IMMUNOCHEMICAL STUDIES OF THE O-ANTIGENS OF *VIBRIO CHOLERAE**. THE CONSTITUTION OF A LIPOPOLYSACCHARIDE FROM *V. CHOLERAE* 569B (Inaba)

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Received 11 January 1971

1. Introduction

In recent years the compositions of the lipopolysaccharides of Gram-negative organisms, especially *Salmonella* species, have been studied extensively. As a result, there has emerged a general concept of the structures of these immunologically important macromolecules [1]. The corresponding cell wall components of *Vibrio cholerae* have, however, received scant attention. This report presents preliminary results concerning the composition of a lipopolysaccharide of *Vibrio cholera* 569B (Inaba).

2. Methods and materials

2.1. *Preparation of materials*

Vibrio cholerae 569B (Inaba) cells were grown on nutrient agar in Roux bottles at 37° for 36 hr. The cells were harvested in saline, centrifuged at 10,000 *g* for 20 min, washed three times in saline and once with distilled water. After incubation in a solution of EDTA (0.005 M) in tris-HCl (0.12 M, pH 8.0) at 37° for 30 min [2], the vibrios were again collected by centrifugation and extracted with 45% phenol by the method of Westphal and Jann [3]. The resultant lipopolysaccharide fraction was further purified by ultracentrifugation of an aqueous solution (81,000 *g* 2 hr). The lipopolysaccharide product was obtained in approx. 1% yield, based on dry weight of organisms. It was free from nucleic acid (lack of absorption at 260 nm).

Cell-wall polysaccharide was prepared as by Freeman [4] and Lipid A as by Nowotny [5]. Lipopolysaccharide was treated with periodate as by Nikaido [6]. Perseitol was purchased from Sigma Chemical Company.

2.2. *General analytical methods*

Neutral sugars were estimated by gas chromatography of their alditol acetates [7]. The polysaccharide or lipopolysaccharide (2 to 5 mg) was treated with Dowex-50 (H^+) in 0.01 N hydrochloric acid (1 ml) in a sealed fusion tube at 105°. Where appropriate, xylose was incorporated as an internal standard. Hydrolysis was complete in 7 days. The hydrolysate was passed through a short column of Dowex-1 (HCO_3^-) and treated with sodium borohydride in the usual way. Acetylation was achieved using sodium acetate/acetic anhydride at 120° for 2 hr. The excess anhydride was removed in a gentle stream of nitrogen and the acetates extracted into ether, filtered and the solution concentrated before examination by gas chromatography (conditions 1).

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The amino sugar, estimated by the modified Elson-Morgan procedure of Gatt and Berman [8], was shown to consist entirely of glucosamine by the amino acid analyser and by the method of Stoffyn and Jeanloz [9]. Uronic acid was estimated by the Dische carbazole method [10] and 2-keto-3-deoxyoctonate (KDO) by that of Osborn [11]. Glycerol was detected by paper chromatography [12] and estimated semi-quantitatively by gas chromatography of the tribenzoate (conditions 2) [13] and the tris (trimethylsilyl) derivative (conditions 3) [14]. Accurate quantitation by the enzymic procedure of Spinella and Mayer [15], was carried out on a 7-day Dowex-50 hydrolysates. Parallel runs demonstrated the lack of destruction of glycerol, as well as its quantitative release from glycerophosphate, under these conditions. Ethanolamine was detected by paper chromatography (system D) [16] and estimated semi-quantitatively by gas chromatography of its dibenzoate (conditions 2) and by the colorimetric procedure of Dittmer and Wells [16–18]. The protein content of the preparations was determined by the Folin method [19] and the constituent amino acids by the Technicon system. Total fatty acids were estimated as by Duncombe [20] using palmitic acid as standard and total phosphorus as by Ames [21]. Nitrogen and acetyl analyses were carried out by the Australian Microanalytical Service, Melbourne.

Descending paper chromatography was carried out using Whatman No. 3 paper and (A) butanol–pyridine–water (6:4:3); (B) ethyl acetate–pyridine–water (8:2:1); (C) ethyl acetate–pyridine–water (10:4:3) and (D) propanol–ethyl acetate–water (7:1:2). Sugars were detected with alkaline silver nitrate.

2.3. *Gas chromatography*

Analysis was carried out using a Varian Aerograph Model 1701. Silanized stainless steel tubing was used throughout and the carrier gas nitrogen. Conditions used were 1) column (2 m × 3 mm) packed with fluorinated silicone QF-1 (0.5%) and neopentylglycol succinate (0.5%) on Varaport 30 (100/120 mesh). The oven temperature was raised from 180° to 220° at 1°/min and the nitrogen flow rate was 20 ml/min; 2) column (200 × 3 mm) packed with SE-30 (4%) on Varaport 30. The temperature was raised from 150° to 250° at 10°/min and the flow rate was 30 ml/min; 3) columm (2 m × 3 mm) packed with SE-30 (3%) on Varaport 30. The oven temperature was increased from 100° to 150° at 2°/min and the flow rate was 15 ml/min.

Table 1
Typical analytical results for *Vibrio cholera* 569B lipopolysaccharide and cell-wall polysaccharide.

	Lipopoly-saccharide	Cell-wall polysaccharide
Mannose% *	1.40	3.6
Glucose%	6.69	20.3
Heptose%	6.87	6.2
Glucosamine% *	6.1	
Galactose% *	0	2
Glycerol% *	1.1	0
Ethanolamine% * approx.	3	
Fatty acids% *	18.5	
Protein	2	
Lipid A%	30	
Total P%	2.2	0
Total N%	2.7	4.1
Acetyl%	2.6	
Loss on drying **	14	

* expressed as anhydro residues.
** dried at 0.2 mm Hg, 70°, 30 hr.

2.4. *Serological methods*

Passive haemagglutination was carried out as by Auzins [22] using alkali-treated LPS. Antiserum was prepared as by Neoh and Rowley [23]; inhibition assays were carried out using 4 haemagglutinating doses of the antiserum.

3. Results and discussion

Many different procedures have been used to isolate serologically active components from cell walls of *Vibrio cholerae*. These include phenol/water extraction [24], treatment with mineral acid [26], and ammonium sulphate fractionation [25]. Treatment of other Gram-negative bacteria with EDTA/tris HCl causes release of lipopolysaccharides [2]. Attempted preparation of cholera lipopolysaccharide by this

mild procedure gave erratic results: often no product was obtained. Subsequent extraction of the recovered vibrios with 45% phenol, however, led to a superior lipopolysaccharide, free from nucleic acid and agar.

The gas chromatogram of the alditol acetates of the neutral sugars present in the lipopolysaccharide is extremely clean. Apart from glucose, mannose and heptose (see table 1), only a trace amount of arabinose (approx. 0.1%) and a small unassigned peak (approx. 1%), eluted closely after the glucose derivative, were noted. Galactose, previously reported as a component of cholera polysaccharide materials [24, 26] is absent, while mannose and heptose, previously unrecognized, are clearly present. The heptose is believed to be L-*glycero*-D-*manno*-heptose (or its enantiomer) on the basis of its paper chromatographic behaviour. It has the expected mobility using solvent system A [29]. Using systems B and C, the component has R_fs identical to that of the main heptose of *Salmonella typhimurium* C5. Moreover, the corresponding alditol acetate has gas chromatographic behaviour identical to that of the corresponding *Salmonella* component as well as to that of authentic perseitol hepta-acetate.

The occurrence of heptose as a component of a lipopolysaccharide is to be expected [1]. Mannose, however, may be present in a contaminant. Gas chromatographic analyses of lipopolysaccharides of *Salmonella* rough mutants constantly show traces of mannose. The source of the sugar is by no means understood [30, 31].

Our lipopolysaccharide differs in two other respects from previously described cholera materials: 1) The content of amino sugar is much lower and 2) uronic acid is absent. We have also carried out uronic acid estimations on whole vibrios and have been unable to detect this component. There is, however, strong, non-specific absorption at the wavelength used for the test and earlier workers may have been led to attribute this to the presence of uronic acid [24, 26].

It will be noted that, by comparison with the lipopolysaccharide, the cell-wall polysaccharide is deficient in heptose. The mild conditions (0.2 M acetic acid, 100°, 2 hr) used in the preparation of cell-wall polysaccharide are not expected to cause cleavage of pyranosidic linkages. Unless, therefore, heptose is involved in novel furanosidic bonding [32], it is likely that at least some of the residues are linked by other labile bonds, e.g. phosphodiester bonds.

Table 2
Haemagglutination inhibition assays of polysaccharide preparations.

Inhibitor	Amount to inhibit 4 HA doses (μg)
Lipopolysaccharide	0.02
Cell-wall polysaccharide	1.0 – 2.0
Periodate-treated lipopolysaccharide	3.1–6.2

This speculation may also be applied to glycerol, which is absent from the cell-wall polysaccharide.

It is clear that we have not yet recognized all the constituents of our lipopolysaccharide. Indeed some 30% of its weight is still unaccounted for. The possibility then exists that the immunologically most important component still evades our attention. It is important to note, moreover, that the cell-wall polysaccharide shows only 1/50 of the haemagglutination inhibitory activity of the lipopolysaccharides (table 2). It would seem that an important antigenic determinant has been lost or destroyed during the acetic acid treatment. Heptose or glycerol may be implicated.

The standard assay [11] for KDO is negative for both preparations. It is therefore necessary to postulate a fundamentally different lipopolysaccharide structure from that of the *Salmonella spp.* where KDO is responsible for linking the polysaccharide and Lipid A moieties [11]. Galactose, a component of the usual 'core' lipopolysaccharide [27] is also absent.

The proportion of lipid in our lipopolysaccharide material is high. Initial studies have shown the presence of glucosamine, phosphorus and a wide range of fatty acids in Lipid A, but no neutral sugars are present in this part of the complex. We tentatively propose a broad similarity of Lipid A of other species [1]. The results of the Folin protein assay and amino acid analysis are consistent. Surprisingly, the major amino acids are glycine and methionine.

While the presence of glycerol and ethanolamine is unequivocal, their role is not entirely clear. The amino acid analyser has, however, shown the presence of small amounts of phosphoethanolamine and glycerophosphoethanolamine in the acid hydrolysate (6 N HCl, 110°, 24 hr). A structural function for the components may therefore be suggested. Adams,

Tornabene and Yaguchi [28] have recently reported isolation of glycerophosphoethanolamine from the lipopolysaccharide of Neisseria catarrhalis.

Acknowledgements

This work was jointly supported by a grant from the Australian Research Grants Committee (G.D.F.J.) and a Queen Elizabeth II Fellowship (to J.W.R.). Miss Judith Wright rendered excellent technical assistance.

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