Acta Biochimica Polonica

Vol. 44 No. 2/1997

293–300

QUARTERLY

Immunochemical characterization of lipopolysaccharide from glucose-nonfermenting Gram-negative clinical bacterial isolate*

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Received: 15 November, 1996; revised: 23 April, 1997; accepted: 12 May, 1997

Key words: *Flavobacterium*, *Sphingomonas*, lipopolysaccharide, sphingosine, clinical strain

A glucose-nonfermenting Gram-negative bacterial strain isolated from bronchofiberoscope used for examination of the patients suffering from pulmonary diseases was subjected to phenol-water extraction. Lipopolysaccharides (LPS) isolated from the water and the phenol phase differed in fatty acid composition. Both contained xylose, glucose, glucosamine and components typical for LPS, namely heptose, 3-deoxyoctulosonic acid (Kdo) and 3-hydroxymyristic acid. The presence of sphingosine in all LPS preparations classifies the strain to the genus *Sphingomonas*.

Nonfermenting Gram-negative bacilli were isolated from clinical material accumulated in bronchofiberoscope lavages derived from patients suffering from lung diseases. The bacteria appeared to be resistant to disinfectants used for sterilization of medical devices. On the basis of biochemical studies the strain has been primarily classified to the genus *Flavobacterium* [1]. According to Bergey's classification [2] this genus accomodates Gram-negative, non-spore-forming, yellow-pigmented rods slight acid producers. Because of this broad definition, the genus rapidly appeared to be very heterogeneous. Since publication of Bergey's manual in 1984 the genus comprised only nonmotile low G+C strains [3] therefore the strains with high G+C content of DNA (63–70 mol%) belonging to the genus *Flavobacterium* have been reclassified and included into new or different genera, exemplified by a newly formulated genus *Sphingomonas* [4]. The cells of the species belonging to this genus are aerobic Gram-negative, non-spore-forming straight rods with characteristic cellular lipids containing glycosphingolipids. Recently a new family *Flavobacteriaceae* has been proposed which includes the genera together with *Flavobacterium* genus, with the G+C content of DNA ranging from 29 to 45 mol% [5]. Since our isolate has 66.6 mol% of G+C [1], it should be excluded from that family and, due to the recently detected sphingosine, should be included into the genus *Sphingomonas*.

Preliminary results presented at the V Symposium on Glycoconjugates, XXXII Meeting of the Polish Biochemical Society, Kraków (Poland), September 17–20, 1996

*This work was supported by the State Committee for Scientific Research (KBN), grant No. 4 P05A 002 10, for M.M.

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The present study describes a preliminary structural and serological characterization of glycoconjugates obtained after phenol-water extraction conventionally used for isolation of lipopolysaccharides from Gram-negative bacteria. On the basis of the results obtained taxonomy of the clinical isolate is discussed.

MATERIALS AND METHODS

Bacterial strain and preparation of lipopolysaccharide. A clinical isolate from bronchoalveolar lavage of the patients with pulmonary diseases was cultivated in a nutrient broth at 37°C as previously described [1]. Other bacterial strains used in this work were obtained from the collection of the Institute of Immunology and Experimental Therapy (Wrocław, Poland). The bacterial pellet, collected by centrifugation, lyophilized or dried with acetone was powdered and subjected to phenol-water extraction according to Westphal & Jann [6]. Material from the water and phenol phases, was separated from nucleic acids by repeated ultracentrifugation at 100000 × ***g*** for 5 h at 4°C using Beckman L7-55 centrifuge until the ratio A_{240}/A_{260} exceeded one. The phenol phase lipopolysaccharide was then fractionated on Sephadex G-200 column in the presence of sodium deoxycholate as previously described [7].

Analytical methods. Sphingosine was determined according to Lauter & Trams [8]. Glucose was quantitated without hydrolysis by the colorimetric method of Gamian *et al.* [9]. Methylation analysis was performed using methyl sulphinyl base; the assay of sugars as alditol acetates, estimation of phosphorus and 3-deoxy-D-*manno*-octulosonic acid (Kdo) were carried out as previously described [7]. Gas-liquid chromatography-mass spectrometry (GLC-MS) of the derivatives was performed using a Hewlett-Packard MSD 5971A instrument equipped with HP-1 column (0.2 mm × 12 m) with the temperature program: 150–270°C, 8°C/min. For the analysis of fatty acids and sphingosines the method of Yabuuchi *et al.* [4, 10] was employed. Samples of LPS (2 mg) were methanolyzed in 1 M methanolic HCl at 80°C for 5 h. The samples evaporated with nitrogen stream were extracted into ethyl acetate and the fatty acid methyl esters were analyzed by GLC-MS. Sphingoid bases were trimethylsilylated with Sil-A reagent (Sigma) at 90°C for 30 min and analyzed by GLC-MS as above.

Serological methods. Preparation of rabbit antiserum, SDS/polyacrylamide gel electrophoresis and immunoblotting were as previously reported [11]. Double immunodiffusion test was performed in 1% agarose with 1% solution of antigen in phosphate buffered saline (PBS), pH 7.0 containing polyethylene glycol 6000 (1%) and sodium deoxycholate (1%).

Adherence measurement. The experiment was carried out according to Christensen *et al.* [12]; the adherence of bacteria to the 96-well tissue culture polystyrene plate was estimated.

RESULTS

Dry bacterial cell mass was subjected to phenol-water extraction used for isolation of lipopolysaccharides. The components from water and phenol phases purified by ultracentrifugation gave two fractions designated LPS-W and LPS-P, respectively. LPS-W and LPS-P constituted 0.7% and 0.8% of dry cell weight, respectively. LPS-P was further fractionated by gel filtration on Sephadex G-200 column in the presence of sodium deoxycholate into three fractions (I–III) which differed in their molecular masses (Fig. 1). SDS-polyacrylamide gel electrophoresis with silver staining of all products: LPS-W and LPS-P I–III, gave the same ladder-like pattern resembling that of the S-type bacterial lipopolysaccharides [11] (Fig. 2A).

For serological analysis an antiserum was obtained by immunization of rabbits with whole bacterial cells. In double immunodiffusion test this serum reacted with all LPS preparations (not shown). Furthermore, immunoblotting of these lipopolysaccharides with rabbit antiserum indicated that specific antibodies reacted with all regions of the LPS molecule. The exemplified immunoblotting of LPS-P I–III is shown at Fig. 2B.

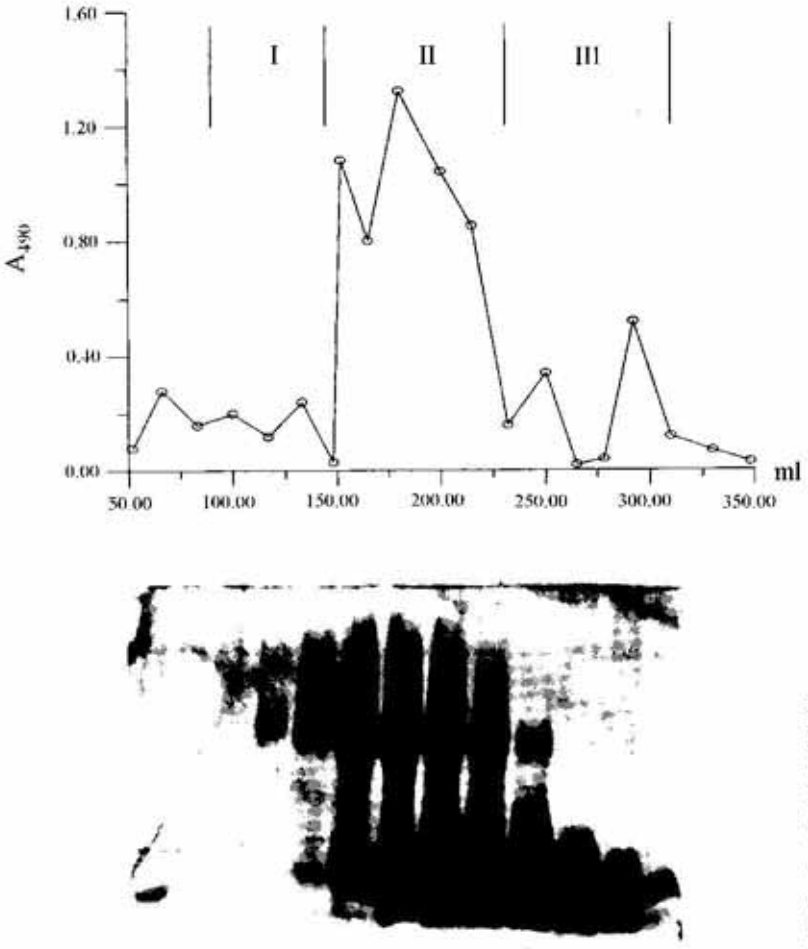

Figure 1. Fractionation of phenol phase LPS on Sephadex G-200 in the presence of sodium deoxycholate.

The dialyzed fractions were analyzed for total sugar content at 490 nm [7]. Photos of silver stained electrophoregrams of the column eluates are aligned under their appropriate fraction number.

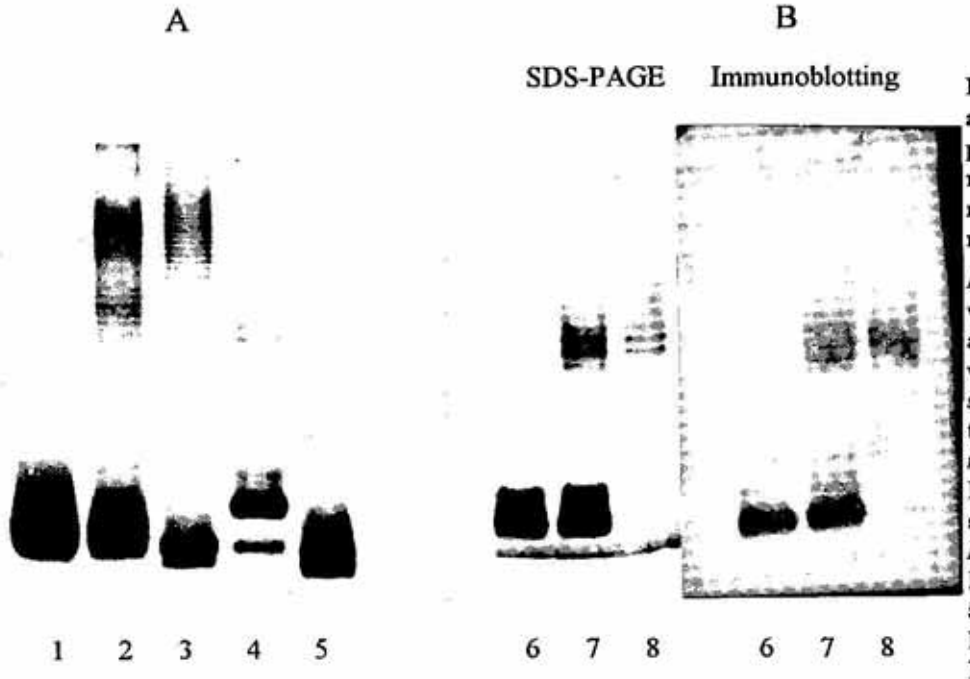

Figure 2. SDS/polyacrylamide gel electrophoresis and immunoblotting of bacterial lipopolysaccharides.

A, Electrophoregrams were stained with silver, and B, immunoblotted with homologous rabbit serum. Strain designations are: 1, LPS *Escherichia coli* K12 C600; 2, LPS *Klebsiella oxytoca* strain 666; 3, LPS *Pseudomonas cerasi*; 4, LPS-W of clinical isolate; 5, LPS *Vibrio cholerae* NAG; 6, LPS-P-III; 7, LPS-P-II; 8, LPS-P-I.

Table 1. Gas-liquid chromatography-mass spectrometry and chemical analysis (in molar ratio) of lipopolysaccharides isolated after phenol/water extraction.

Components from water phase: LPS-W; components from phenol phase: LPS-P-I, II and III. t_R, retention time. Molar ratios are arbitrarily referred to glucosamine or palmitic acid residues.

Component	t_R (min)	LPS-W	LPS-P-I	LPS-P-II	LPS-P-III
Xylose	7.14	0.4	1.5	1.4	0.5
Glucose	9.97	5.9	22.0	6.9	7.5
Glucosamine	11.59	1.0	1.0	1.0	1.0
Heptose	12.56	2.8	2.2	2.7	1.7
3-deoxyoctulosonic acid[a]		0.3	0.3	0.2	0.3
Phosphorus[a]		3.1	nd[b]	1.4	2.4
Fatty acid					
Sphingosine[a]		0.2	0.4	0.2	0.4
n C14:0	5.25	traces	0.2	0	traces
2-OH C14:0	6.75	0.3	0.2	0	0
3-OH C14:0	7.10	2.5	1.3	0	0
C16:0	7.93	1.0	1.0	1.0	1.0
C18:1	10.12	0.7[c]	traces	0	0
X	10.22		0.4	3.0	1.1
C18:0	10.42	0.4	3.2	2.4	0.9
X	16.65	1.1	traces	0	0

[a]Determined colorimetrically; [b]not determined; [c]poorly resolved

Table 2. Methylation analysis of lipopolysaccharides isolated from phenol/water extract

Water phase: LPS-W; phenol phase: LPS-P-II and III.

Methylated sugar	t_R*	Molar ratio in		
		LPS-W	LPS-P-II	LPS-P-III
2,3-Me_2Xyl	0.89	0.8	0.8	0.3
2,3,4,6-Me_4Glc	1.00	1.0	1.0	1.0
2,3,6-Me_3Glc	1.24	1.1	1.3	1.2
2,3,4-Me_3Glc	1.28	–	0.4	0.3
3,6-Me_2GlcNAc	1.93	0.4	0.2	0.2

*t_R, retention time for the corresponding alditol acetate referred to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol

Table 3. Adhesion of bacterial cells to polystyrene plate (Nunc)

Bacteria		A_{570}	
		Exp.1[a]	Exp.2[b]
Staphylococcus epidermidis strain	186	0.151	0.343
	187	0.189	0.290
	627	0.082	0.282
	628	0.075	0.400
Staphylococcus pulvereri strain	215	0.103	0.233
Escherichia coli K12 C600		0.092	0.129
Clinical isolate		**0.517**	**0.541**
Pseudomonas aeruginosa		0.015	0.198

Incubation of bacteria for a, 18 h or b, 24 h.

Chemical analysis of lipopolysaccharide preparations revealed the presence of xylose, glucose, glucosamine, heptose, 3-deoxyoctulosonic acid (Kdo), phosphorus, sphingosine, palmitic, stearic and 3-hydroxymyristic acid in the molar ratios presented in Table 1. Small amount of 2-hydroxymyristic acid is also present. The retention time of the heptose derivative obtained in the GLC-MS analysis differs from that of L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptose. The gas chromatograms of the trimethylsilyl ether derivatives of long-chain bases from all LPS preparations revealed two peaks in almost equal proportion. The mass spectra of both compounds were closely similar to each other. One of the component was identified as dihydrosphingosine, with major fragments of m/z 132, 313, and the second one, with ions of m/z 132 and 341, is a two carbon longer homologue of dihydrosphingosine.

Methylation analysis carried out on the whole LPS preparations evidenced the presence of terminal glucose, 5-substituted pentose, 4-substituted glucose and non-stoichiometric amounts of 6-substituted glucose and 4-substituted glucosamine (Table 2). Lack of the methylated heptose derivative could be due to its phosphorylation.

The adherence of different bacterial species to polystyrene plates is presented in Table 3. The cells of our clinical isolate adhered more strongly than any other strain tested in the present study.

DISCUSSION

The clinical isolate temporarily classified as *Flavobacterium* sp. IIb [1] contains a lipopolysaccharide giving a ladder-like electrophoretic pattern and classical LPS constituents, such as heptose, Kdo and 3-hydroxymyristic acid. Lipopolysaccharides isolated from water and from phenol phase differ in their fatty acid composition, however both contain an additional component — sphingosine. In this respect our lipopolysaccharide resembles the glycosphingolipids which have been found by Kawahara *et al.* [13] in *Sphingomonas paucimobilis*, previously known as *Flavobacterium devorans*. Glycosphingolipids are ubiquitous components of the plasma membrane of eucaryotic cells. They are located in the outer leaflet of the membrane and function in cellular interactions, differentiation, and immuneresponses [14]. However, they are rarely found in procaryotic cells. In bacteria the sphingosine-containing compounds were reported in the genus *Sphingobacterium* showing low G+C content [5] and in *Sphingomonas* of high G+C content [10, 15]. According to Kawahara *et al.* [13] the outer membrane of *Sphingomonas paucimobilis* is built of phospholipids and glycosphingolipids where the latter are playing a similar role as LPS in other Gram-negative bacteria.

The results of chemical analysis suggest that our bacterial isolate synthesizes a

unique lipopolysaccharide that contains sphingosine. High content of G+C (66.6 mol%) [1] and the presence of sphingosine may justify reclassification of the strain to the genus *Sphingomonas*. Moreover, 2-hydroxymyristic acid present in *Sphingomonas* species [4, 10, 13, 15] has been found also in LPS of our clinical isolate. This group of microorganisms has been known to persist in hospital environment and cause meningitis, bacteremia, septicemia, wound infection and peritonitis [4]. They have been isolated from infusion and irrigation fluids, "sterile" water used in the surgical operation room, from vaginal swabs, urine, sputum and spinal fluid [4]. Furthermore, these bacteria are resistant to many antibiotics prescribed for infections caused by aerobic, Gram-negative rods [16] and to other chemotherapeutics and disinfectants [1]. A related pathogen, *Chryseobacterium (Flavobacterium) meningosepticum* survived the pasteurization process as long as it was carried out at a temperature lower than 62°C [17]. All these factors emphasize the importance of these bacteria as nosocomial pathogens, particularly dangerous for immunocompromised patients.

The measurement of bacterial adherence to polystyrene plates was applied by Christensen *et al.* [12] to distinguish between slime-producing and non-slime-producing staphylococcal strains. Clinical studies reported that the adherence positively correlates with pathogenicity enabling bacterial colonisation of catheters, valves, artificial joints and other foreign-body elements transplanted to patients and making the bacteria resistant to many antibiotics [18]. Strong adherence of the clinical isolate studied is in accordance with colonisation of medical devices by this strain and its resistance to disinfectants used for sterilization.

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