Structural and functional features of *Rhodococcus ruber* lipoarabinomannan

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The genus *Rhodococcus* is part of the phylogenetic group nocardioform actinomycetes, which also includes the genus *Mycobacterium*. Members of this phylogenetic group have a characteristic cell envelope structure, which is dominated by various complex lipids. Among these, lipoglycans are of particular interest since mycobacterial lipoarabinomannans are important immunomodulatory molecules that are likely to be involved in the subsequent fate of mycobacterial bacilli once inside phagocytic cells. *Rhodococcus ruber* is a species closely related to an established opportunistic human pathogen, *Rhodococcus equi*. This paper reports the isolation and characterization of *R. ruber* lipoarabinomannan, designated as RruLAM. SDS-PAGE and gas chromatography analyses revealed that RruLAM was of an intermediate size between *Mycobacterium tuberculosis* lipoarabinomannan and lipomannan. Using a combination of chemical degradation and 1H, 13C-NMR experiments, the carbohydrate structure of RruLAM was unambiguously shown to be composed of a linear (α1→6)-Man backbone substituted at some O-2 positions by a single t-α-Araf sugar unit. Integration of the anomeric proton signals provided an indication of the degree of branching as approximately 45%. The RruLAM structure is much simpler than that established for *M. tuberculosis* lipoarabinomannan but is also different from that determined for the closely related species and opportunistic human pathogen, *R. equi*. RruLAM was unable to induce the production of TNF-α by either human or murine macrophage cell lines, suggesting that more sophisticated structures, such as phosphoinositol capping motifs, are required for such activity.

INTRODUCTION

*Rhodococcus ruber*, a slow-growing species, is closely related to an established opportunistic human pathogen, *Rhodococcus equi*; both are classified within the phylogenetic group nocardioform actinomycetes (Goodfellow, 1986). This group also includes genera such as *Mycobacterium, Nocardia, Corynebacterium* and *Tsukamurella*. Typically, these species are Gram-positive, non-sporulating and partially acid-fast (Goodfellow, 1987). Members of this phylogenetic group have a characteristic cell envelope structure, composed primarily of branched long-chain lipids termed mycolic acids (Brennan & Nikaido, 1995). Information about the potential virulence of *R. ruber* is somewhat limited. However, it has been shown that immunosuppressed guinea pigs inoculated with *Rhodococcus* spp. develop visible granulomas (Haburchak et al., 1978). In addition, Osoagbaka (1989) isolated and cultured *R. ruber* from infected human sputa and subsequently infected immunosuppressed mice, finding the pathological appearance of the lungs similar to that observed in humans (Osoagbaka, 1989). Furthermore, mycolyl glycolipid fractions purified from *R. ruber* have been shown to activate the alternative complement pathway and induce granuloma formation in
immunosuppressed mice (Matsunaga et al., 1996; Yasuda, 1999).

Intense research into the architecture of the mycobacterial cell envelope has revealed an abundance of polysaccharides and lipoglycans, the latter consisting of phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM) and liparabinomannan (LAM) (Brennan & Nikaido, 1995). LAM is a largely heterogeneous molecule that possesses three distinct domains. Firstly, there is a carbohydrate backbone composed of two homopolysaccharides, D-mannan and D-arabian. The D-mannan segment exists as a linear (α1→6)-Manp backbone substituted at the O-2 position by single Manp residues; the D-arabian domain consists of a linear (α1→5)-Araf backbone punctuated by branching from 3,5-O-linked α-D-Araf residues (Chatterjee & Khoo, 1998; Vercellone et al., 1998). Secondly, there is a mannosyl-phosphatidyl-myo-inositol (MPI) anchor, and thirdly, various capping motifs.

Comparative analyses of LAMs from different mycobacterial strains have shown that within this common structure there is a great deal of heterogeneity, including variations in the acylation state of the MPI anchor (Nigou et al., 1999a), the presence of additional succinate residues (Delmas et al., 1997), methylipentose and arabinose residues (Treumann et al., 2002) and finally capping motifs on the non-reducing termini of the Araf residues (Chatterjee et al., 1992; Khoo et al., 1995). In slow-growing mycobacteria, such as Mycobacterium tuberculosis, these motifs are characterized by Manp residues (Chatterjee et al., 1992; Venisse et al., 1993), whereas fast-growing mycobacteria, such as Mycobacterium smegmatis, possess phosphoinositol capping motifs (Khoo et al., 1995; Gilleron et al., 1997), resulting in LAM being termed either ManLAM or PILAM, respectively. These subtle differences in the capping motifs are thought to explain the different immunomodulatory functions of ManLAM and PILAM. ManLAMs have the ability to inhibit the production of pro-inflammatory cytokines, such as IL-12 and TNF-α (Knutson et al., 1998; Nigou et al., 2001) and conversely PILAM stimulates the production of these pro-inflammatory cytokines (Adams et al., 1993; Gilleron et al., 1997). These facts not only corroborate the findings that slow-growing mycobacteria have the ability to exist and multiply within phagocytic cells and fast-growing mycobacteria do not, but illustrate the importance of ManLAM as a key virulence factor enabling the persistence of slow-growing mycobacteria within phagocytic cells (Nigou et al., 2002). Lipoglycans structurally related to mycobacterial LAM have been identified in a number of other actinomycetes, including Corynebacterium matruchotii (Sutcliffe, 1995), Dietzia maris (Sutcliffe, 2000), Gordonia rubropertincta (Flaherty & Sutcliffe, 1999) and Rhodococcus rhodnii (Flaherty et al., 1996), but these studies failed to show whether these lipoglycans possessed in vitro biological activities typical of mycobacterial LAM. However, recent work by Garton et al. (2002) described the structure of a novel lipoglycan from R. equi. The study demonstrated that the purified lipoglycan, termed ReqLAM, induced an immune response in equine macrophages that was equivalent to virulent R. equi, suggesting that the early macrophage cytokine response to the bacteria can be attributed to ReqLAM (Garton et al., 2002). We report here the isolation and characterization of a lipoglycan from R. ruber, a close relative of R. equi, and provide evidence for structural differences compared to ReqLAM. The relationship between their structures and their ability to induce inflammatory responses is discussed.

**METHODS**

**Bacteria and growth conditions.** R. ruber type strain DSM 43338 was purchased from the DSMZ, Braunschweig, Germany. It was routinely grown at 30 °C in GYM streptomycetes medium, which contained 4 g glucose, 4 g yeast extract and 10 g maltose extract per litre of deionized water, supplemented with 0-05 % Tween 80 (http://www.dsmz.de). Cells were grown to late exponential phase, harvested by centrifugation, washed and lyophilized.

**Purification of RruLAM.** Purification procedures were adapted from protocols established for the extraction and purification of mycobacterial lipoglycans (Nigou et al., 1997). Briefly, the cells were delipidated at 60 °C by using CHCl3/CH3OH (1:1, v/v). The delipidated biomass was resuspended in deionized water and disrupted by sonication (MSE Soniprep, 12 micro amplitude, 60 s on, 90 s off for 10 cycles, on ice). The cellular glycans and lipoglycans were further extracted by refluxing the broken cells in 50 % ethanol at 65 °C. Contaminating proteins and nucleotides were removed by enzymic degradation using z-amylose and proteases followed by dialysis. The resulting extract was resuspended in buffer A, 15 % propan-1-ol in 50 mM ammonium acetate, and loaded onto an octyl-Sepharose CL-4B column (50 × 2-5 cm) and eluted with 400 ml buffer A at 5 ml h⁻¹, enabling the removal of non-lipidic moieties (Nigou et al., 2001). The retained lipoglycans were eluted with 400 ml buffer B, 50 mM ammonium acetate and 50 % propan-1-ol. The resulting lipoglycans were resuspended in buffer C, 0-2 M NaCl, 0-25 % (w/v) sodium deoxycholate, 1 mM EDTA and 10 mM Tris, pH 8, to a final concentration of 200 mg ml⁻¹, and loaded onto a Sephacryl S-200 HR column (50 × 2-5 cm) and eluted with buffer C at a flow rate of 5 ml h⁻¹. Fractions of 1-25 ml were collected and analysed by SDS-PAGE followed by silver staining, which contained periodic acid. Lipoglycan fractions identified in this way were pooled, dialyzed extensively against water, lyophilized and stored at −20 °C.

**Fatty acid analysis.** A 200 μg sample of of RruLAM was decayed by strong alkaline hydrolysis (200 μl 1 M NaOH at 110 °C for 2 h). The reaction mixture was neutralized with HCl and the liberated fatty acids were extracted three times with chloroform and, after drying under nitrogen, were methylated using three drops of 10 % (w/v) BF3 in methanol (Fluka) at 60 °C for 5 min. The reaction was stopped by the addition of water and the fatty acid methyl esters extracted three times with chloroform. After drying, the fatty acid methyl esters were solubilized in 10 μl pyridine and trimethylsilylated using 10 μl hexamethyldisilazane and 5 μl trimethylchlorosilane at room temperature for 15 min. After drying under a stream of nitrogen, the fatty acid derivatives were solubilized in cyclohexane before analysis by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

**Analysis of glycosidic linkages.** Glycosyl linkage composition was analysed after methylation of RruLAM according to a modification of the procedure of Ciucanu & Kerek (1984). The methylated polysaccharide was hydrolysed with 300 μl 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h, reduced using 350 μl of a 10 mg ml⁻¹ solution...
of NaBD₄ (NH₄OH 1 M/C₂H₅OH, 1:1, v/v) and peracylated with 300 μl acetic anhydride for 1 h at 110 °C. The resulting alditol acetates were solubilized in cyclohexane before analysis by GC and GC/MS.

**APTS derivatization and capillary electrophoresis.** RruLAM (1 μg) was hydrolysed using strong acid hydrolysis (30 μl 2 M TFA at 110 °C for 2 h). The samples were dried and mixed with 0·3 μl 0·2 M 1-aminopyrene-3,6,8-trisulfonate (APTS) (Interchrom, Montluçon, France) in 15% acetic acid and 0·3 μl of a 1 M sodium cyanoborohydride solution dissolved in tetrahydrofuran. The reaction mixture was heated at 55 °C for 90 min and subsequently quenched by the addition of 20 μl water. A 2 μl sample of the APTS-derivatized solution was diluted 10-fold before being subjected to capillary electrophoresis. Analyses were performed on a P/ACE capillary electrophoresis system (Beckman Instruments) with the cathode on the injection side and the anode on the detection side (reverse polarity). The electropherograms were acquired and stored on a Dell XPS P60 computer using the System Gold software package (Beckman Instruments). APTS derivatives were loaded by applying 0·5 p.s.i. (3·45 kPa) vacuum for 5 s (6·5 ml injected). Separations were performed using an uncoated fused-silica capillary column (Sigma, Division Supelco) of 50 μm internal diameter with 40 cm effective length (47 cm total length). Analyses were carried out at a temperature of 25 °C with an applied voltage of 20 kV using 1% (w/v) acetic acid, 30 mM triethylamine in water, pH 3·5, as running electrolyte. The detection system consisted of a Beckman laser-induced fluorescence (LIF) detector equipped with a 4 mW argon-ion laser with an excitation wavelength of 488 nm and emission wavelength filter of 520 nm.

**NMR spectroscopy.** NMR spectra were recorded on a Bruker DMX-500 spectrometer equipped with a double resonance (1H/13C)-BBI z-gradient probe head. Around 5 mg RruLAM was exchanged in D₂O (D, 99-97% from Euriso-top, Saint-Aubin, France), with intermediate lyophilization, and then redissolved in 0·4 ml D₂O. Data were processed on a Bruker-X32 work station using the xwinnmr program. One-dimensional (1D) proton (1H) spectra were recorded at 308 K using a 90° tipping angle for the pulse and 1 s as recycle delay between each of the 256 acquisitions of 1·64 s. The spectral width of 4310 Hz was collected in 16k complex points that were transformed to 32k real points in the frequency domain. After Fourier transformation, the spectra were base-line corrected with a fourth-order polynomial function. The 1H NMR chemical shifts were referenced relative to internal acetone at 2·225 p.p.m.

All two-dimensional (2D) spectra were recorded without sample spinning and data were acquired in the phase-sensitive mode using the time-proportional phase increment (TPPI) method. The 2D 1H-13C heteronuclear multiple quantum correlation (HMOC) spectra were recorded in the proton-detected mode with a Bruker 5 mm 1H broadband tunable probe with reverse geometry. The globally optimized alternating-phase rectangular pulses (GARP) sequence (Shaka et al., 1985) at the carbon frequency was used as a composite pulse decoupling during acquisition. The spectrum was obtained according to the Bax and Subramanian pulse sequence (Bax & Subramanian, 1986). Spectral widths of 25 154 Hz in 13C and 4310 Hz in 1H dimensions were used to collect a 2048 × 512 (TPPI) point data matrix with 44 scans/tl increments expanded to 4096 × 1024 by zero filling. The relaxation delay was 1 s. A sine bell window shifted by π/2 was applied in both dimensions. The 2D 1H-13C HOHAHA spectra were recorded using an MLEV-17 mixing sequence of 112 ms (Bax & Davies, 1985). The spectral width was 4310 Hz in the F2 dimension and 4223 Hz in the F1 dimension; 512 spectra of 4096 data points with 24 scans/tl increments were recorded. The 2D 1H-1H NOESY spectra were acquired at different mixing time (100, 200 and 400 ms) using the sequence of Jeener et al. (1979). The spectral width was 1502 Hz in both dimensions; 512 spectra of 4096 data points with 32 scans/tl increments were recorded. The spin-lock field strength corresponded to a 90° pulse with 30 μs. Data were processed as above.

**TNF-α production by macrophages.** THP-1 and J774 monocyte/macrophage human and murine cell lines, respectively, were maintained in continuous culture with RPMI 1640 medium (Life Technologies), 10% fetal calf serum (Life Technologies) in an atmosphere of 5% CO₂ at 37 °C. THP-1 as non-adherent and J774 as adherent cells. Purified RruLAM was added in duplicate to monocyte/macrophage cells (5 × 10⁶ cells per well) in 24-well culture plates and then incubated for 6 h at 37 °C. Stimuli were previously incubated for 1 h at 37 °C in the presence or absence of 10 μg ml⁻¹ polymyxin B (Sigma), which is known to inhibit the effect of contaminating LPS (Adams et al., 1993). The assays were conducted independently and in triplicate. Supernatants were assayed for TNF-α using the previously described cytotoxic assay against WEHI164 clone 13 cells (Espevik & Nissen-Meyer, 1986). Basically, 50 μl supernatant was added to 50 μl of WEHI cells (5 × 10⁶ cells ml⁻¹) in flat-bottom 96-well plates and incubated for 20 h at 37 °C, then 50 μl tetrazolium salts (MTT, 1 mg ml⁻¹ in PBS) was added to each well and incubated for 4 h. Formazan crystals were solubilized with 100 μl lysis buffer [N,N-dimethyl formamide, 30% SDS (1:2), adjusted to pH 4·7 with acetic acid] and the absorbance read at 570 nm with an ELISA plate reader (Bio-Tek Instruments). The TNF-α content of supernatants was determined by comparison with a reference curve obtained using serial dilutions of human recombinant TNF-α (Life Technologies). LPS was from *Escherichia coli* O55:B5 (Sigma) and ManLAM from *Mycobacterium bovis* BCG.

**RESULTS**

**Purification and general structural features of RruLAM**

*R. ruber* lipoglycans were purified by the methods previously reported for mycobacterial lipoglycans (Nigou et al., 1997). The following critical steps were performed: (i) ethanol/water extraction of the lipoglycans from delipidated cells, (ii) enzymic degradation of protein contaminants, (iii) separation of lipoglycans from glycans by hydrophobic interaction chromatography, and (iv) size fractionation of lipoglycans by gel-permeation chromatography. This procedure allowed the recovery of a lipoglycan that migrated between *M. tuberculosis* LAM and LM as revealed by SDS-PAGE analysis (Fig. 1a). The lipoglycan was subsequently subjected to total acid hydrolysis (2 M TFA for 2 h at 110 °C) in the presence of mannoheptose as an internal standard. The resulting sugar hydrolysates were then converted to either APTS or TMS derivatives. The APTS derivatives were analysed by capillary electrophoresis monitored by laser-induced fluorescence (CE-LIF). The electropherogram (Fig. 1b) showed three peaks assigned as Ara-APTS (I), Man-APTS (II) and mannoheptose-APTS (III). From the peak integration, the relative composition of the lipoglycan polysaccharide was determined to be 23% Ara and 77% Man. In addition, preparation of TMS derivatives and subsequent analysis by GC and GC/MS revealed the presence of glycerol (Gro) and inositol (Ins) (not shown) and established the ratio of Ara/Man/Ins as 11:29:1. The presence of fatty acids was investigated by
alkaline hydrolysis followed by GC/MS analysis of fatty acid methyl esters. The predominant fatty acids identified were palmitic acid (C16:0) (56%) and tuberculostearic acid (10-methyloctadecanoic acid, C19:0) (33%), while hexadecenoic acid (C16:1), stearic acid (C18:0) and octadecenoic acid (C18:1) were present in smaller amounts (3%, 6% and 3%, respectively). In addition, high-field $^1$H-NMR resonances at 0.9 and 1.3 p.p.m. were assigned to –CH$_3$ and –(CH$_2$)$_n$– groups, respectively, confirming the presence of fatty acids and in agreement with GC/MS analysis (Nigou et al., 1999a).

Altogether, the presence of Gro, Ins and fatty acids, the basic components of a phosphatidyl-$\text{myo}$-inositol anchor, with Ara and Man, provided the signature of a lipoglycan related to mycobacterial LAM. The lipoglycan was subsequently termed RruLAM. Fig. 2(a) shows the 1D $^1$H-NMR spectrum of RruLAM; the spectrum is typified by an anomeric region which is much simpler than that observed for mycobacterial LAM (Venisse et al., 1993), but also significantly simpler than that of ReqLAM (Garton et al., 2002) (Fig. 2b), indicating a simpler carbohydrate structure. Indeed, the RruLAM 1D $^1$H spectrum (Fig. 2a) exhibits three major well-defined resonances, whereas the ReqLAM spectrum (Fig. 2b) is characterized by several overlapping resonances arising from six different glycosidic residues (Garton et al., 2002).

**Carbohydrate backbone**

The glycosidic linkages present in the RruLAM were analysed qualitatively by per-O-methylation analysis. The particularly dominant per-O-methylated alditol acetates identified by GC/MS corresponded to t-Araf, 2,6-Manp and 6-Manp residues (not shown). Beside these major products, minor traces of 5-Araf, t-Manp and 2-Manp were detected. A quantitative analysis of the glycosidic linkages was deduced from NMR data. The anomeric proton resonance region of RruLAM is dominated by three intense signals at $\delta$ 5.5-19 (I$_1$), $\delta$ 5.0-4 (II$_1$) and $\delta$ 4.9-1 (III$_1$) (Figs 2 and 3a). As revealed by $^1$H-13C HMQC experiment (Fig. 3b), their corresponding anomeric carbons resonate at $\delta$ 112-3 (I$_1$), $\delta$ 101-8 (II$_1$) and $\delta$ 102-4 (III$_1$), respectively. Anomeric proton and carbon resonances of the different spin systems were assigned from $^1$H-13C HMQC and $^1$H-1H HOHAHA experiments (Figs 3b and 4b, respectively) based on our previous studies with mycobacterial LAMs (Venisse et al., 1993; Gilleron et al., 2000) and LAM-related structures (Gilleron et al., 1999; Nigou et al., 1999b, 2002). The assignments are summarized in Table 1. Spin systems I, II and III were unambiguously assigned to t-Araf, 2,6-$\alpha$-Manp and 6-$\alpha$-Manp respectively, according to the following lines of evidence. Methylation analysis indicated the presence of one type of Araf and two distinct Manp moieties. The downfield resonance at 112-3 p.p.m. (I$_1$) was in agreement with an Araf with an $\alpha$-configuration. Indeed, the $\alpha$-anomeric configuration of t-$\alpha$-Araf (I) was deduced from its C-1 chemical shift at $\delta$ 112-3 compared to $\beta$-Araf units in mycobacterial LAM ($\alpha$Araf $\delta_{C-1}$ 108–110; $\beta$Araf $\delta_{C-1}$ 103) (Venisse et al., 1993; Gilleron et al., 1997) and by the presence of an intense intra-residue nOe contact between its H-1 (H-1) and H-2 (H-2) protons observed on the NOESY spectrum (Fig. 4c). The chemical shift of the C-4 carbon at $\delta$ 86.8 (I$_4$, Fig. 3b; Table 1) correlated with a furanose ring form. Resonances of C-2, C-3 and C-5 (I$_2$, I$_3$ and I$_4$ Fig. 3b, Table 1) were not deshielded, in agreement with an unsubstituted $\alpha$-Araf unit. Glycosylation at position O-6 of 2,6-$\alpha$-Manp (II) and 6-$\alpha$-Manp (III) was shown by the deshielding of their C-6 resonances at $\delta$ 68-6 (II$_6$ and III$_6$, Fig. 3b; Table 1).
Fig. 2. 1D $^1H$ spectrum of RruLAM (a) and ReqLAM (b) in D$_2$O. Full spectra ($\delta$ $^1H$ 5-50–0-50) are shown. RruLAM and ReqLAM (Garton et al., 2002) spectra were recorded at 308 K and 313 K, respectively.

Fig. 3. 1D $^1H$ (a) and 2D $^1H$-$^13C$ HMQC (b) spectra of RruLAM in D$_2$O at 308 K. Expanded regions ($\delta$ $^1H$ 3-67–5-25) (a) and ($\delta$ $^1H$ 3-67–5-25, $\delta$ $^{13}C$ 61–115) (b) are shown. Glycosyl residues are labelled in Roman numerals and their carbons and protons in Arabic numerals. I, t-$\alpha$-Araf; II, 2,6-$\alpha$-Manp; III, 6-$\alpha$-Manp; IV, t-$\alpha$-Manp.
compared to unsubstituted t-α-Manp in mycobacterial LAM (Δδ 4-4 p.p.m.) (Venisse et al., 1993; Gilleron et al., 1997). Glycosylation at position O-2 of 2,6-α-Manp (II) was determined by the deshielding of its C-2 resonance at δ 80-4 (II2, Fig. 3b; Table 1) compared to unsubstituted t-α-Manp and to the C-2 resonance of 6-α-Manp (III2, Fig. 3b; Table 1) at δ 73-0 (Δδ 7-4 p.p.m.). The α-anomeric configuration of both units was confirmed by the presence of an intense intra-residue nOe contact between their H-1 at δ 5-04 (II) and δ 4-91 (III) and their H-2 at δ 4-02 (II) and δ 4-00 (III) protons and the absence of intra-residue H-1/H-3 nOe contact (Fig. 4c). Beside the three major signals, a weak C-1 resonance at 105-2 p.p.m. (IV1) was detected on the HMQC spectrum (Fig. 3b). Its H-1 resonance at δ 5-05 (IV1) partially superimposed with H-1 of 2,6-α-Manp units (II1) (Figs 3a and 4a). Based on its C-1 and C-2 resonances at δ 105-2 and δ 72-9, respectively (IV1 and IV2, Fig. 3b; Table 1), spin system IV was tentatively attributed to t-α-Manp units detected by methylation analysis. Another spin system typified by its H-1 weak resonance at δ 5-15 (Fig. 4a) and composed of six protons (labelled with *) could only be characterized in the 1H-1H HOHAHA experiment (Fig. 4b). Due to the presence of six protons, this spin system was tentatively attributed to an Ara residue. The remaining non-labelled signals on the 1H-1H HOHAHA could not be assigned.

The sequence of the different units was investigated from the 1H-1H NOESY spectrum (Fig. 4c). Interestingly, H-1 of the t-α-Araf unit (I1) at δ 5-19 showed an intense inter-residue nOe contact with H-2 of the 2,6-α-Manp unit (II1) at δ 4-02, indicating that t-α-Araf units glycosylate 2,6-α-Manp units at O-2. H-1 of the 2,6-α-Manp unit (II1) and the 6-α-Manp unit (III1) showed inter-residue nOe contacts with protons between 3-74 and 3-81 p.p.m., tentatively attributed to their own H-6 (not shown). These data indicate that 2,6-α-Manp units on the one hand and 6-α-Manp units on the other hand are interconnected by (x1→6) linkages.

**Table 1.** Proton and carbon chemical shifts of RruLAM

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift (p.p.m.)*</th>
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<tbody>
<tr>
<td></td>
<td>H-1 C-1</td>
</tr>
<tr>
<td>t-α-Araf  (I)</td>
<td>5-19</td>
</tr>
<tr>
<td></td>
<td>112-3</td>
</tr>
<tr>
<td>2,6-α-Manp (II)</td>
<td>5-04</td>
</tr>
<tr>
<td></td>
<td>101-8</td>
</tr>
<tr>
<td>6-α-Manp  (III)</td>
<td>4-91</td>
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<tr>
<td></td>
<td>102-4</td>
</tr>
<tr>
<td>t-α-Manp  (IV)</td>
<td>5-05</td>
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<td>105-2</td>
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</tbody>
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ND, Not determined.

*Chemical shifts were measured at 308 K in D₂O and are referenced relative to internal acetone at δ₄H 2-225 and δ₄C 34-00.
†The chemical shifts of these protons could not be determined precisely and are given within an interval.
Mycobacterial ManLAMs are characterized by the presence of mannose caps, i.e. Man residues that glycosylate some Ara units. In order to investigate the presence of such structural features in RruLAM, the lipoglycan was submitted to the procedure previously developed for cap analysis: mild acidic hydrolysis (0.1 M HCl for 30 min at 110°C) followed by APTS derivatization and CE-LIF analysis (Nigou et al., 2000). The electropherogram (not shown) exhibited two main peaks assigned to Ara-APTS and Man-APTS but no peaks corresponding to oligosaccharide units, supporting the absence of (Manp)x→Araf→ sequences in RruLAM.

Altogether, the data allowed us to propose that the RruLAM carbohydrate backbone is a multibranched structure composed of a (α1→6)-Manp chain substituted at some O-2 positions by a single t-α-Araf unit (Fig. 5). A small number of 2,6-α-Manp units may also be glycosylated by a single t-α-Manp unit. Indeed, CE data indicate that the t-α-Manp units detected by methylation and NMR analyses do not glycosylate the Araf units, suggesting that these units directly substitute the (α1→6)-Manp chain. Integration of the anomeric proton signals yielded a ratio t-α-Araf/(2,6-α-Manp + t-α-Manp)/6-α-Manp of 1.0/1.0/1.0/1.2. The established structure implies that the number of t-α-Araf units plus t-α-Manp units should be equal to the number of 2,6-α-Manp units. The NMR integration data indicate that the contribution of the t-α-Manp units to the (2,6-α-Manp + t-α-Manp) resonance is very weak, since the t-α-Araf signal on the one hand and the (2,6-α-Manp + t-α-Manp) signal on the other hand exhibit the same intensity. Consequently, considering that the area arising from t-α-Manp units is negligible, a branching degree [2,6-α-Manp/(2,6-α-Manp + 6-α-Manp)] ratio] of 45% was calculated.

**TNF-α production by macrophages**

The potency of RruLAM to stimulate the production of TNF-α was investigated using murine (J774) and human (THP-1) macrophage cell lines. As expected, LPS, used as a positive control, induced a huge production of TNF-α by J774 macrophage cell lines (Fig. 6). The production was completely abrogated by adding polymyxin B. RruLAM, when tested at concentrations of 10 μg ml⁻¹ (Fig. 6) and 20 μg ml⁻¹ (not shown), was not able to significantly induce the production of TNF-α by J774 macrophage cell lines (Fig. 6). Indeed, the amount of TNF-α elicited by RruLAM was even weaker than that induced by ManLAM (Fig. 6), which is already known to be a poor inducer of pro-inflammatory cytokines (Gilleron et al., 1997; Nigou et al., 2002). Identical results were obtained with THP-1 macrophage cell lines (not shown), except that the overall amount of TNF-α released in these experiments was weaker than that obtained with J774 cell lines.

**DISCUSSION**

Mycobacterial LAMs are large heterogeneous lipoglycans of approximately 17 kDa that possess three distinct domains: a carbohydrate backbone, an MPI anchor and various capping motifs. The carbohydrate backbone is composed of two distinct homopolysaccharides, D-mannan and D-arabinitan. The structure of the capping motifs consists of either oligomannosyl units or phosphoinositol units and allows LAM to be divided into two subclasses, ManLAM and PILAM, respectively (Chatterjee & Khoo, 1998; Vercellone et al., 1998).

In the present report we investigated the occurrence, followed by the purification and characterization, of LAM from *R. ruber*, a species closely related to the established opportunistic human pathogen *R. equi*. In particular, we focused on elucidating the structure of the carbohydrate backbone. RruLAM was found by SDS-PAGE analysis to migrate between ManLAM (17 kDa) and LM (6 kDa), suggesting a smaller LAM-like molecule. This was confirmed by GC analysis, which revealed a ratio of Ara/Man/Ins of 11:29:1, indicating a molecular mass of around 7 kDa. The structure of the RruLAM carbohydrate backbone was shown to consist of a multibranched structure...
of a linear (z1→6)-Manp chain substituted at some O-2 positions by a single t-3-Araf unit (Fig. 5). The analyses also revealed that a weak number of 2,6-α-Manp units may be glycosylated by single t-3-Manp units. RruLAM is an original structure in that it is much simpler than those established for mycobacterial LAMs (Chatterjee & Khoo, 1998; Vercellone et al., 1998), but is also slightly different from that determined for the closely related species and opportunistic human pathogen R. equi (Garton et al., 2002). Indeed ReqLAM, which has a similar molecular mass (8 kDa), possesses Manp units that glycosylate the (z1→6)-Manp backbone at some O-2 positions, some of these side chain Manp units themselves being substituted at their O-2 position by t-Araf units (Garton et al., 2002). The data show that despite being closely related to R. equi, the R. ruber lipoglycans are significantly different. Paradoxically, the RruLAM structure is closely related to that of Turicella otitidis LAM, which is composed of an (z1→6)-Manp chain substituted at all O-2 positions by t-α-Araf units (N. Garton, M. Gilleron, G. Puzo, I. Sutcliffe, unpublished), giving rise to a fully substituted chain, whereas RruLAM exhibits a varying degree of branching which is approximately 45%. All the structures of LAM established so far, from mycobacterial species or related genera, possess an (z1→6)-Manp backbone which appears to be a basic core structure (Chatterjee & Khoo, 1998; Vercellone et al., 1998). Nevertheless, the complexity of the molecules is dramatically different between mycobacteria and the other strains investigated, mycobacteria being able to elaborate and produce the most complex structures. This suggests the presence of a considerable panel of glycosyltransferases dedicated to the production of lipoglycans within mycobacterial proteomes (Cole et al., 1998, 2001).

In contrast to mycobacterial PILAM, RruLAM was not able to induce the production of TNF-α by human or by murine macrophage cell lines. Its activity was even weaker than that observed for ManLAM, which is known to be a poor inducer of pro-inflammatory cytokines. The ability of PILAM to induce the production of TNF-α has been attributed so far to the presence of phosphoinositol caps (Gilleron et al., 1997) and to its capacity to elicit a TLR-2-dependent signalling in macrophages (Means et al., 1999). The molecular basis of PILAM pro-inflammatory effects are not fully understood; the present study provides an original structure, which may help in deciphering the biosynthesis and structure–function relationships of mycobacterial LAMs.

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