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Immunochemical Studies of the Lipopolysaccharides of *Vibrio cholerae*: Constitution of O-Specific Side Chain and Core Polysaccharide

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Lipopolysaccharides from various strains of *Vibrio cholerae* have been found to consist of distinct O-specific side chain and core polysaccharide regions in their degraded polysaccharides. The major identifiable components in the core polysaccharide were phosphorus, glucose, heptose, fructose, and ethanolamine phosphate, with small amounts or traces of mannose, rhamnose, and D-perosamine. On the other hand, glucose, fructose, mannose, rhamnose, glucosamine, D-quinovosamine, and D-perosamine were identified from O-specific side chain polysaccharide. The amounts of each component have been determined from O-specific side chain and core polysaccharides of the lipopolysaccharides from these bacterial strains.

The polysaccharide moieties from a number of gram-negative bacteria have been well characterized (5, 7, 11, 12, 14, 25), but relatively little work has been done with *Vibrio cholerae* (9, 10, 20). Recently, we initially fractionated and identified core polysaccharide and O-specific side chains from lipopolysaccharides of *V. cholerae* (17). Now we report detailed comparative analysis of the sugars and aminosugars of O-specific side chain and core polysaccharide regions from several strains of *V. cholerae.*

MATERIALS AND METHODS

Bacterial strains, growth conditions, and preparation of lipopolysaccharides. Lipopolysaccharides were isolated from the cell walls of *V. cholerae*, Inaba (66/64)., Inaba (569 B)., Inaba (35 A3)., Ogawa (NIH 41)., Ogawa (NIH 90)., biotype *eltor* (OE/27)., and Nag (4715); grown in a synthetic medium (19) for 18 h at 37°C; treated with aqueous 45% (wt/vol) phenol for 15 min at 72°C by the method of Westphal and Jann (24); and purified by repeated ultracentrifugation at 150,000 × *g* for 2 h as described previously (17, 19).

Polysaccharide and lipid A moiety of lipopolysaccharide were cleaved as described by Wilkinson and Galbraith (25). Samples of lipopolysaccharides (100 mg) were hydrolyzed with 1% acetic acid (7 ml) at 105°C for 3 h. Each hydrolysate was separated into chloroform-soluble and water-soluble fractions (aqueous layer) by thorough mixing with an equal volume of chloroform, followed by low-speed centrifugation. The water-soluble fraction (aqueous layer) containing the degraded polysaccharide moiety was separated, washed three or more times with chloroform, and dried in vacuo over P_2O_5 to obtain degraded polysaccharides. The degraded polysaccharide was further fractionated by using Sephadex G-50 column (1.5 by 200 cm) with pyridine-acetic acid-water (10:4:1, vol/vol/vol), pH 5.4, as eluant giving two major peaks.

Chromatographic techniques. For paper chromatography, carried out on Whatman no. 1 filter paper, the following solvents were used: (i) butanol-pyridine-water-benzene (5:3:3:1, vol/vol/vol/vol), organic phase, descending; (ii) butanol-pyridine-water (6:4:3, vol/vol/vol), descending; (iii) ethyl acetate-acetic acid-88% (vol/vol) formic acid-water (18:3:1:4, vol/vol/vol/vol), descending; (iv) butanol-pyridine-water (6:5:5, vol/vol/vol), ascending; (v) methanol-water-pyridine-10 M HCl (32:7:4:1, vol/vol/vol/vol), ascending. Thin-layer chromatography was done on Stahl silica gel H (E. Merck AG, Darmstadt, Germany) with ethyl acetate-pyridine-acetic acid-water (5:5:1:2, vol/vol/vol/vol) as solvent. For paper electrophoresis, on Whatman 3MM filter paper, the following buffers were used: pH 3.5 (pyridine-acetic acid-water, 6.5:62.5:931, vol/vol/vol); pH 7.5 (NaH_2PO_4, 22 g, and $Na_2HPO_4 \cdot 12H_2O$, 301 g, in water, 10 liters).

Reducing sugars were detected with triphenyltetrazolium chloride or alkaline silver nitrate, vicinal diols with pyriodate-benzidine, primary amines with ninhydrin, and organic material (on silica gel) with 2% sulfuric acid in ethanol as described earlier (19).

Quantitative estimations of neutral sugars were done by the method of Dubois et al. (6), heptose (with D-glycero-L-manno-heptose as standard) by the Osborn modification (15) of the method of Dische (2), hexoses by the method of Dische and Danilchenko (3), and hexosamines by the method of Rondle and Morgan (21). Reducing sugars were estimated by the method of Nelson (13) as modified by Somogyi (22). Fructose was determined after hydrolysis of the samples in 0.2 M acetic acid (1 mg/0.1 ml) at 100°C for 8 h; after centrifugation the supernatant was freeze-dried and the residue was taken up in water (1 mg/0.1 ml). Aliquots (60 μl) of this solution were analyzed in the optical test with hexokinase-adenosine triphosphate-phospho-glucose isomerase-6-phospho-glucose dehydrogenase-nicotinamide adenine dinucleotide

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phosphate (Boehringer Mannheim Corp., GmbH, Germany). Rhamnose was estimated in the same experiment from the difference between E 402 and E 415 for the spectrum in cysteine/H_2SO_4. Mannose was determined with glucose-6-phosphate dehydrogenase (Boehringer Mannheim Corp.) used in conjunction with phospho-mannose-isomerase and phospho-glucose-isomerase (19). Ethanolamine was estimated by the colorimetric procedure of Dittmer and Lester (4). Total phosphorus was determined by the method of Bartlett (1) and inorganic phosphorus by the method of Parvin and Smith (16).

Monosaccharides were reduced to alditols with $NaBH_4$ and converted into alditol acetates of O-trimethyl-silyl derivatives. Alditol acetates were characterized on columns containing 3% (wt/wt) ECNSS-M as stationary phase, and O-trimethyl-silyl derivatives were characterized on columns containing 3% (wt/wt) OV-225 by gas-liquid chromatography as described earlier (17). For analysis of amino compounds (with a Technicon Auto-Analyzer), samples were first hydrolyzed with 5 M HCl, 105°C for 6 h, followed by repeated drying in vacuo over P_2O_5 and KOH.

RESULTS

Table 1 shows the sugar and aminosugar compositions of the isolated lipopolysaccharides from eight different strains of *V. cholerae*. All the lipopolysaccharides contained phosphate, glucose, and heptose as D-glycero-M-manno-heptose, fructose, mannose, rhamnose, glucosamine, D-quinovosamine (2-amino-2,6-dideoxy-glucose), and D-perosamine (4-amino-4,6-dideoxy D-mannose). In general, these components were present in almost similar amounts but there were some differences. For example, Ogawa (NIH 90) and Ogawa (5321) contained more glucose than most of the other lipopolysaccharides; the glucosamine contents of Inaba (35 A3) and *eltor* (OE/27) were unusually high and that of Nag (4715) was unusually very low. Characteristically these lipopolysaccharides contained aminosugars D-quinovosamine and D-perosamine in addition to the usual regular glucosamine in almost similar amounts. All lipopolysaccharides contained phosphorus in about the same amounts (3.8 to 6.0%). Small amounts of ethanolamine were detected in hydrolysates (0.5 N HCl at 105°C for 30 min) of the lipopolysaccharides.

In most lipopolysaccharides, the lipid A and polysaccharide moieties are apparently linked via a ketosidic bond from 2-keto-3-deoxyoctulosonic acid (KDO) (11). Attempts to detect KDO in lipopolysaccharides of all these strains employing the thiobarbituric method of Weissbach and Hurwitz (23), and gas-liquid chromatography by using authentic KDO standards, failed.

As described previously (19), mild acid hydrolysis of the lipopolysaccharides gave rise to fractions consisting of degraded polysaccharides (water soluble), lipid A (chloroform soluble), and interfacial materials. Chloroform-soluble fractions consisting of lipid A and interfacial materials were not studied further. The isolated, degraded polysaccharides were fractionated on a column of Sephadex G-50 as described above. By monitoring the eluates for phosphorus and carbohydrate two main peaks differing in molecular size were obtained from all degraded polysaccharide fractions. (A typical chromatographic pattern of degraded polysaccharide is shown in Fig. 1. Almost identical patterns were obtained from all the strains of *V. cholerae* studied.) The first of these was eluted immediately after the void volume of the column and was followed by a second peak. When these two peaks were rerun separately on the same column, both moved in their original positions. Detailed analysis of these two peaks showed that high-molecular-weight fractions do not contain phosphorus and heptose, whereas all the phosphorus and heptose of the degraded polysaccharides were detected in low-molecular-weight fractions. On the basis of hemagglutination assays high-molecular-weight heptose-free fractions (peak 1) were characterized as O-specific side chain polysaccharide and low-molecular-weight heptose-containing fractions (peak 11) as core polysaccharide regions of the lipopolysaccharides as described

TABLE 1. *Sugar analysis of lipopolysaccharides from V. cholerae*

Strain	Lipopolysaccharide[a] (% weight)										
	P	Glc	Hep	Fru	Man	Rha	GlcN	Quin	Pero	Ethanolamine	Total sugars
Inaba (66/64)	4.8	8.6	6.5	3.8	2.2	1.8	7.2	1.2	3.0	1.8	34.3
Inaba (569 B)	5.2	7.2	7.0	4.2	1.8	1.6	6.5	0.8	2.8	2.6	37.1
Inaba (35 A3)	4.2	10.5	8.5	3.0	3.6	1.2	13.6	1.8	2.3	1.2	44.5
Ogawa (NIH 41)	4.8	8.8	9.2	2.8	2.5	1.0	8.8	1.0	2.4	0.8	36.5
Ogawa (NIH 90)	3.8	13.4	8.8	4.8	3.2	3.0	6.6	1.2	2.8	2.3	41.8
Ogawa (5321)	6.0	14.5	10.2	3.2	1.2	3.2	6.5	2.2	1.3	1.8	42.3
Biotype *eltor* (OE/27)	4.8	9.2	7.6	3.8	3.2	2.0	12.6	1.2	3.4	2.2	43.0
Nag (4715)	4.2	8.4	5.2	3.2	2.6	2.8	3.8	1.6	2.8	1.6	29.4

[a] P, Phosphorus; Glc, glucose; Hep, heptose; Fru, fructose; Man, mannose; Rha, rhamnose; GlcN, glucosamine; Quin, quinovosamine; Pero, perosamine.

previously (17). The results of analysis of these O-specific side chain polysaccharide and core polysaccharide from various strains of *V. cholerae* are given in Table 2. Glucose, fructose, mannose, rhamnose, and D-perosamine made up both of these fractions in the majority of strains, although there were large variations in individual quantities of these components in the two fractions. Glucose, heptose, fructose, phosphate, and ethanolamine-phosphate were concentrated in core polysaccharide, whereas mannose, rhamnose, glucosamine, D-quinovosamine, and D-perosamine were concentrated in O-specific side chain polysaccharide. In particular, glucosamine and D-quinovosamine were absent from core polysaccharide, and heptose, phosphate, and ethanolamine phosphate were absent from O-specific side chain polysaccharide. In core polysaccharide of biotype *eltor* (OE/27) and Nag (4715), mannose was absent and, in all other strains, present in small amounts or traces.

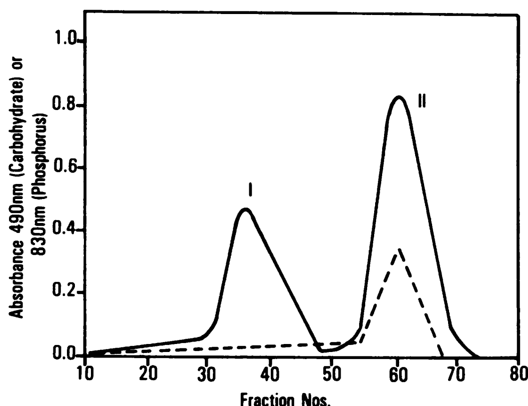

FIG. 1. *Typical chromatographic elution profile of degraded polysaccharide of V. cholerae on Sephadex G-50 column (1.5 by 200 cm). Eluant, Pyridine-acetic acid-water (10:4:1, vol/vol/vol), pH 5.4; flow rate, 15 ml/h. Fractions (1 ml) were screened for total phosphorus* (-----) *and for total carbohydrates* (——).

The core fractions of the degraded polysaccharides containing ethanolamine phosphate were hydrolyzed vigorously by using 5 M HCl at 105°C for 6 h in sealed ampoules, and the resultant hydrolysates were analyzed by paper chromatography in solvents iv and v and thin-layer chromatography on Stahl silica gel H in ethyl acetate-pyridine-acetic acid-water (5:5:4:3, vol/vol/vol/vol). Ethanolamine phosphate, ethanolamine, and inorganic phosphorus were identified from the hydrolysates (Table 3). On autoanalysis, with a synthetic standard, ethanolamine phosphate was identified as ethanolamine triphosphate. These results indicated that ethanolamine is present in the form of ethanolamine triphosphate in core regions of the lipopolysaccharides. Ethanolamine triphosphate decomposed during storage. Paper chromatography and thin-layer chromatography indicated that the initial decomposition products were ethanolamine pyrophosphate and inorganic phosphorus, whereas the products of virogous acid hydrolysis were ethanolamine phosphate, etha-

TABLE 2. *Distribution of sugars in O-specific side chain and core polysaccharide regions of the degraded polysaccharide of V. cholerae*[a]

Strain	Preparation	Lipopolysaccharide (% weight)									
		P	Glc	Hep	Fru	Man	Rha	GlcN	Quin	Pero	Ethanolamine phosphate
Inaba (66/64)	O-PS	0	4.8	0	2.4	3.4	3.8	4.6	2.4	4.8	0
	C-PS	4.0	14.2	16.2	5.2	Tr	Tr	0	0	Tr	3.6
Inaba (569 B)	O-PS	0	3.2	0	1.8	4.2	2.8	6.2	3.8	3.0	0
	C-PS	5.2	16.4	19.8	6.5	0.8	1.2	0	0	1.2	3.8
Ogawa (NIH 41)	O-PS	0	2.8	0	2.7	6.2	3.8	7.2	2.6	3.2	0
	C-PS	5.6	11.3	13.2	5.2	Tr	Tr	0	0	1.2	5.2
Ogawa (5321)	O-PS	0	3.2	0	1.8	4.6	3.2	5.1	2.8	3.6	0
	C-PS	4.8	19.8	18.8	6.8	0.8	Tr	0	0	2.4	4.4
Biotype *eltor* (OE/27)	O-PS	0	2.8	0	0.8	4.0	6.2	7.3	3.6	2.8	0
	C-PS	3.8	18.2	15.8	6.2	0	Tr	0	0	1.9	3.8
Nag (4715)	O-PS	0	2.8	0	3.2	5.2	4.8	9.6	3.8	4.8	0
	C-PS	4.6	17.3	12.8	7.8	0	3.8	0	0	Tr	4.3

[a] Tr, Traces. Degraded polysaccharide was obtained from lipopolysaccharide by heating in 1% acetic acid at 105°C for 3 h. O-specific side chain polysaccharide (O-PS) and core polysaccharide (C-PS) were obtained by fractionation of the degraded polysaccharide on Sephadex G-50. See Table 1 footnote for explanations of abbreviations.

TABLE 3. *Percent composition of phosphorus and ethanolamine in core polysaccharide of V. cholerae*[a]

Component	Inaba (66/64)	Inaba (569 B)	Ogawa (NIH 41)	Ogawa (5321)	*eltor* (OE/27)	Nag (4715)
P (total)	4.0	5.2	5.6	4.8	3.8	4.6
Pi	1.8	2.3	1.2	2.2	0.8	1.2
Ethanolamine phosphate	3.6	3.2	4.6	3.8	2.8	3.8
Ethanolamine	0.6	0.8	1.2	0.8	0.6	0.4

[a] Ethanolamine phosphate, ethanolamine, and Pi (inorganic phosphate) were estimated after the samples were hydrolyzed with 5 M HCl at 105°C for 6 h by autoanalysis.

nolamine, and inorganic phosphorus. Recently, we reported that the de-O-acylated lipid A (water-soluble products of lipid A) obtained as a result of mild alkaline methanolysis was proved to be a mixture of phosphorylated oligosaccharides from *V. cholerae* Inaba (569 B) and Inaba (biotype *eltor*) (17). All fractions of the de-O-acylated lipid A obtained after gel filtration on Sephadex G-25 contained different concentrations of phosphate from these strains (17, 18). Thus, the occurrence of substantial amounts of phosphate residues in lipid A moiety and core polysaccharide region of the degraded polysaccharides could provide evidence for the possible sites for the attachment of ethanolamine triphosphate residues to either moiety by means of acid-labile phosphate bonds.

DISCUSSION

The results reported here indicate that it is possible to separate O-specific side chain and core polysaccharide regions of the lipopolysaccharides on Sephadex G-50 from *V. cholerae*. These studies showed an interesting and characteristic chemical constitution of O-specific side chain and core polysaccharide from Inaba (66/64), Inaba (569 B), Ogawa (NIH 41), Ogawa (5321), *eltor* (OE/27), and Nag (4715) strains of *V. cholerae* generally used in the preparations of vaccines. Glucose and heptose were the main sugars of the lipopolysaccharides, and glucose was present in both O-specific side chain and core polysaccharide, whereas heptose was detected only in core polysaccharides. Chemical studies have revealed that the polysaccharide component of lipopolysaccharide derived from different genera vary widely with respect to composition and structure (11). It has also been shown clearly that discrete structures in the polysaccharide portion of lipopolysaccharide carry the serological determinants responsible for antibody specificity (11, 12). It will be noted that, by comparison with the core polysaccharide, the O-specific side chain is deficient in phosphate, ethanolamine phosphate, and heptose, and core polysaccharide is deficient in glucosamine from all strains of *V. cholerae* examined. This observation holds promise of a future role for ethanolamine phosphate, heptose, or glucosamine in specific immunological specificity and structure-function relationship of the polysaccharide moiety from these bacteria.

Redmond (20) and Jann et al. (10) previously identified D-quinovosamine and D-perosamine as a constituent of lipopolysaccharide from *V. cholerae* Inaba (569 B). We have confirmed this and, moreover, we found that D-quinovosamine is present exclusively in O-specific side chain polysaccharide, whereas D-perosamine is concentrated in O-specific side chain, with small amounts or traces in core polysaccharides from all the *V. cholerae* strains tested. Although the structure of O-specific side chain varies widely among the different species and serotypes of gram-negative bacilli, the structure of the core region appears to be similar in most species, especially in the "back bone" containing only heptose, phosphate, hexosamine, and KDO (11). The fact that KDO is completely absent from the polysaccharide moiety of the lipopolysaccharide of *V. cholerae* is of interest. It points to a core structure different from those of *Escherichia coli* and *Salmonella* species (11, 12). The important questions left unanswered are the sequence and the nature of linkages of the sugar residues in the core structure and O-specific side chain polysaccharide of these bacterial species.

The cholera vaccines now available consist of killed vibrios and give rise to only limited protection of short duration. Recently, Holmgren and Sevennerholm (8) showed that a combination of *V. cholerae* subunit L-toxoid and lipopolysaccharide antigen from Inaba (569 B) induced a more than 100-fold-higher degree of immunity in rabbits against challenge with vibrios than did immunization with either of the two antigens alone. It has been well recognized that lipid A moiety represents the toxic center of the lipopolysaccharide, and the polysaccharide, on the other hand, is associated with O-antigenicity and phage-receptor properties rather than endotoxicity. The substitution of O-specific side chain or core polysaccharide of defined structure into subunit L-toxoid offers the particular advantage of preparing a better and

nontoxic vaccine against cholera infection, consisting of immunodeterminant regions of the degraded polysaccharide and toxoid. Further studies in progress will show whether the promise of these initial results can be substantiated.

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