COMPARISON OF VARIOUS METHODS OF LIPOPOLYSACCHARIDE ISOLATION FROM *COXIELLA BURNETII* STRAIN PRISCILLA IN THE VIRULENT PHASE I

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Summary. – Four methods of isolation of a lipopolysaccharide (LPS) from *Coxiella burnetii* strain Priscilla in virulent phase I have been examined. Each isolation method afforded a specific LPS portion of the polydisperse LPS system present in the outer membrane of *C. burnetii* cell. The hot phenol/water method was found the most efficient with respect to yield and chemical composition of the smooth (S) LPS that was shown to prevail in the outer membrane LPS macromolecules of *C. burnetii.*

Key words: *Coxiella burnetii* in virulent phase I; isolation methods; lipopolysaccharides; chemical composition

Introduction

In Gram-negative bacteria, LPSs are built according to a common structural principle. They consist of a lipid moiety called lipid A, which is covalently linked to a heteropolysaccharide chain and are associated with other outer membrane components through hydrophobic and ionic interactions (Helander, 1985). In *C. burnetii,* an obligatory, intraphagolysosomal parasiting bacteria, a LPS is present as a structural component of the outer membrane of the cell envelope (Williams and Waag, 1991). It plays an essential role in the interaction between the microbe and host including the pathogenicity and immunogenicity of the agent, is capable to induce antibody response, and is considered as the protective immunogen as well (Kazár *et al.,* 1978). In order to elucidate the biological function of *C. burnetii* LPS in relation to its structure, the LPSs from both virulent and avirulent phases are being investigated in detail for their composition and chemical structure. The data obtained thus far have been published recently (Toman and Kazár, 1991; Toman *et al.*, 1993; Toman and Škultéty, 1994).

For structural studies, highly purified and homogeneous LPSs are needed, and therefore, application or development of a suitable isolation procedure, fulfilling the above criteria, is of great importance. In principle, LPS macromolecules are extractable from the bacteria under relatively mild conditions, which do not permit cleavage of covalent bond between lipid A and the heteropolysaccharide chain. Numerous procedures have been developed in this respect. Of them, the phenol/water method of Westphal and Jann (1965) and the phenol/chloroform/petroleum ether (PCP) method of Galanos *et al.* (1969) have been most extensively used. In addition to the latter methods, we examined and evaluated two other isolation procedures, namely, the EDTA/Triton X–100/magnesium ions method of Uchida and Mizushima (1987) and the Empigen BB method of Lowell *et al.* (1988). The four methods were compared in terms of their efficiency, yields of isolated *C. burnetii* LPSs and their chemical composition. The results are reported herein.

Materials and Methods

C. burnetii growth and isolation. C. burnetii strain Priscilla, serologically in phase I (yolk sack passage 3), was propagated in chick embryo yolk sacks. The cells were killed with formalin and purified by differential centrifugation and ether treatment (Schramek *et al.*, 1978).

LPS isolation methods. Four different isolation methods were used. Of them, the phenol/water method of Westphal and Jann (1965) (**1**) has been the oldest. In this procedure, purified *C. burnetii* cells (1100 mg) were first treated with chloroform/met-

hanol (2:1; v/v) mixture at room temperature overnight to remove phospholipids, and then extracted with 45% aqueous phenol three times at 68 °C for 20 mins. Upon cooling and subsequent centrifugation of the mixture, a hydrophilic LPS was isolated by dialysis from the aqueous phase.

The PCP procedure according to Galanos *et al.* (1969) (**2**) was modified as follows. Phospholipid-free dried cells (1000 mg) were extracted three times for 15 mins below 20 °C with 30 ml of a monophasis mixture of liquid phenol, chloroform, and petroleum ether (2:5:8, v/v/v). After removal of chloroform and petroleum ether from collected supernatant, the LPS was precipitated from the remaining liquid phenol by addition of water or acetone and dialyzed.

The method of Uchida and Mizushima (1987) (**3**) involves protein denaturation to disrupt a possible interaction with the LPS, extraction of the LPS with EDTA/Triton X-100 (Serva, Heidelberg, FRG), and its isolation as a precipitate in the presence of magnesium ions. Briefly, dried phospholipid-deprived bacterial cells (1000 mg) were suspended in 50 ml of distilled water. To the suspension, 8 ml of 100 mmol/l Tris-HCl pH 8.0, 8 ml of 0.5 mol/l $MgCl_2$, and 20 ml of 8% Triton X-100 were added successively and the suspension was heated in boiling water for 10 mins. After cooling and centrifugation, the precipitate was washed once with 80 ml of 10 mmol/l Tris-HCl pH 8.0 and 10 mmol/l $MgCl_2$. To the precipitate, 20 ml of each, distilled water, 0.2 mol/l EDTA pH 8.0, 2 mol/l NaCl, and 8% Triton X-100 were added and the mixture was shaken at 37 °C for 60 mins.. After centrifugation , 10 ml of 1 mol/l $MgCl_2$ were added to the supernatant under stirring and the mixture was incubated at 37 °C for 60 mins. The resulting LPS was obtained by centrifugation.

LPSs (1 mg each) were hydrolyzed with 2 mol/l trifluoroacetic acid (0.2 ml) at 100 °C for 2 hrs. After evaporation, each residue was dissolved in water (0.2 ml) containing $NaBD_4$ (3 mg) and kept at 20 °C overnight. Salts were removed with the mixed-bed ion exchanger (Ionenaustauscher V, Merck, Darmstadt, FRG) and the solution was evaporated to dryness. The residue was co-distilled three times with methanol. Acetic anhydride (50 µl) and pyridine (50 µl) were added, and the sample was heated at 100 °C for 1 hr. After cooling, water was added and the solvent was removed by evaporation. The residue was dissolved in chloroform and injected directly to a gas chromatography column.

Gas chromatography-mass spectrometry (GC-MS) of alditol acetates was carried out on a Hewlett-Packard Model 5971 A mass selective detector connected to a Hewlett-Packard Model 5890 A chromathograph equipped with a SP-2330 capillary column (30 m × 0.25 mm; Supelco, Bellefonte, USA). The column temperature program was 80 °C (2 mins) to 235 °C at 30 °C/min. Electron impact (EI) mass spectra were recorded at 70 eV.

Results and Discussion

After removal of phospholipids from the purified *C. burnetii* cells, strain Priscilla in phase I, with the chloroform/methanol mixture, the LPSs were isolated by methods **1**, **2**, **3**, and **4** respectively. The cells treated according to **2-4** were extracted subsequently by **1**. In Table 1, the yields of LPSs isolated by these methods are given. It is evident that **1** was the most efficient method giving 15.4% yield of

Table 1. Yields of LPSs isolated from C. burnetii strain Priscilla in phase I by various isolation methods

Method	1	2	2 and 1*	3	3 and 1*	4	4 and 1*
Yield of LPS [%]	15.4	1.9	9.5	1.5	11.5	1.5	7.6

1, phenol/water; **2**, PCP; **3**, EDTA/Triton X-100/magnesium ions; **4**, Empigen BB; *The first method was followed by the second method.

The isolation method of Lowell *et al.* (1988) (**4**) was performed as follows. The purified, phospholipid-deprived cells (1000 mg) were suspended in 50 ml of PBS pH 7.4, and for LPS and protein solubilisation, 1.6 ml of 1% Empigen BB (Calbiochem, La Jolla, CA, USA) were added. The mixture was shaked at 37 °C for 60 mins. After cooling and centrifugation, the treatment of cells with Empigen BB was repeated three times. The supernatants were pooled and 75 g of $(NH_4)_2SO_4$ were added. The mixture was kept at 4 °C overnight and after centrifugation of proteins, the LPS was obtained from the dialyzed supernatant by lyophilisation.

Analytical methods. Protein content was determined by the method of Hartree (1972). Total hexosamine content was analyzed by the Morgan-Elson reaction as modified by Swann and Balazs (1966), 3-deoxy-D-manno-2-octulosonic acid (Kdo) by the thiobarbituric acid assay (Brade *et al.*, 1983), and phosphate according to Lowry *et al.* (1954).

LPS which might represent the whole LPS content of the outer membrane of *C. burnetii* judging from the data given for the LPSs present in Gram-negative bacteria (Škárka and Ferenčík, 1987). Lower yields were obtained when **1** followed the methods **2-4.** The latter were of low efficiency in terms of the yields of isolated LPSs which represented only one tenth of that obtained by **1**. An explanation could be found in a deep, outer membrane penetrating effect of the extractant used in **1** that results in an exhaustive extraction of a hydrophilic LPS. It is assumed that the outer membrane of *C. burnetii* is largely disrupted in this process. With **2**, a hydrophobic LPS should be extracted preferentially, and in **3** and **4**, surface exposed LPSs are isolated by surface active substances without cell wall disruption (Vávreková *et al.*, 1992). Thus,

Table 2. Analytical data for LPSs of C. burnetii obtained by various isolation methods

Methods	Protein [%]	Phosphate [nmol/mg]	Hexosamine [nmol/mg]	Kdo [nmol/mg]	
				total*	acetate**
1	7.6	608.1	274.3	138.1	63.8
2	7.4	554.2	255.8	145.9	66.8
2 and 1	6.9	618.5	261.2	159.2	85.9
3	13.8	120.3	158.4	13.8	11.8
3 and 1	8.5	589.7	205.7	134.5	71.3
4	9.6	256.7	308.5	25.5	12.6
4 and 1	8.7	605.3	282.1	113.6	56.3

*Released by hydrolysis with 1 mol/l HCl; **Released in 100 mmol/l sodium acetate buffer pH 4.4.

each isolation method should afford a specific LPS portion of the polydisperse LPS system present in the outer membrane of *C. burnetii* cell in phase I.

Colorimetric assays (Table 2) give some basic information on the chemical composition of the isolated LPSs. The protein content in each LPS indicates the degree of LPS contamination with proteins and possibly with some of their degradation products. Therefore, their contents in a LPS is highly individual but might depend on the isolation method applied. In contrast, the phosphate content may give an information on the nature of the isolated LPS as it is well known that phosphoryl groups are present mainly in lipid A and core region of LPSs. The highest phosphate values were found in the LPSs isolated by **1** or when **1** followed the methods **2-4.** This is consistent with the above discussed exhaustive extraction of a typical S-LPS from the *C. burnetii* outer membrane (see below). Surprisingly, the LPS isolated by **2** had not only the phosphate but also hexosamine and Kdo contents similar to those of LPSs isolated by **1** or where **1** followed the other isolation technique. This may indicate absence of a hydrophobic LPS in the outer membrane of *C. burnetii* in phase I and/or the fact that extraction of a small amount of the hydrophilic LPS also occurred by using **2.** Substantially lower phosphate contents were found in the LPSs isolated by **3** and **4,** which apparently represent a distinct species in the family of LPSs located in the *C. burnetii* outer membrane. The distinct character of both LPSs reflect also their Kdo and hexosamine contents. The total amount of Kdo released by hydrolysis with 1 mol/l HCl was found to be approximately ten and six times lower for LPSs isolated by **3** and **4,** respectively, than that estimated in other LPSs. Likewise, substantially lower values were obtained for Kdo released in 100 mmol/l sodium acetate buffer

Table 3. GC-MS analysis of alditol acetates derived from LPSs of C. burnetii isolated by various methods

Methods	Composition (peak area % of alditol acetates)								
	Ara	Vir	Xyl	Man	Gal	Glc	Strep	U	Hep
1	1.6	13.4	4.9	32.3	1.1	1.4	15.9	0.8	28.6
2	6.0	9.9	1.3	31.2	0.8	3.4	15.1	1.7	30.6
2 and 1	5.2	11.8	2.9	30.5	0.5	3.2	13.7	1.6	30.6
3	13.1	1.3	8.5	25.0	9.2	28.0	3.7	tr	11.2
3 and 1	2.0	12.0	2.7	28.9	0.8	1.4	18.1	6.5	27.6
4	24.1	6.1	6.6	20.1	4.3	16.8	7.8	3.8	10.4
4 and 1	4.7	11.1	4.2	29.7	1.2	2.0	13.2	5.6	28.3

Vir, virenose (6-deoxy-3-C-methyl-gulopyranose); Strep, dihydrohydroxystreptose (3-C-(hydroxymethyl)-lyxofuranose); Hep, D-glycero-D-mannoheptose; U, unknown compound; tr, traces.

Kdo released in 100 mmol/l sodium acetate buffer

pH 4.4 where the ketosidic linkages should be cleaved only. Of interest are relatively high values of hexosamines as compared to the total Kdo. Furthermore, the phosphate, hexosamine, and total Kdo contents were 2 times higher in the LPS isolated by **4** than those found in the LPS obtained by **3**.

The sugar analysis of the individual LPSs by GC-MS (Table 3) revealed a clear dependence of the constituent sugar composition on the isolation method applied. When **1** was used or **1** followed **2-4**, virenose (6-deoxy-3-C-methyl-gulopyranose) and dihydrohydroxystreptose (3-C-(hydroxymethyl)-lyxofuranose) were found in the LPSs in the amounts ranging from 11.1 to 13.4%, and from 13.2 to 18.1%, respectively. Based on passive hemolysis tests with rabbit anti-phase I cell serum both sugars represent the immunodominant sugars of the *C. burnetii* LPS (Schramek *et al.*, 1985). They are located in O-polysaccharide chain of the LPS (Toman and Kazár, 1991) in terminal positions (Schramek *et al.*, 1985; Toman, 1991). Thus, the presence of virenose and dihydrohydroxystreptose is characteristic for a typical, heterogenic S-LPS of *C. burnetii* in phase I. In addition to the latter sugars, high contents of mannose and heptose (D-glycero-D-mannoheptose) are found. Mannose is almost exclusively terminally linked in the LPS, too (Toman, 1991). Arabinose, xylose, galactose, and glucose were detected in variable amounts. At present, it is not known with certainty whether they are constituents of the major S-LPS. As expected from the results of colorimetric assays, the LPS isolated in a low yield (1.9%) by **2** had the composition of the major constitutent sugars similar to that of the S-LPS discussed above.

The sugar analysis of the LPSs isolated by **3** and **4** also confirmed their distinct character. In both LPSs, a noticable increase in the composition of those sugars is observed that are present in minor amounts in other LPSs, and on the contrary, virenose, dihydrohydroxystreptose, and heptose, typical for those LPSs, are found in relatively low proportions, especially in the LPS isolated by **3**. Moreover, the sugar composition of both LPSs differed considerably. E.g., in the LPS isolated by **3**, the highest content of glucose (28%) followed mannose and arabinose while in the LPS isolated by **4**, arabinose (24.1%) followed mannose, and glucose.

In conclusion, each isolation method applied afforded a specific LPS portion of the polydisperse system of the *C. burnetii* surface LPS macromolecules. The most striking differences were observed among the LPSs isolated by the methods **1**, **3**, and **4** in all parameters followed. The method **1** appears to be the most efficient in terms of the yield and chemical composition of the S-LPS that prevails in the outer membrane LPS macromolecules of *C. burnetii* in phase I.

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