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THE EFFECT OF REMOVAL OF D-FRUCTOSE ON THE ANTIGENICITY OF THE LIPOPOLYSACCHARIDE FROM A ROUGH MUTANT OF *Vibrio cholerae* OGAWA*

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ABSTRACT

The lipopolysaccharide (LPS) of a rough mutant (95R) of *Vibrio cholerae* Ogawa has been investigated chemically and serologically. D-Fructose was released from LPS under conditions (10mM trifluoroacetic acid, 60°, 1 h) that liberated no other sugar constituent of the LPS (2-amino-2-deoxy-D-glucose, D-glucose, L-*glycero*-D-*manno*-heptose). Upon periodate oxidation, D-fructose and D-glucose were oxidised quantitatively, whereas ~50% of heptose was periodate-resistant. The data indicate that D-fructose does not link the polysaccharide and lipid A portion as proposed earlier, and suggest that D-fructose is present as a branch. By passive hemolysis inhibition, it was shown that the release of D-fructose paralleled the exposure of an antigenic determinant cryptic in LPS.

INTRODUCTION

The lipopolysaccharide (LPS) of *Vibrio cholerae* contains D-fructose[1] but appeared to lack 3-deoxy-D-*manno*-2-octulosonic acid (KDO)[1–3] which, in enterobacterial and other LPS, links the polysaccharide and the lipid A component. Thus, the possibility was discussed that D-fructose provided this linkage. Recently, a phosphorylated KDO derivative was identified[4] as a constituent of *V. cholerae* LPS, and, moreover, a common antigenic determinant requiring the presence of KDO for its expression was also present[5,6]. These observations shed doubt on the earlier considerations[1].

The present study was performed to determine, by chemical and immunochemical methods, whether or not D-fructose is part of the linkage region of the polysaccharide–lipid.

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EXPERIMENTAL

Bacteria and LPS. — *V. cholerae* strain 95R (a rough mutant of serotype Ogawa 162) was obtained from G. D. F. Jackson (Kensington, Australia) and grown aerobically in a 14-L fermenter. Dry bacteria were extracted by the phenol–chloroform–light petroleum method[7], purified, and converted into the triethylammonium salt after electrodialysis[8]. The yield of purified LPS was 2.6% of the dry bacteria.

Chemical analyses. — Neutral sugars were determined after hydrolysis with 0.1M hydrochloric acid (100°, 48 h) by g.l.c. of their alditol acetates with xylose as internal standard, and hexosamines by the Morgan–Elson reaction[9]. Fructose was determined as mannitol and glucitol hexa-acetates by g.l.c. after reduction and acetylation, and colorimetrically using a phenol–acetone–boric acid reagent[10], and as its methoxime-acetate derivative[11]. D-Fructose, D-sorbose, D-tagatose, or the sample to be investigated (500 μg) was dried and a solution (100 μL) of methoxyamine · HCl (30 mg) in pyridine (1 mL) was added. The vial was closed with a Teflon-lined cap and the formation of methoximes allowed to proceed at 60° for 2 h. Acetic anhydride (100 μL) was then added and the mixture was kept at 100° for 30 min. The solvents were evaporated under reduced pressure, and a solution of the residue in chloroform was subjected to g.l.c. and g.l.c.–m.s.

Smith degradation of LPS. — To a solution of LPS (20 mg) in water (2 mL) was added 0.1M sodium metaperiodate (4 mL). Oxidation was allowed to proceed at 4° in the dark for 5 days. Ethylene glycol (50 μL) was added, and the sample was kept at room temperature for 1 h and then treated with sodium borohydride (100 mg) at room temperature for 1 h. The reduction was quenched with acetic acid (50 μL), and the mixture was dialysed against distilled water, acidified with aqueous 1% acetic acid, stored at 37° for 1 h, dialysed, and freeze-dried to give Smith-degraded LPS.

Hydrolysis kinetics. — Portions (5 mg each) of LPS were hydrolysed with 10 and 100mM trifluoroacetic acid (1 mL) in sealed ampoules at 60° for various times. Each ampoule was cooled in an ice-bath, neutralised with triethylamine, and dialysed to equilibrium against water (3 × 5 mL). The dialysate and retentate were lyophilised separately, and a solution of each residue in water (1 mL) was subjected to chemical and serological analysis.

Serology. — The presence of antigenic specificities was determined by the passive hemolysis test using antigen-coated sheep erythrocytes. The common LPS specificity was assayed in the system of alkali-treated[12] *Acinetobacter* lipid A/normal mouse serum[5,6], whereas the antigenic determinant of bisphosphorylated lipid A was tested as described previously[13]. Inhibition is expressed as the amount of antigen causing 50% of hemolysis.

RESULTS

Determination of fructose. — Determination of the fructose in the LPS of *V. cholerae* by g.l.c. of the derived alditol acetate or colorimetrically using the phenol–acetone–boric acid reagent was found not to be suitable. The LPS contains glucose, which interferes in the former assay, and KDO-phosphate, which could also react in the latter assay. Therefore, a method which involves the conversion of neutral ketoses into the acetylated methoxime derivative was used. The method was found to be sensitive, reproducible, and rapid, yielding well-resolved peaks in g.l.c. Two peaks from each ketose (fructose, sorbose, and tagatose) of comparable area were obtained which gave identical e.i. and c.i. mass spectra (not shown). C.i. (ammonia)-m.s. gave the expected molecular weight of 421 as indicated by peaks at *m/z* 422 $(M + 1)^+$ and at 439 $(M + 18)^+$; the two peaks represent the *syn-* and *anti-*methoxime derivatives. The retention times for standards and the LPS-derived sample are listed in Table I; fructose and tagatose could be separated from sorbose, but not from each other. The hydrolysate of LPS yielded two peaks which were indistinguishable from those corresponding to fructose derivatives and thus confirm the presence of fructose in the LPS.

Acid-catalysed hydrolysis of LPS. — The LPS was hydrolysed with trifluoroacetic acid (10 and 100mM) at 60° and the release of fructose was monitored. The acid-degraded LPS obtained after various intervals was isolated after dialysis and its inhibition capacity in a passive hemolysis assay for the common LPS specificity was determined. The results are combined in Fig. 1. In 100mM acid, ~65% of the fructose had been released after 10 min and the release was complete after 60 min. In 10mM acid, release was not complete after 90 min.

The native LPS did not express the common LPS specificity as indicated by an inhibition value of >1000 ng. The antigenic determinant was exposed during hydrolysis and the inhibition value reached a plateau of 16 ng after 10 min (100mM acid) or 60 min (10mM). The exposure of the antigenic determinant was paralleled

TABLE I

G.L.C. RETENTION TIMES OF METHOXIME-ACETATE DERIVATIVES OBTAINED FROM HEXULOSES AND HYDROLYSATES OF *V. cholerae* 95R LPS

Methoxime-acetate of	*Absolute retention time obtained by g.l.c.*[a] *(min)*
D-Fructose	10.31/10.59
D-Sorbose	11.06/11.42
D-Tagatose	10.23/10.89
LPS hydrolysate[b]	10.32/10.60

[a]Using a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54, and H_2 as carrier gas. Analysed at 160°→200° at 1°/min. [b]After hydrolysis in 0.1M trifluoroacetic acid at 60° for 1 h.

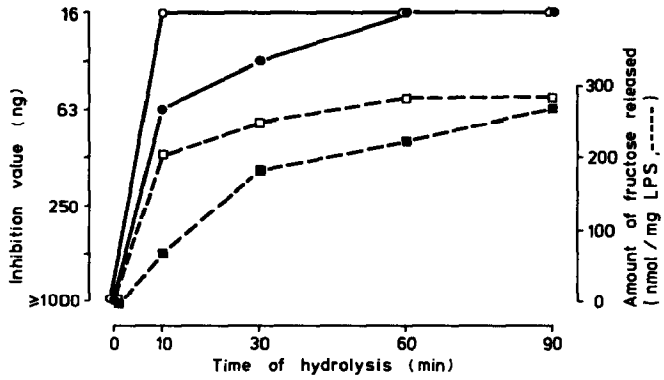

Fig. 1. Release of fructose (---) and the exposure of the common LPS specificity (—) upon hydrolysis in 10 (■, ●) and 100mM (□, ○) trifluoroacetic acid at 60° (see Experimental).

by the release of fructose (Fig. 1). The lipid A antigenic determinant was detected neither in native nor in partially degraded LPS, but treatment with 100mM acid at 100° for 1 h exposed lipid A antigenicity (data not shown).

Analysis of LPS, acid-degraded LPS, and Smith-degraded LPS. — Acid-degraded LPS was prepared by hydrolysis of LPS in 10mM trifluoroacetic acid at 60° for 1 h. After dialysis against water, the retentate and dialysate were analysed for free sugar components in comparison with native LPS. The results together with those for Smith-degraded LPS are summarised in Table II. LPS contained fructose, glucose, heptose, and 2-amino-2-deoxyglucose in the molar ratios ~1:2:2.5:2. The heptitol acetate had the same retention time in g.l.c. as the corresponding derivative of L-*glycero*-D-*manno*-heptose. No other hexosamines were present (amino acid analyser). For acid-degraded LPS, fructose was exclusively present in the dialysate, whereas glucose, heptose, and 2-amino-2-deoxyglucose were found in the retentate. On Smith degradation, fructose and glucose were oxidised completely, whereas one-third (235 nmol) of the LPS-bound heptose resisted oxidation.

TABLE II

SUGAR COMPOSITION OF NATIVE, SMITH-DEGRADED, AND ACID-DEGRADED LPS OF *V. cholerae* 95R

Sample	*Fru*	*Glc*	*Hep*	*GlcN*
	(nmol/mg of sample)			
LPS	275	575	680	510
Smith-degraded LPS	—[a]	—	235	390
Acid-degraded LPS[b]				
Retentate	—	520	625	520
Dialysate	270	—	—	—

[a]<10 nmol/mg. [b]Hydrolysed in 0.1M trifluoroacetic acid at 60° for 1 h.

DISCUSSION

Chemical and immunochemical methods were employed to investigate the LPS of *V. cholerae* in which an acid-labile D-fructose residue was proposed[1] as the sugar linking the core oligosaccharide and the lipid A component. Fructose has now been shown to be released quantitatively from LPS by mild acid hydrolysis without cleaving the main polysaccharide chain, since fructose was the sole dialysable sugar. Periodate oxidation destroyed the fructose in the LPS, whereas the heptose was partly unchanged. These data indicate that fructose cannot be the link between the core and lipid A regions. The serological data further support this conclusion. Lipid A antigenicity is cryptic in all the LPS studied so far, and is expressed only by free lipid A obtained after cleavage of the core oligosaccharide–lipid A linkage[14]. The fact that lipid A antigenicity was not detected in samples of LPS which had been hydrolysed under conditions that released the fructose quantitatively strongly indicated that free lipid A was not present. As with other LPS, lipid A antigenicity was detected after more vigorous hydrolysis of *V. cholerae* LPS. KDO-phosphate has been shown[4] to be a constituent of *V. cholerae* LPS and it is postulated that, as in other LPS[15], KDO in *V. cholerae* LPS links the polysaccharide and lipid A components.

The common LPS specificity (an antigenic determinant present in all LPS containing KDO and neutral sugars)[6] was present but masked in the LPS of *V. cholerae*. It was exposed during mild acid hydrolysis and the rate of exposure paralleled the release of fructose. A similar phenomenon has been observed[6] with the LPS of a *Salmonella minnesota* Rb_2 mutant, where a KDO disaccharide was released as the antigenic determinant was exposed. In *Enterobacteriaceae*, this determinant resides in the heptose/KDO region. The constituents of this inner core region are also present in *V. cholerae*. Therefore, it is assumed that the fructose which masks the common LPS specificity is linked as a branch to the inner core region of the LPS.

The data presented here, together with those for the lipid A component[16] and the presence of KDO[4], show that, in its general architecture, the inner core region of the LPS of *V. cholerae* is similar to the of *Enterobacteriaceae* and other Gram-negative bacteria[15].

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