, as revealed by comparative genomics of BCG daughter substrains (5). We investigated the relatedness of the Tice substrain to two other BCG daughter substrains, namely the Pasteur and Connaught substrains, which have also previously been used for the isolation of anti-neoplastic glucans (13, 25), by well established molecular techniques. Among these is lipid analysis, a method routinely used for the identification and reclassification of mycobacterial strains, because of the diversity and uniqueness of mycobacterial lipids (26). Accordingly, lipids from the three substrains were extracted with organic solvents and comparatively analyzed. The lipid profiles of all three substrains of BCG were similar to each other and to that of *M. bovis* (data not shown) as expected. The extractable lipids include the mycobacterial ubiquitous lipid compounds such as triacylglycerol and phospholipids (26) and the species- and type-specific dimycocerosates of phthiocerol and phenolic glycolipid of *M. bovis* (20, 27). The fatty acid profiles of the BCG daughter substrains were also similar, consisting of C_{16}-C_{26} fatty acids and dicyclopropanated α-mycolates and ketomycolates. All substrains, however, were devoid of the methoxymycolates that typify *M. bovis*, *M. tuberculosis*, and some other slow growing mycobacterial species (21). This observation was consistent with the previous report of the occurrence of mutations in the *cmaB* gene that encodes the methyltransferase that *O*-methylates the hydroxymycolate precursors to yield methoxymycolates (28). These data also confirmed the proposed genealogy of BCG substrains, which suggests that the Tice, Pasteur, and Connaught substrains derived from a recent evolution from the original BCG strain (5, 29).

Comparative PCR analysis using specific primers that detect large chromosomal regions present in *M. bovis* but absent from some BCG substrains (RD) since their original derivation (2–5) was performed to identify the three substrains. RD08 (5), also called RD15 (30), is missing from the Connaught substrain, whereas RD14 is specifically depleted in the Pasteur daughter strain of *M. bovis* BCG. The two regions were preserved in the Tice substrain (5). Two primer pairs were used for both RDs, one for amplifying internal sequences and other for the detection of flanking sequences. The latter primer allows the amplification of a 500-bp segment only when a gap was left in the chromosome by the corresponding RD. Application of this strategy showed that although the Tice substrain has retained both RD08 and RD14, the Connaught and Pasteur daughter substrains were devoid of RD08 and RD14, respectively (Table I), in agreement with published data (5). It was, thus, concluded that the observed genotypes and phenotypical characters of the daughter substrains of BCG examined in the present study corresponded to the standard substrains.

Purification of Glucans from the Culture Filtrates and Boiling Water Extracts of M. bovis BCG—One possibility that may explain the structural differences between the CFGs of mycobacteria other than *M. bovis* and the BWG of the Tice substrain of *M. bovis* BCG was the occurrence of two types of α-glucans differently located in the cell envelope of mycobacteria. To test this hypothesis, we isolated the glucans from both the culture filtrate and boiling water extract of the Tice substrain of BCG. To exclude strain variability, the CFGs and BWGs were also purified from the Pasteur and Connaught daughter substrains of *M. bovis* BCG. Thus, the unfractionated culture filtrates and the boiling water extracts from the three substrains of *M. bovis* BCG were precipitated by ethanol. The precipitates were first fractionated by ion-exchange chromatography (8–10). The glucans were then purified from the neutral fractions of the DEAE-Trisacryl column by gel filtration chromatography on a Bio-Gel P-100 column. They were eluted at the void volume of the Bio-Gel columns and represented the main constituents of the neutral fractions from both the extracellular materials of all the substrains (data not shown). The quantitatively minor additional peaks were composed of arabinomannans and mannans (as determined by GC analysis of the acid hydrolysis products). The elution profiles of the neutral polysaccharides

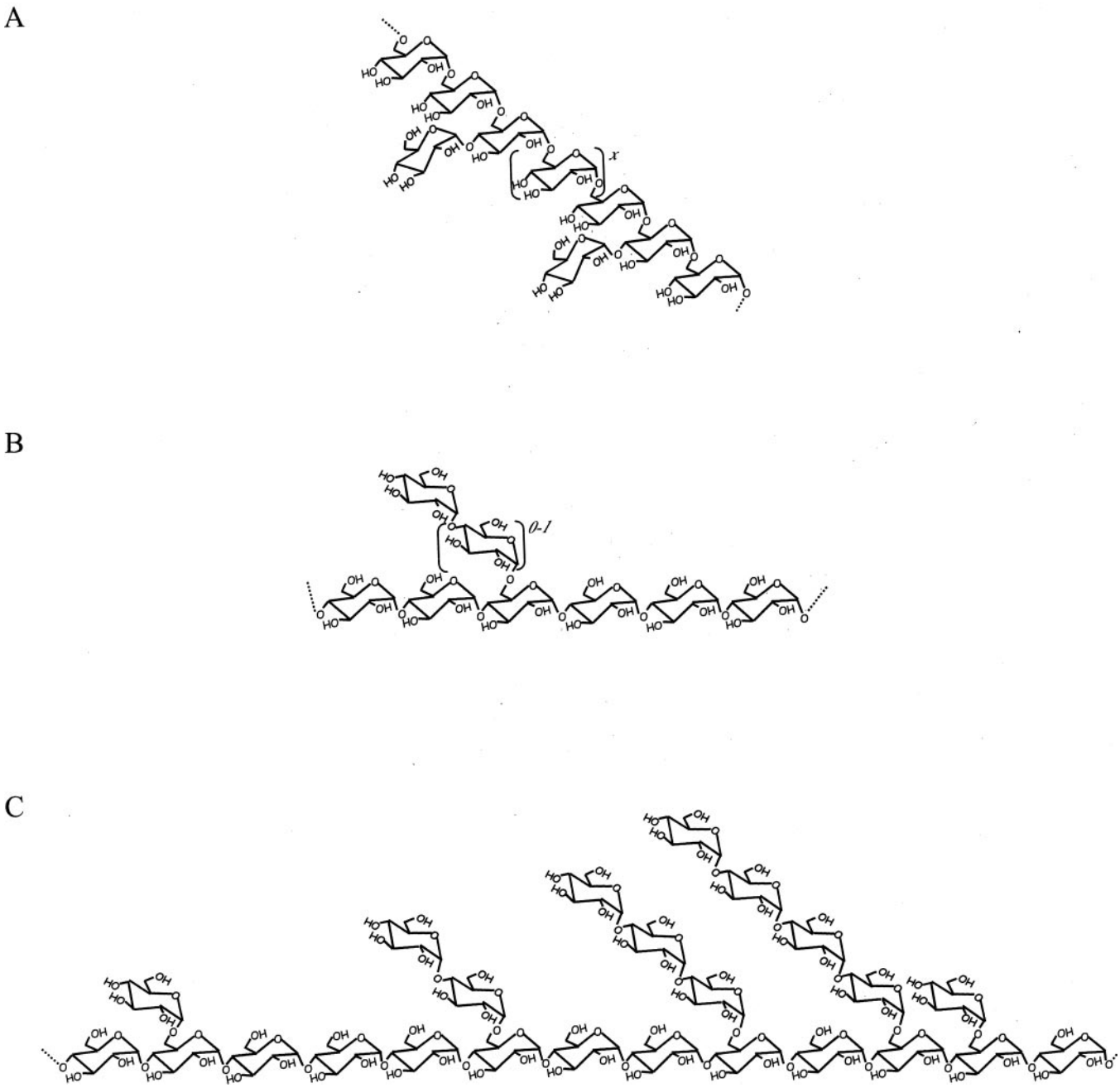

FIG. 1. **The repeating units proposed for the mycobacterial anti-neoplastic α-glucans.** *A*, the glucan purified from the boiling water extract of the Tice strain of *M. bovis* BCG (7); *x*, unknown. *B*, the glycogen-like glucan purified from the culture filtrate and surface-exposed materials of *M. tuberculosis* (9,10). *C*, a tentative structural model for the repeating unit of the glycogen-like α-glucan of *M. bovis* BCG based on the data from MALDI-TOF spectrometry and the glycosyl-linkage composition (terminal -Glc*p*:4-Glc*p*:4,6-linked-Glc*p* in a 1:3:1 ratio). An alternative model would be a mixture of repeating units with homogeneous branched chains, *i.e.* mono, di-, tri-, and tetra-glucosyl residues branched at some positions 6 of the linear 4-linked-Glc*p* chain.

from the boiling water extracts were qualitatively similar to those derived from the culture filtrates ,but the latter materials contained more glucans than the former ones (data not shown).

The homogeneity of the glucans from both sources was determined by dynamic light scattering. The polysaccharides purified from the culture fluids and boiling water extracts of the BCG daughter substrains contained most exclusively (98–100%) a unique molecular species (data not shown) exhibiting similar conformational features as ascertained by their average radius (R = 28 nm ± 2.6).

Structural Features of the Glucans Revealed by NMR Analysis—Consistent with the above data the ^{1}H NMR spectrum of the purified CFG from the Tice substrain of *M. bovis* BCG (Fig. 2*A*) was superimposable both to those of the BWG and CFG isolated from other BCG substrains. The ^{1}H NMR spectra of the purified CFGs were very similar to that of the α-glucans of *M. tuberculosis* (10). They showed two main anomeric signals at 5.38 and 4.99 ppm. The chemical shift (above or around 5.0 ppm) and coupling constant (3–4 Hz) values indicated an α-configuration for both types of sugar residues (31).The remaining upfield signals were attributed to the proton resonances of H-2s to H-6s. The chemical shift values and the relative intensities of these resonances were similar to those observed in the spectrum of type III rabbit liver glycogen (32); in contrast, the intensities of these signals were reversed in the spectrum of the BWG isolated by Wang *et al.* (7).

TABLE I
Molecular identification of the Pasteur, Tice, and Connaught daughter substrains of M. bovis BCG

	RD[a]			
	RD14 (9.0 kilobases from Rv1765c to Rv1773c)[b]		RD08 (3.4 kilobases from Rv0309 to Rv0312)[c]	
Primers[d]	Internal[c]	Flanking[e]	Internal[f]	Flanking
Forward	Primer 1	Primer 3	Primer 5	Primer 7
Reverse	Primer 2	Primer 4	Primer 6	Primer 8
	PCR amplification of approximately 500-bp fragments in BCG strain			
Connaught	+	−	−	+
Pasteur	−	+	+	−
Tice	+	−	+	−

[a] RD are designed as described in Behr *et al.* (5) and Brosch *et al.* (30).
[b] Rv numbers refer to open reading frames form the *M. tuberculosis* H37Rv genome. Sequences are available at www.pasteur.fr/recherche/unites/Lgmb.
[c] RD08 in Behr *et al.* (5) corresponds to RD15 in Brosch *et al.* (30).
[d] Primer sequences: Primer 1, GTGGAGCACCTTGACCTGAT; Primer 2, CGTCGAATACGAGTCGAACA; Primer 3, TTGATTCGCCAACAACTGAA; Primer 4, GGGCTGGTTAGTGTCGATTC; Primer 5, CCATCTTCAAGGGGCTACAG; Primer 6, ATTTGCAGGCAGACCTCATC; Primer 7, TGACCACACGAGGTGATTGT; Primer 8, GATGGCTGTCAACGTCAATG.
[e] Available from supporting information in Brosch *et al.* (30).
[f] Sequence generously communicated by Dr. Roland Brosch (Institut Pasteur).

To assign the ^{1}H NMR resonances of the CFGs and BWGs analyzed herein, the two-dimensional COSY, TOCSY, and HMQC spectra of the purified polysaccharides were recorded and analyzed. Based on the chemical shifts recorded for different types of glucosyl residues from various glucans (7, 9, 31, 32), three types of glycosyl residues could be identified by NMR analysis of the mycobacterial α-glucans shown in Fig. 1: (i) terminal Glc*p*-(1→4)- residues, (ii) →4)-Glc*p*-(1→4)-, and →4,6)-Glc*p*-(1→4)- residues, and (iii) terminal Glc*p*-(1→6)- and →4)-Glc*p*-(1→6)- residues. Analysis of the two-dimensional COSY spectra of the purified glucans (Fig. 3) showed that the H-1 signal resonances between 5.33 and 5.45 ppm correlate with several H-2 resonances. Starting from the most prominent anomeric resonance at 5.38 ppm, it was possible to find two series of H-1/H-2 cross-peaks. The cross-peak of the H-1s resonance of one series with the corresponding H-2s resonance was slightly shifted downfield from that of the H-1s resonance of the second series, allowing the identification of the resonances of the two H-2s at 3.66 and 3.62 ppm, respectively. Because of the overlapping of cross-peaks, however, it was difficult to assign with certainty the H-3 through H-6 resonances of the two series. Two-dimensional TOCSY experiments were used to solve the question of identifying the resonances of the two series. The TOCSY spectrum showed that resonances at 3.66, 3.85, and 3.98 ppm belonged to the same spin system of one series, whereas resonances at 3.44, 3.62, 3.72, 3.73, 3.80, and 3.90 ppm belonged to the spin system of the other series (Fig. 4). Thus, starting from two well identified signals isolated from the bulk of peaks, namely the resonance at 3.98 ppm of one series and that at 3.44 ppm of the other series, it was possible to identify most of the resonances of the two series that have their H-1s resonance at 5.38 ppm by two-dimensional COSY (Fig. 3, Table II). Thus, the proton resonances of the H-3s of the two series were identified at 3.98 and 3.72 ppm, and those of H-4s were seen at 3.66 and 3.44 ppm. Two-dimensional TOCSY experiments were also helpful in the assignment of the proton resonances belonging to the series that has its H-1 resonance at 4.99 ppm, for which no information was obtained by the analysis of the two-dimensional COSY spectrum, probably because of the small intensities of the corresponding proton resonances. By improved varying of the mixing times in the TOCSY experiments it was also possible to assign most of the resonances of the spin system (Fig. 4, Table II).

The ^{13}C NMR spectra of the α-glucans of *M. bovis* BCG substrains were superimposable with that of the corresponding polysaccharides from *M. tuberculosis* (10). The anomeric resonance signal was seen at 100.1 ppm, which can only have resulted from the resonances of C-1s of α-Glc*p*, with those of β-Glc*p* and of α-Glc*f* expected at a lower field (104–110 ppm; see Bradbury and Jenkins (33)). A signal was observed at 78.0 ppm and assigned to the resonance of C-4s of 4-linked-α-D-Glc*p* because *O*-substitution of glycosyl residues at a given position is known to cause the corresponding carbon chemical shift to move upfield by 5–10 ppm (9, 32, 33). Thanks to the identification of the resonance of C-4s of 4-substituted α-D-Glc*p* residues it was possible, using two-dimensional HMQC experiments (Fig. 5), to attribute the resonances of the corresponding H-4s (at 3.66 ppm) and thereby to confirm the assignment of the resonances of the protons of 4-substituted α-D-Glc*p* (Table II), in full agreement with data obtained for rabbit liver glycogen (32). The unsubstituted C-2s, C-3s, and C-5s had their resonances between 70 and 74 ppm, whereas the resonance of unsubstituted C-6s was easily identified by the DEPT experiment at 61.3 ppm (33). The attribution of the various carbon resonances was performed by two-dimensional HMQC experiments (Fig. 5) and is summarized in Table II. Thus, two-dimensional NMR experiments performed on both CFGs and BWGs purified from the Tice, Connaught, and Pasteur substrains of *M. bovis* BCG demonstrated that the polysaccharides from both sources share their major structural features. It was, thus, concluded that the α-glucans isolated from the extracellular materials and boiling water extracts of the three substrains of *M. bovis* BCG were glycogen-like polysaccharides consisting of linear 4-linked-α-D-Glc*p* residues substituted at position 6 with mono- or oligo-α-D-Glc*p* units.

Determination of the Chain Lengths of Branched Residues—The chain lengths of the α-glucans were determined by enzyme and chemical degradations. For this purpose standard polysaccharides, *i.e.* pullulan (positive control) and maltoheptaose (negative control), and the mycobacterial α-glucans were both acetolysed and treated with pullulanase, an enzyme that specifically hydrolyzes (1→6)-α-D-Glc*p* linkages. The completeness of the enzyme degradation was checked by the disappearance of the ^{1}H NMR signal attributed to the α-D-Glc*p*-(1→6)-linked residues at 4.99 ppm, and the resulting oligosaccharides were per-*O*-acetylated. The per-*O*-acetylated oligoglucosides obtained by both chemical and enzymatic degradations were analyzed by MALDI-TOF MS and found to be similar in composition. As expected, triglucosides were released from pullulan, whereas no digestion was observed for maltoheptaose. The mass spectra of the per-*O*-acetylated oligoglucosides liberated from the α-glucans showed intense pseudomolecular $(M + Na)^+$ peaks at 701, 989, 1277, 1565, and 1853 *m/z* (data not shown). They corresponded to di-, tri-, tetra-, penta-, and hexa-

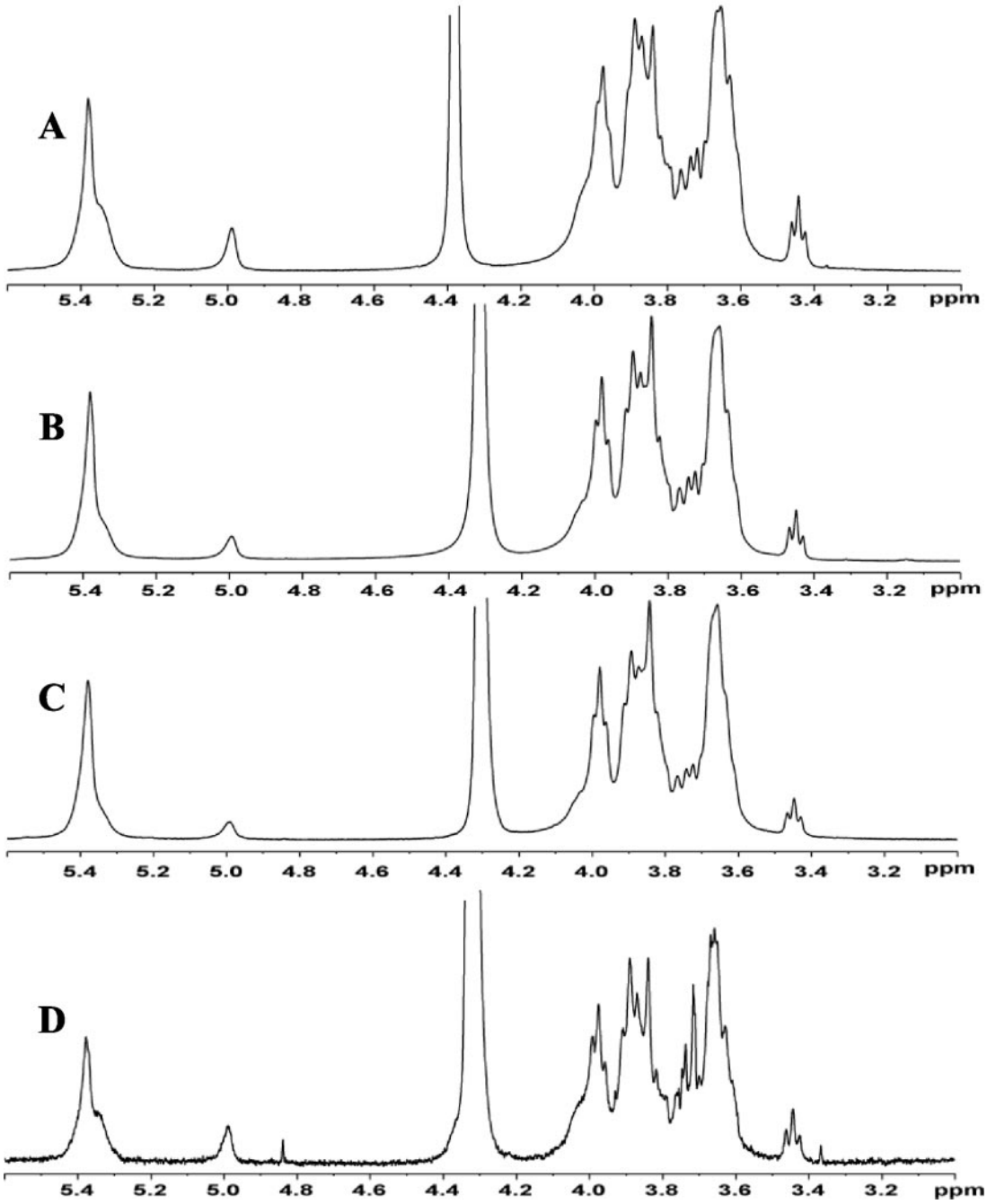

FIG. 2. **^{1}H NMR spectra of glucans purified from the culture filtrates of the Connaught (*A*), Pasteur (*B*), and Tice (*C*) substrains of *M. bovis* BCG and from the boiling water extract of the Tice strain (*D*).** Spectra were recorded in D_2O (99.9% D) at 500.13 MHz and 333 K (*A*, HOD at 4.40 ppm) or 343 °K (*B–D*, HOD at 4.30 ppm).

oligoglucosides. TLC analysis of the per-*O*-acetylated oligoglucosides confirmed the above data and showed the occurrence of monoglucoside derivatives in the mixture of degradation products (data not shown), the corresponding $(M + Na)^+$ peaks, expected at 413 at *m/z*, being masked by the matrix ion peaks. These results support a structure for the α-glucans from *M. bovis* BCG based on a linear 4-linked-α-D-Glc*p* substituted by oligoglucosyl residues at some 6 positions.

Glycosyl-linkage Analysis of the Purified Glucans of M. bovis BCG—The possible cyclic nature of the glucans was examined by labeling the polysaccharides with APTS, a fluorescent probe that can be covalently linked to a carbohydrate provided that it possesses a reducing end that is first reduced by $NaBH_3CN$ before reacting with APTS. Thus, the purified CFG of the Pasteur substrain of *M. bovis* BCG was incubated with APTS and $NaBH_3CN$, and the reaction products were chromatographed on a PD-10 column. The excluded fractions were found to contain both Glc and APTS, indicating that glucans from both culture filtrates and boiling water extracts were linear polysaccharides and possess a free reducing end (data not shown). Analysis of the partially *O*-methylated alditol acetate derivatives of CFGs and BWGs by GC-MS (in the electron impact mode) revealed the occurrence of three types of constituents. The major molecular species (60%) corresponded to 4-linked-Glc*p*, with mass peaks at 117, 161, 233, and 277 *m/z*; the two other, representing 20% each, corresponded to terminal Glc*p*, with mass peaks observed at 117, 161, 205, and 249 *m/z*, and 4,6-linked-Glc*p*, typified with mass peaks at 117, 161, 261, and 305 *m/z* (data not shown). Importantly, no traces of ions assignable to the fragmentation of an alditol acetate derived

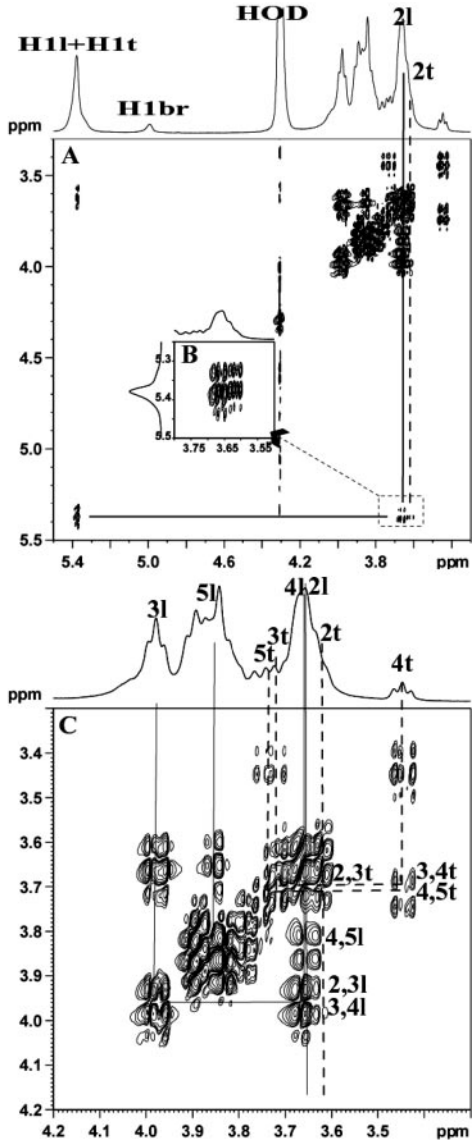

FIG. 3. **Two-dimensional COSY-double quantum-filtered NMR spectrum of the glucan purified from the culture filtrate of the Tice strain of *M. bovis* BCG (*A*) and expansion of the H-1/H-2 (*B*), and ring proton (H-3 through H-6) (*C*) cross-peak regions.** The spin system for both the terminal (*t*) α-D-Glc*p*-(l→4) and linear (*l*) →4)-α-D-Glc*p*-(l→ residues are drawn (*C*) with the help of the TOCSY spectrum (see Fig. 4). Only the anomeric proton of the →4-linked-α-D-Glc*p*-(l→6)- (*br*) is visible on the COSY spectrum (*A*); the other proton resonances of this spin system were attributed by two-dimensional TOCSY experiments (see Fig. 4 and Table II). Acquisition parameters were as described under "Experimental Procedures."

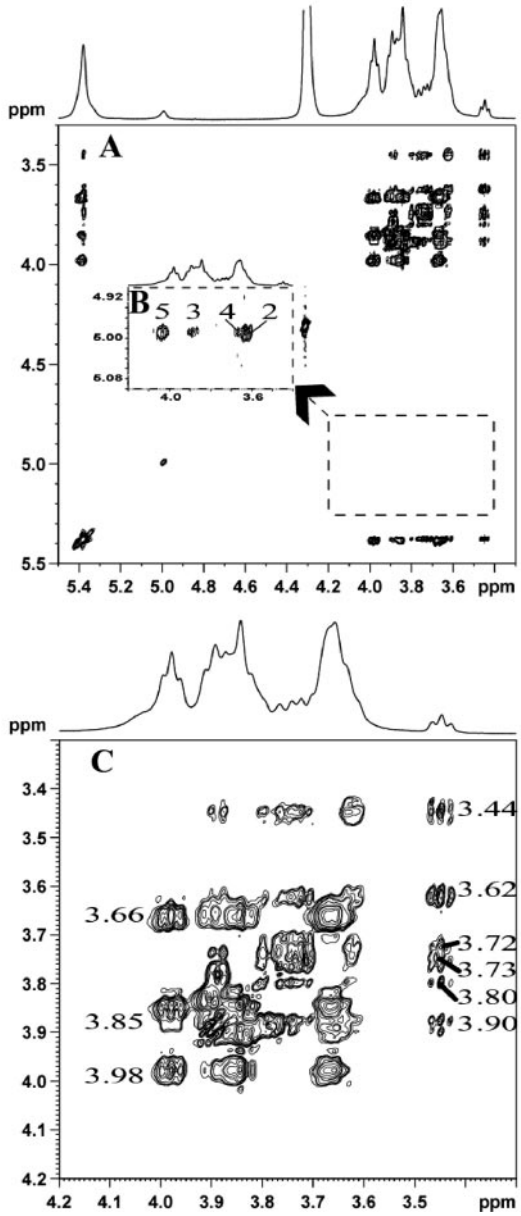

FIG. 4. **Two-dimensional TOCSY NMR spectrum of the glucan purified from the culture filtrate of the Tice strain of *M. bovis* BCG (*A*).** The magnification of the H-1/H-2 cross-peak region of the spin system of the -α-D-Glc*p*-(l→6)- residues (*B*) and the expansion of the ring proton (H-3 through H-6) cross-peak region (*C*) are shown. The H-2 through H-5 of the spin system of the →4-linked-α-D-Glc*p*-(l→6)-branching residues (*B*) and the chemical shift values of the ring proton resonances (in ppm) of the terminal and linear →4)-α-D-Glc*p*-l→ Glc*p* residues (*C*) are also indicated. Acquisition parameters were as described under "Experimental Procedures."

from 6-linked-Glc*p* was observed in the glucans of *M. bovis* BCG analyzed herein; this latter partially *O*-methylated alditol acetate derivative was the prominent component of the alditol derivatives detected in the BWG from the Tice substrain of BCG published by Wang *et al.* (7).

To further ascertain the absence of 6-linked-Glc*p* residues in the glucans of substrains of *M. bovis* BCG, a standard linear polysaccharide composed of 6-linked-Glc*p* residues, *i.e.* T-40 dextran, was analyzed by mono-dimensional ^{1}H NMR (Fig. 6A) and two-dimensional COSY, TOCSY and HMQC (data not shown). The chemical shift values of the glucosyl residues of dextran were significantly different from those of the α-glucans isolated from *M. bovis* BCG (Table II). Furthermore, the down-shifted value of the chemical shift of H-4 (at 3.66 ppm) of the Glc*p*-(1→6)-branching residues in the α-glucans isolated from *M. bovis* BCG compared with those of unsubstituted H-4 in terminal Glc*p* (at 3.44 ppm) and Glc*p*-(1→6) in dextran (at 3.57 ppm) demonstrated that the branched chains contained only 4-linked-Glc*p* but not 6-linked-Glc*p*.

Altogether these data clearly indicated that the glucans purified from both CFGs and BWGs of *M. bovis* BCG daughter substrains contained the three types of glycosyl residues found in the CFG of *M. tuberculosis* (9). The structures of the glucans from both *M. tuberculosis* and *M. bovis* BCG substrains analyzed herein differed from that previously proposed for the neoplastic glycan isolated from the boiling water extract of the Tice substrain of *M. bovis* BCG (7). The structure proposed for the glucans of *M. bovis* BCG (Fig. 1*C*) is similar to that of

TABLE II

Chemical shifts (in ppm) of the proton and carbon resonances of the glycosyl residues that composed the α-glucans purified from the extracellular material and boiling water extract of the Tice strain of Mycobacterium bovis BCG and chemical shifts of the proton resonances of the glucosyl residues of dextran

Chemical shift values of the different proton and carbon resonances were deduced from the interpretation of the monodimensional ^{1}H NMR and ^{13}C NMR and the two-dimensional COSY-DQF, TOCSY, and HMQC spectra. The types of residues refer to Fig. 1.

H and C atoms	Residues			
	→4)-α-Glc*p*-(1→4)-linear Glc*p* units	α-Glc*p*-(1→4)-terminal Glc*p* units	→4)-Glc*p*-(1→6)-branching Glc*p* units	→6)-α-Glc*p*-(1→6)-linear units in dextran
H-1	5.38	5.38	4.99	5.05
C-1	100.1	100.1	98.5	99.8
H-2	3.66	3.62	3.64	3.65
C-2	72.0	72.0		73.5
H-3	3.98	3.72	4.03	3.80
C-3	73.8	73.4		75.5
H-4	3.66	3.44	3.66	3.57
C-4	78.0	70.0		71.8
H-5	3.85	3.73	3.88	3.98
C-5	72.0			72.3
H-6		3.8/3.9		3.85/4.04
C-6	61.3	61.3		67.9

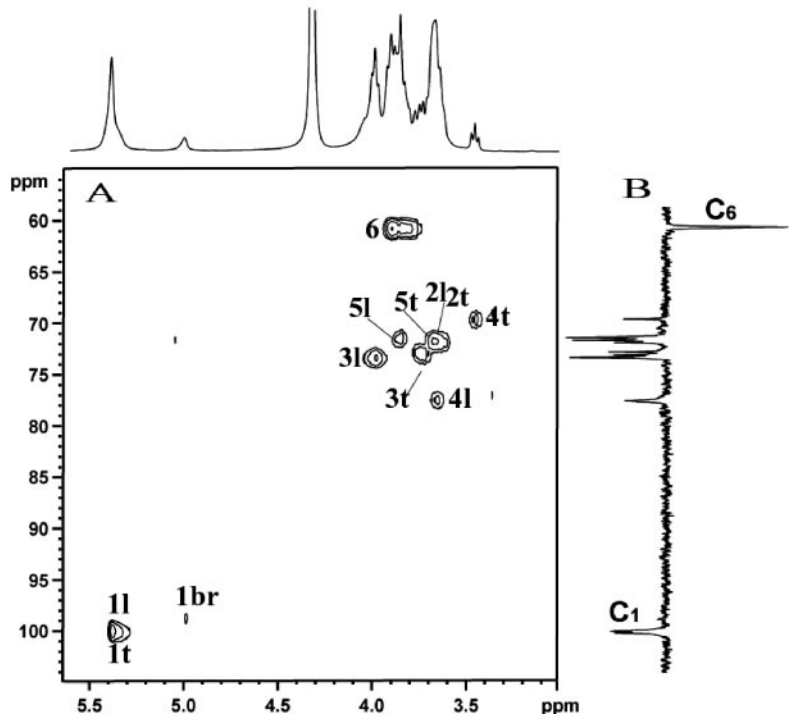

FIG. 5. **Two-dimensional HMQC NMR spectrum of the glucan purified from the culture filtrate of the Tice strain of *M. bovis* BCG Pasteur.** The ^{13}C NMR spectrum was obtained using the Bruker® DEPT sequence. Acquisition parameters were as described under "Experimental Procedures." The assignments of the main cross-peaks are indicated. *t*, terminal Glc*p* residues; *l*, linear →4)-α-D-Glc*p*-(l→6) residues; *br*, branching 4)-α-D-Glc*p*-(1→6).

glucans from *M. tuberculosis* (9, 10); it differed from the latter by the occurrence of longer branched chains in *M. bovis* BCG than in *M. tuberculosis*.

A Possible Explanation for the Occurrence of 6-Linked α-Glucans in Mycobacterial Products—When the Glc-rich fractions originated from the ethanol precipitates of *M. bovis* BCG daughter substrains were fractionated by gel chromatography on Sephadex columns, occasionally several peaks were observed, all composed of α-glucans (as determined by GC analysis of the acid hydrolysis products) eluted from the gel at different positions, including that of the mycobacterial glycogen-like α-glucans (data not shown). Examination of the ^{1}H NMR spectra of the various compounds showed that they were all superimposable with that of the standard dextran, notably with anomeric signals at 5.05 ppm, attributable to the resonances of H-1s of 6-linked-α-D-glc*p* residues (Fig. 6*A*). The origin of these polysaccharides was ascertained by irrigating a Sephadex column by the solvent alone and analyzing the eluted compounds (Fig. 6*B*). Analysis of the two-dimensional NMR spectra and glycosyl-linkage compositions of these polysaccharides (data not shown) confirmed their structural identity with dextrans. We, thus, concluded that the occurrence of 6-linked α-D-Glc*p* residues in the glucan isolated from the Tice strain of *M. bovis* BCG (7) is probably due to the use of Sephadex columns to purify the polysaccharide.

DISCUSSION

Structural studies have demonstrated that the α-glucans isolated from both the culture fluids and cell surface of *in vitro* grown *M. tuberculosis* are glycogen-like polysaccharides (Fig. 1*B*) composed of repeating units of 5–6 →4-α-D-glucosyl residues substituted at position 6 with oligoglucosyl residues (9, 10). α-Glucans isolated from some other mycobacterial species (8) exhibited structural features similar to those determined for that of *M. tuberculosis* (9, 10). These polysaccharides are also produced by intracellular tubercle bacilli (11) and have been shown to possess various biological properties. Among these an anti-neoplastic activity against superficial bladder cancer has been observed for the glucans isolated from the culture fluid of the Pasteur substrain of *M. bovis* BCG (13).

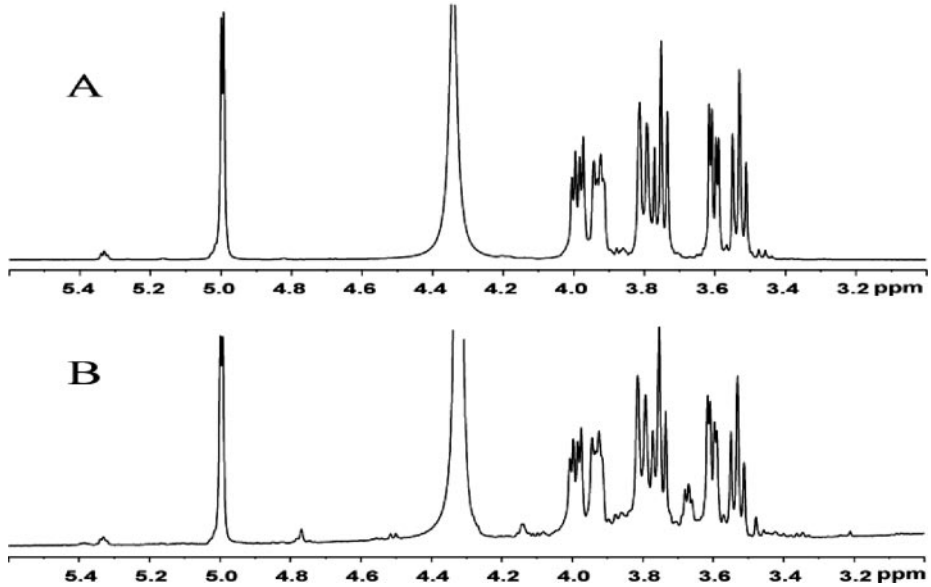

FIG. 6. **^{1}H NMR spectra of dextrans.** *A*, a standard dextran (T-40). *B*, the polysaccharide fraction leaked from a Sephadex G-200 column irrigated with the solvent alone (1% acetic acid in 0.1 M NaCl). Spectra were recorded in D_2O (99.9% D) at 500.13 MHz at 343 K (*B*, HOD at 4.30 ppm).

What may be similar material has been identified in cultures of the Tice substrain of *M. bovis* BCG (7), but the structure proposed for the polysaccharide isolated from this strain was different from that of the glycogen-like α-glucans produced by *M. tuberculosis* and consists primarily of repeating units of →6-α-D-glucosyl residues (Fig. 1*A*). This observation prompted us to address the question of the singularity of *M. bovis* BCG and, alternatively, the occurrence of two structurally unrelated families of glucans in mycobacteria or at least in the Tice substrain of *M. bovis* BCG.

M. tuberculosis and *M. bovis* are two species that are members of the *M. tuberculosis* complex. Based on the structural identity between the various α-glucans isolated from phylogenetically distant mycobacterial species such as *M. kansasii*, *M. smegmatis*, and *M. avium* (8), the possibility that the closely related *M. tuberculosis* and *M. bovis* differ in terms of synthesis of polysaccharides was unlikely. Nevertheless, the hypothesis was examined by analyzing the glucans isolated from three substrains of *M. bovis* BCG. The structural features determined for the α-glucans purified from the extracellular materials of the three substrains were found to be very similar to those of the glycogen-like polysaccharides previously isolated from other mycobacteria (8–10). A more probable hypothesis that may explain the structural difference between the α-glucans isolated from *M. tuberculosis* and *M. bovis* consisted of different locations and/or extractabilities of the polysaccharides. Although the α-glucans of *M. tuberculosis* and other species were purified from the extracellular and surface-exposed materials, that of *M. bovis* was purified from a boiling water extract of BCG cells. Therefore, the latter aggressive procedure may extract polysaccharides originating from various bacterial cell envelope compartments. Structural analysis of the α-glucans purified from the boiling water extracts of three substrains of *M. bovis* BCG demonstrated that the polysaccharides shared the same structural features with the extracellular α-glucans isolated from the substrains which corresponded to those of the glycogen-like α-glucans, in apparent conflict with the data published by Wang *et al.* (7) but consistent with the previous characterization of structurally similar molecules from the cell walls of an unspecified strain of *M. bovis* BCG (14). Altogether, the present work demonstrates that various compartments of the mycobacterial cell envelope contain the same type of glucans composed of repeating units of →4-α-D-glucosyl residues substituted at position 6 with oligoglucosyl units. The reason for the isolation from the Tice strain of *M. bovis* BCG of polysaccharides consisting of repeating units of →6-α-D-glucosyl residues (7) is unclear. One possibility resides in the elution of dextrans, *i.e.* linear 6-substituted α-D-glucans, derived from Sephadex gels during the purification procedure, a phenomenon we have occasionally observed. In this connection it is interesting to note that the glucans isolated from boiling water extract from the Tice strain of *M. bovis* BCG have been purified by gel chromatography on Sephadex columns (7). This may explain the presence of 6-linked-α-D-glucan in what was considered as polysaccharides purified from *M. bovis* BCG. Accordingly, the anti-tumor activity that has been found for the glucans isolated from the boiling water extract of the Tice strain of *M. bovis* BCG (7) would correspond to the anti-tumor activity subsequently described for the glucans produced by the Pasteur strain of *M. bovis* BCG (11), glucans that are shown herein to correspond to a glycogen-like polysaccharides. Construction of glycogen-like glucan-defective strains of *M. tuberculosis* and *M. bovis* are under progress; the analysis of the resulting mutant strains should definitely clarify the question of the production by mycobacteria of structurally different glucans.

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Revisiting the Structure of the Anti-neoplastic Glucans of *Mycobacterium bovis* Bacille Calmette-Guérin: STRUCTURAL ANALYSIS OF THE EXTRACELLULAR AND BOILING WATER EXTRACT-DERIVED GLUCANS OF THE VACCINE SUBSTRAINS

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