Volume 216, number 2, 261-264 FEB 04746 June 1987

The structure of the lipopolysaccharide O-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16M

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Received 31 March 1987

The surface M antigen of *Brucella* species has been identified as the lipopolysaccharide O-polysaccharide component composed of a repeating pentasaccharide unit containing a sequence of one 1,3- and four 1,2-linked , 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units. A neutral polysaccharide produced by *Brucella* species and referred to as polysaccharide B (poly B) has been identified as a family of circular 1,2-linked polymers of β-D-glucopyranosyl units ranging in ring size from 17 to 24 glucosyl units.

Polysaccharide B; ^{13}C-NMR; Cyclic β1,2-glucan; O antigen structure; M antigen; (*Brucella melitensis*)

1. INTRODUCTION

In 1932, Wilson and Miles [1] indicated that the major serological differences between *Brucella abortus* and *B. melitensis*, the causative agents of brucellosis, could be attributed to two surface antigens, an A antigen being predominant in *B. abortus* and an M antigen in *B. melitensis*. Subsequent studies showed that the A and M antigens were associated with the O-chain polysaccharide components of *Brucella* smooth lipopolysaccharides (LPS) [2], and that they showed extensive serological cross-reactivities. The two antigens have formed the basis for the majority of serodiagnostic tests for brucellosis infections.

Until the recent elucidation of the structure of the *Brucella* A antigen as a linear unbranched homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units forming the O-chain of the LPS [3], the molecular basis for the relationship between the A and M antigens was unknown. Our present work on the *Brucella* M antigen has now revealed that the O-chain of the LPS produced by *B. melitensis* 16M is also an unbranched linear polymer of 4,6-dideoxy-4-formamido-α-D- units but, unlike the A antigen, it is composed of a repeating pentasaccharide unit containing a sequence of one 1,3- and four 1,2-linked aminoglycosyl units. The structural knowledge of the *Brucella* A and M antigens now permits a molecular interpretation of their serological reactivities in terms of common epitope features as well as their serospecific differentiation because of the structural differences in the two antigens.

A previously poorly defined glycan produced by *Brucella* species termed polysaccharide B (poly B) has now been identified as a mixture of nonreducing circular D-glucans composed of 1,2-linked β-D-glucopyranosyl units ranging in size from 17 to 24 glucosyl units. The immunological significance of these glucans which have been widely found in other bacteria [4] remains unknown.

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2. MATERIALS AND METHODS

2.1. *Cell production and LPS and glycan isolation*

B. melitensis 16M (supplied by Dr M. Meyer, University of California at Davis, USA) was grown

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

on potato infusion agar in Roux flasks for 48 h at 37°C. The bacteria (15 g dry wt), harvested in 0.1 M Tris-HCl buffer (pH 7.2) containing 1% (w/v) NaCl and 2% (w/v) phenol, were kept at 22°C for 6 days. Following the removal of cells by low-speed centrifugation, the supernatant was dialyzed against water and the concentrated retentate was subjected to ultracentrifugation (105 000 $\times g$, 4°C, 12 h) to yield LPS (1.3 g) as a deposited clear gel.

The supernatant obtained from the above ultracentrifugation was digested with proteinase K, ribonuclease and deoxyribonuclease and, following dialysis, the contentrated retentate was fractionated on Sephadex G-50 (2 × 90 cm) using 0.05 M pyridinium acetate (pH 4.7) as the eluant, and the major carbohydrate-containing peak (K_{av} 0.68) was collected (poly B, 0.36 g).

Polysaccharide O-chain was obtained in the void volume fraction obtained on gel filtration (Sephadex G-50) of the water-soluble product released by fission of the LPS with 5% acetic acid for 2 h at 100°C.

2.2. *Analytical methods*

SDS-PAGE analyses were performed as absorbed by Tsai and Frasch [5]. Methylations were according to Hakomori [6]. Gas-liquid chromatography (GLC) and GLC mass spectrometry (GLC-MS) were carried out as in [3] using the programs: (A) OV-17 capillary column (25 m), 180–250°C at 2°C/min. (B) 3% (w/w) SP2340 on Supelcoport (2 mm × 180 cm) column, 180–240°C at 1°C/min. Retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}).

^{13}C NMR measurements were made on polysaccharide solutions (80 mg/ml) in D_2O at 37°C using a Bruker AM-500 spectrometer operating at 125 MHz in the pulsed Fourier transform mode as described [7].

3. RESULTS AND DISCUSSION

The major portion of the LPS and poly B produced by *B. melitensis* 16 M was released from cells on standing in Tris-HCl buffer. LPS (8.8% yield) obtained following ultracentrifugation of the dialyzed buffer extract gave a banding pattern indicative of an S-type LPS composed of an O-chain of repeating pentasaccharide units on SDS-PAGE analysis [8]. Hydrolysis of the LPS with hot 5% (v/v) acetic acid gave an insoluble lipid A (8%) and an O-polysaccharide (83%) isolated by gel filtration of the water-soluble hydrolysis product. The O-polysaccharide had $[\alpha]_D$ +56.2° (*c* 1.1, water), gave a single precipitin line in immunodiffusion against monoclonal antibodies specific for *Brucella* M antigen (Bundle, D.R., et al., in preparation), and on elemental analysis gave C, 44,51; H, 6.30; N, 6.60; and ash, 0.0%.

Fission of the specifically *N*-acetylated O-chain M antigen by anhydrous hydrofluoric acid [3] gave 4-acetamido-4,6-dideoxy-D-mannose (95%) characterized by specific optical rotation, ^{13}C NMR, and GLC-MS as previously described in the analysis of the *Brucella* A antigen [3]. Fission of the methylated original M antigen with anhydrous hydrofluoric acid or 10 M HCl gave two methylated aminoglycosyl derivatives which were identified by GLC-MS (program A) as their acetylated products 1,2-di-*O*-acetyl-4,6-dideoxy-3-*O*-methyl-4-(*N*-methylformamido)-D-mannose (T_{GM} 1.86, 70.4%) and 1,3-di-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-methylformamido)-*D*-mannose (T_{GM} 1.78, 17.2%) in a molar ratio of 4:1. On the basis of the optical rotation, composition, SDS-PAGE, and methylation results, the M antigen would appear to be an unbranched linear polymer of a repeating pentasaccharide unit composed of one 1,3- and four 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units, a conclusion entirely consistent with subsequent high-resolution NMR studies.

The ^{13}C NMR spectrum (125 MHz) of the M antigen (fig.1B) was that expected of a polymer of the proposed pentasaccharide repeating unit and showed greater complexity than the spectrum of the A antigen (fig.1A) which is a homopolymer of only 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units. The proposed M antigen structure was also consistent with extensive two-dimensional NMR studies made on the free amino form of the O-polysaccharide and its *N*-acetylated derivative.

A polysaccharide released from *B. melitensis* 16M cells in 2.4% yield was identified as a cyclic D-glucan. Gel fractionation of the concentrated supernatant of Tris-HCl extract from which the LPS had been removed by ultracentrifugation gave a sharp major glycose-containing peak ($M_r \sim 3300$)

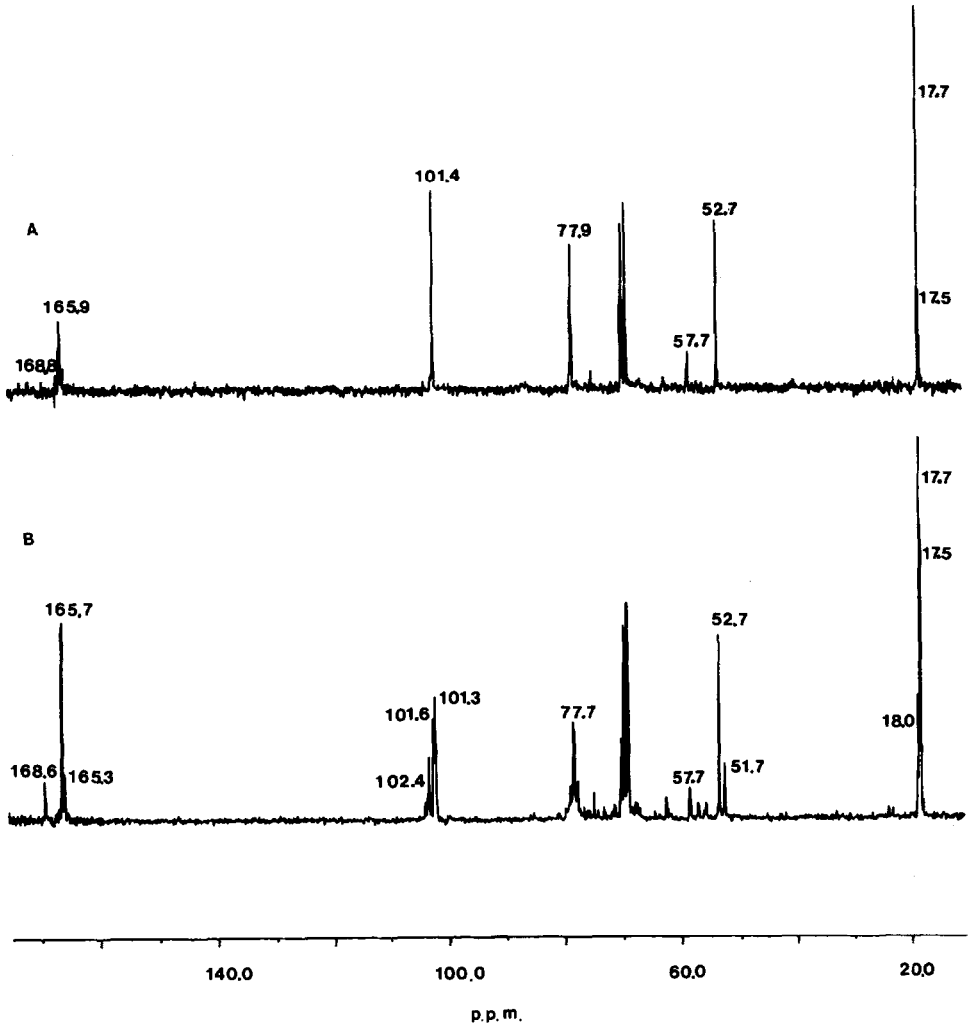

Fig.1. ^{13}C NMR spectrum (125 MHz) of (A) LPS O-polysaccharide of *B. abortus* 1119-3 and (B) LPS O-polysaccharide of *B. melitensis* 16M recorded at 310 K. Chemical shifts are expressed relative to internal 1,4-dioxane (δ 67.4 ppm).

which was collected. The fraction had $[\alpha]_D$ −16.03° (*c* 2.3, water) and an analysis gave C, 43.90; H, 6.11; N, 0.02; and ash, 0.02%.

Hydrolysis (1 M H_2SO_4, 8 h, 100°C) of the fraction gave only D-glucose (97.8%), identified by GLC of its trimethylsilylated (−)-2-butyl glycoside derivatives [9] and as its D-glucitol hexaacetate derivative. Hydrolysis of the methylated D-glucan (2 M trifluoroacetic acid, 6 h, 100°C) gave only 3,4,6-tri-*O*-methyl-D-glucose, identified by GLC-MS (program B) as its 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol-1-d (T_{GM} 1.63) derivative.

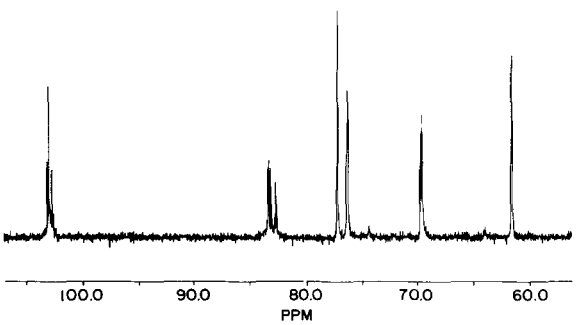

Fig.2. ^{13}C NMR spectrum (125 MHz) of the cyclic β-D-glucan from *B. melitensis* 16M.

The absence of any tetra-*O*- or di-*O*-methylglucose derivatives in the hydrolysis product indicated that the polysaccharide is probably a circular polymer of 1,2-linked β-D-glucopyranosyl residues. This conclusion was supported by the absence of glucitol in the hydrolysate of the sodium borohydride-treated D-glucan. High-performance liquid chromatography showed the D-glucan to be composed of a mixture of cyclic forms, the major portion of which were composed of between 17 and 24 glucosyl units. The ^{13}C NMR (125 MHz) spectrum of the D-glucan (fig.2) showed multiple resonance signals for each of the carbon atoms of the D-glucopyranosyl rings, corresponding to individual cyclic structures of different sizes. The ^{13}C NMR spectra of isolated fractions of unique M_r each showed only six carbon signals. These findings are in agreement with those obtained in definitive studies made on other bacterial 1,2-linked cyclic β-D-glucans by high-resolution NMR and fast-atom bombardment MS methods [4,10,11].

Analysis of the literature indicates that the identified β-D-glucan produced by *B. melitensis* 16M, as well as by other *Brucella* species, is the poly B product described by many authors. However, since physical and chemical data were not provided and the poly B was only defined by its method of isolation, we believe that the attributed serological reactions of poly B with *Brucella* anti A and M sera were due to the presence of contaminating O antigens and a reevaluation of the immunological role of poly B is required.

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