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EVIDENCE FOR THE STRUCTURAL HETEROGENEITY OF THE POLYSACCHARIDE COMPONENT OF *COXIELLA BURNETII* STRAIN NINE MILE LIPOPOLYSACCHARIDE

R. TOMAN, J. KAZÁR

Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 46 Bratislava, C.S.F.R.

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Summary. - Highly purified lipopolysaccharide (LPS) preparation obtained from *Coxiella burnetii* strain Nine Mile in phase I was used to determine the structure and monosaccharide composition of the polysaccharide component. The procedure included sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by silver staining and gel chromatographic fractionation of acetic acid-hydrolyzed LPS. Five fractions (A-E) were analysed by GLC-mass spectrometry. D-Mannose and D-glycero-D-manno-heptose were present in an appreciable amount in all polysaccharide fractions (A-D), whereas the virenose and dihydrohydroxystreptose contents varied. The highest content of both rhamnose and ribose was found in the low-molecular weight polysaccharide fraction D. The former sugar is being reported for the first time to be a LPS constituent. D-Xylose and D-glucose content varied considerably in the individual fractions and was the highest in fraction A. Glucosamine and galactosaminuronic acid were present in all polysaccharide fractions and, surprisingly, L-glycero-D-mannoheptose was also found, but its presence was limited within the certain degree of polymerisation of the polysaccharide chains. Mild acid hydrolysis of LPS resulted in a partial release of dihydrohydroxystreptose and virenose residues, which were collected and identified in fraction E. The data presented indicate a strong microheterogenity within the individual polysaccharide chains with respect to their sugar composition, size, and shape. Thus, the chemical structure of *Coxiella* LPS appears to represent a significant departure from the structures described for enteric LPSs.

Key words: Coxiella burnetii strain Nine Mile phase I; lipopolysaccharide; heterogeneity; chemical composition; structural features

Introduction

Elucidation of the mechanisms of Q-fever pathogenesis and immunity requires, inter alia, a detailed knowledge of the structural features of surface macromolecules of *Coxiella burnetii*. It has been well known (Mayer *et al.*, 1988) that lipopolysaccharides represent a considerable portion of the surface macromolecules of *Coxiella burnetii*. It has been well known (Mayer *et al.*, 1988) that lipopolysaccharides represent a considerable portion of the surface macromolecules of *C. burnetii*. However, their detailed structures, especially those of virulent phase I are largely unknown (Mayer *et al.*, 1988). In the present study, we report some new data on the more detailed structure of a lipopolysaccharide (LPS I) isolated from virulent *C. burnetii* cells in phase I.

Materials and Methods

C. burnetii strain Nine Mile, serologically in phase I (yolk sac passage 3), was propagated in chicken embryo yolk sacs. The rickettsial cells were killed with formalin and purified as described elsewhere (Schramek *et al.*, 1978).

Purified cells of *C. burnetii* were washed twice with chloroform - methanol (2:1, v/v) at room temperature for 4 hr. The mixture was centrifuged at 4000 x g for 10 min, and the sediment was washed again with chloroform - methanol mixture. Cells were suspended in distilled water, and LPS I was extracted by the phenol - water procedure (Schramek and Galanos, 1981). After dialysis against distilled water for 4 days, a highly purified LPS I material was obtained by ultracentrifugation (120 000 x g, 4 hr). A sample of LPS II was obtained as described (Schramek and Brezina, 1979). LPS from *Salmonella typhimurium* was a gift from Dr. H. Mayer, Max-Planck-Institut für Immunbiologie, Freiburg, Germany.

Hexosamine content was determined by the p-dimethylaminobenzaldehyde reaction (Swann and Balazs, 1966) and KDO-like substance ("KDO") (Mayer *et al.*, 1988) by the thiobarbituric acid method after hydrolysis of samples with 1 mol/l hydrochloric acid at 100 °C for 2 hr (Brade *et al.*, 1983). Protein was analysed according to Bradford (1976) and total phosphate by the method of Lowry *et al.* (1954).

SDS-PAGE electrophoresis of LPS preparations was performed in the discontinuous system described by Laemmli (1970). The separating gel was 10 - 18 % concentration gradient of polyacrylamide. To solutions of LPSs in distilled water equal volumes of LPS solubilization buffer (Laemmli, 1970) were added. Solutions were heated at 100 °C for 10 min, cooled and applied to wells in the concentration of 15 μg of LPS. Electrophoresis proceeded at 20 mA per slab gel until the bromophenol blue dye reached the end of the gel. LPS bands were visualized by silver staining as described by Tsai and Frasch (1982).

Prior to gel chromatography, LPS I (380 mg) was partially hydrolyzed with 1 % (v/v) acetic acid (70 ml) at 100 °C for 165 min and the solution was centrifuged at 10 000 x g for 30 min to remove the lipid A in the pellet. The pellet was washed three times with distilled water followed by centrifugation at 10 000 x g for 30 min in order to remove residual polysaccharide. All of the supernatant fluids containing the polysaccharide portion of LPS I were pooled, concentrated by evaporation and lyophilized. The yield was 270 mg. The lyophilized polysaccharide (263 mg) was dissolved in 50 mmol/l pyridinium-acetate buffer (pH 4.7, 8 ml) and fractionated on a Sephadex G-50 column (2.5x85 cm) using the same buffer as the eluent at a flow rate of 7 ml/hr. The void volume was determined by the use of Blue dextran (Sigma Chemical Company, U.S.A.). The effluent was collected in 3.5 - ml fractions, which were analysed for sugar content by the phenol - H_2SO_4 method (Dubois *et al.*, 1956).

Both LPS I and fractions A - D (Table 1, 500 μg of each) were hydrolyzed with 2 mol/l trifluoroacetic acid (0.7 ml) at 100 °C for 2 hr. After hydrolysis and evaporation, each sample was dissolved in water (500 μl) containing $NaBD_4$ (2 mg) and treated at 20 °C overnight. Salts were removed with mixed - bed ion exchanger (Ionenaustauscher V, Merck, Germany) and the solution was evaporated to dryness. The residue was co-distilled three times with methanol, acetic anhydride (100 μl) and pyridine (100 μl) were added, and the sample was heated at 100 °C for 1 hr. The sample was cooled, water was added (three 500 μl portions), and the solvent was removed by evaporation. The residue was dissolved in chloroform and injected directly to a GLC column.

GLC-mass spectometry of alditol acetates (Lönngren and Svensson, 1974) was performed with a JMS - D 100 (Jeol) spectrometer, using a column (0.3 x 200 cm) packed with 0.124 - 0.147 mm Supelcoport coated with 3 % of SP 2340. The inlet helium pressure was 101.3 kPa at a programmed temperature range of 180 °C (4 min) to 220 °C at 2 °C min^{-1}. The spectra were determined at 23 eV. Osmometric pressure measurements were performed in water at 30 °C, using a Knauer membrane osmometer fitted with a Zweischicht Membrane (Knauer).

Results and Discussion

Using a conventional hot phenol-water procedure LPS I was isolated from the cells in virulent phase I. The material was dialyzed against distilled water

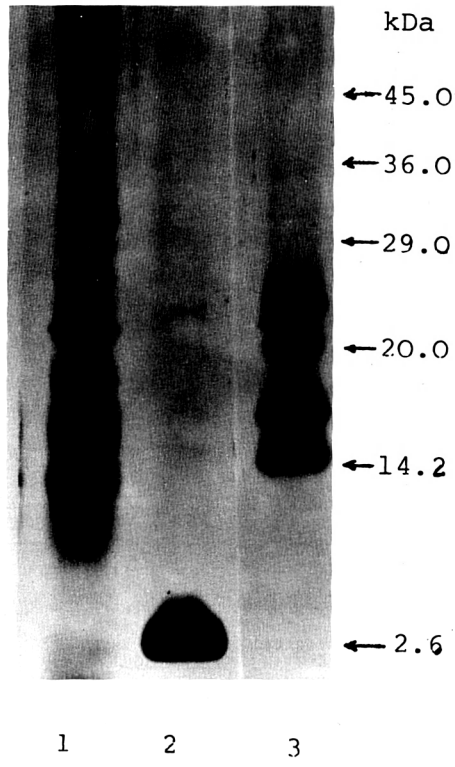

Fig. 1
Silver- stained SDS-polyacrylamide gel of LPS from *S. typhimurium* (lane 1), LPS II and LPS I from *C. burnetii* Nine Mile (lanes 2 and 3)

and finally purified by ultracentrifugation. SDS-PAGE electrophoresis of the LPS I isolated is shown in Fig. 1, lane 3. Its profile is characterized by two strong bands at about 14.2 - 16.0 kD and by a group of unevenly distributed bands in the region of about 20.0 - 29.0 kD. At first sight, the evidently irregular banding was in contrast with the well known ladder-like banding characteristic for most bacterial polysaccharides. The latter is represented here by LPS from *Salmonella typhimurium* (Fig. 1, lane 1) as an example. The irregular banding of LPS I indicates strong microheterogeneity within the polysaccharide macromolecule. Moreover, formation of polysaccharide chains by sugar units with predetermined sequencing appears rather unlikely. It is evident from Fig. 1 that structural features of LPS I should differ considerably from those reported (Mayer *et al.*, 1988) for LPS II isolated from cells in avirulent phase II. LPS II forms only a single band at about 2.6 kD (Fig. 1, lane 2).

It is well known that structural studies require a rather homogeneous polysaccharide material. Therefore, LPS I was subjected to mild acid treatment in order to remove the lipid A moiety and the supernatant polysaccharide solution was fractionated on a column of Sephadex G-50. Five fractions (A-E) were obtained (Table 1) which differed each from the other in both composition of constituent sugars and molecular masses. D-Mannose and D-glycero-D-mannoheptose were present in appreciable amounts in all polysaccharide fractions (A-D). The highest content of both sugars (71.6 %) was found in fraction C with $\bar{M}_n$ 10 200. Virenose and dihydrohydroxystreptose were dominant in the high molecular mass fractions A and B. Mild acid hydrolysis of LPS I resulted in a partial release of these two sugars from the polysaccharide backbone and they were collected and identified in fraction E. The highest content of L - rhamnose was detected in the low-molecular mass polysaccharide fraction D. This sugar is being reported for the first time to be a constituent of LPS I. D-Xylose and D-glucose contents varied considerably in the individual frac-

Table 1. Fractionation of the polysaccharide component of *Coxiella burnetii* strain Nine Mile (phase I) lipopolysaccharide on Sephadex G - 50

Fraction	Yield (%)	Composition (peak area % of alditol acetates)									$\bar{M}_n$
		Rha	Rib	Vir	Xyl	Strep	Man	Gal	Glc	D-Hep	L-Hep	
LPS I	100	3.9	5.9	12.4	4.9	16.5	24.7	-	3.9	24.7	3.1	-
A	18.3	1.8	6.5	15.3	12.3	16.3	18.3	tr	9.1	20.4	-	15 800
B	21.9	0.6	2.6	16.0	-	15.1	29.4	-	2.0	28.4	5.9	13 000
C	24.7	2.1	5.6	6.4	5.0	4.0	36.1	-	1.0	35.5	4.3	10 200
D	14.9	16.2	15.1	9.4	3.8	5.8	21.7	tr	4.1	23.9	-	7 100
E	10.6	-	-	20.1	-	60.4	-	-	-	-	-	-

Vir, L-virenopyranose /6-deoxy-3-C-methyl-L-gulopyranose/; Strep, dihydrohydroxy-L-streptofuranose /3-C-/hydroxymethyl/-L-lyxofuranose/; D-Hep, D-glycero-D-mannoheptose; L-Hep, L-glycero-D-mannoheptose; tr, traces.

tions and were the highest in fraction A. L-Glycero-D-mannoheptose was also identified though its presence was limited within the certain degree of polymerization (DP) of polysaccharide chains. We found that the appearance of D-ribose in total hydrolyzates was an indicator for protein presence in LPS isolates. In this case, however, the amount of D-ribose in the individual fractions (Table 1) did not correlate with the findings for protein contents given in Table 2. Therefore, we cannot exclude a possibility of D-ribose involvement in building-up polysaccharide chains, at least within a certain DP.

Table 2 gives the analytical data for polysaccharide fractions (A-D). The total phosphate content increases with decreasing molecular masses of individual fractions. This indicates, especially in fraction D, an increased presence of core region molecules in the polydisperse polysaccharide system as it has been shown the location of phosphate groups to be restricted mainly to the inner core region of LPSs (Brade *et al.,* 1988). From amino sugars, N-acetyl-D-glucosamine and D-galactosaminuronic acid could be detected but their distribution appears rather uneven within the polysaccharide fractions isolated (Table 2). The highest hexosamine content in fraction B indicates higher frequency of appearance of both amino sugars in certain O-polysaccharide chains with hitherto unknown DP. Most of the protein has been gradually removed in the course of LPS I isolation and purification. After final purification by ultracentrifugation a small amount of protein still persisted in the LPS I preparation. Upon fractionation on Sephadex G-50, fractions A-D had the protein contents given in Table 2. At present, it is not clear whether the protein portions are covalently linked to LPS or represent a contaminating material not removed by purification procedures.

On the basis of the above mentioned results a tentative distribution of constituent sugars can be estimated in LPS I (Fig. 2). It is assumed that the core region of LPS I is much more expanded than reported earlier (Mayer *et al.,* 1988). It consists of lipid A proximal core region (A), having the structure given in Fig. 2, to which an appreciable amount of D-mannose and D-glycero-D-mannoheptose is attached. As the highest content of L-rhamnose has been found in the low molecular mass fraction D, it is highly probable that this sugar constitutes the core region of LPS I, too. Furthermore, it can be assumed from

Table 2. Analytical data for polysaccharide fractions A - D

Fraction	Phosphate (%)	Hexosamine (%)	"KDO" (%)	Protein (%)
A	0.13	2.0	1.4	2.0
B	0.17	7.0	2.1	5.6
C	1.4	3.7	2.6	2.3
D	4.8	2.4	1.9	5.8

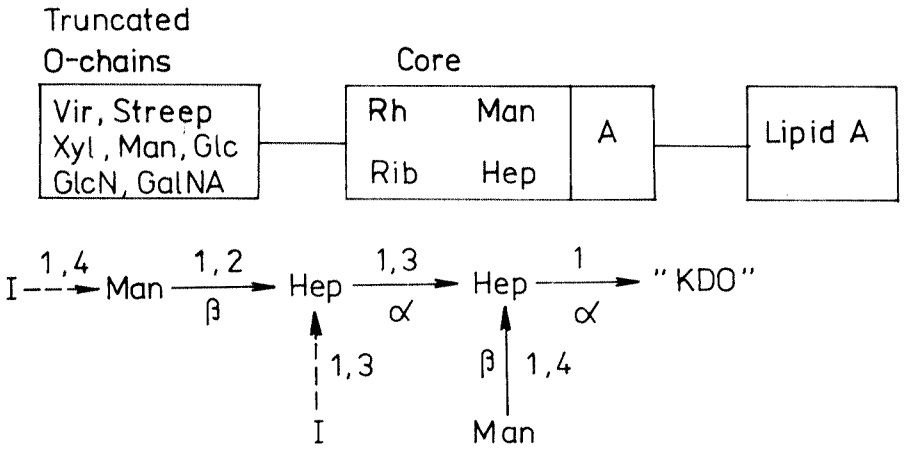

Fig. 2
Tentative distribution of constituent sugars in LPS from *Coxiella burnetii* (9 mi.) in phase I
A - Structure of lipid A, proximal core region with the attachment sites of phase I specific sugars (I- - -); Mayer, H. *et al.* (1988).

both sugar composition and molecular masses of the individual fractions (A-D, Table 1) that LPS I O-polysaccharide chains are truncated and composed mainly of virenose, dihydrohydroxystreptose, D-mannose, and most probably of N-acetyl-D-glucosamine, D-galactosaminuronic acid, D-xylose, and D-glucose. Nevertheless, other variations in the distribution of constitutent sugars within the LPS I chains cannot be excluded. Further studies are in progress to solve this crucial problem of determination of the LPS I structure.

In conclusion, the results reported clearly indicate that the primary structure of LPS I isolated from *C. burnetii* cells in virulent phase I differs considerably from those reported for most bacterial LPSs. Its elucidation seems to be a real challenge for structural chemists and might be of interest also for those who are investigating unusual antigenic properties of this LPS.

References

Brade, H., Galanos, C., and Lüderitz, O. (1983): Differential determination of the 3-deoxy-D--mannooctulosonic acid residues in lipopolysaccharides of *Salmonella minnesota* rough mutants. *Eur. J. Biochem.* **131**, 195-200.

Brade, H., Brade, L., and Rietschel, E. T. (1988): Structure activity relationships of bacterial lipopolysaccharides (endotoxins). Current and future aspects. *Zbl. Bakt. Hyg.* **A 268**, 151-179.

Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein - dye binding. *Analyt. Biochem.* **72**, 248-254.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956): Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* **28**, 350–356.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.

Lönngren, J., and Svensson, S. (1974): Mass spectrometry in structural analysis of natural carbohydrates. *Adv. Carbohydr. Chem. Biochem.* **29**, 41–106.

Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., and Farr, A. L. (1954): The quantitative histochemistry of brain. I. Chemical methods. *J. Biol. Chem.* **207**, 1–17.

Mayer, H., Radziejewska-Lebrecht, J., and Schramek, Š. (1988): Chemical and immunochemical studies on lipopolysaccharides of *Coxiella burnetii* phase I and phase II. *Adv. exp. Med. Biol.* (Mol. Immunol. Complex. Carbohydr.) **228**, 577–591.

Schramek, Š., and Brezina, R. (1979): Isolation of endotoxic lipopolysacharide from phase II *Coxiella burnetii. Acta virol.* **23**, 349.

Schramek, Š., and Galanos, C. (1981): Lipid A component of lipopolysaccharides from *Coxiella burnetii. Acta virol.* **23**, 230–234.

Schramek, Š., Brezina, R., and Kazár, J. (1978): Influence of mild acid hydrolysis on the antigenic properties of phase I *Coxiella burnetii. Acta virol* **22**, 302–308.

Swann, D. A., and Balazs, E. A. (1966): Determination of the hexosamine content of macromolecules with manual and automated techniques using the p-dimethylaminobenzaldehyde reaction. *Biochim. Biophys. Acta* **130**, 112–129.

Tsai, C. M., and Frasch, C. E. (1982): A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analyt. Biochem.* **119**, 115–119.