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Lipopolysaccharides of R Mutants Isolated from *Vibria cholerae*

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The chemical and serological properties of lipopolysaccharides isolated from the S form and from the R form of cholera vibrios were compared. It was found that the S–R mutation of cholera vibrios involves total elimination of the two component amino sugars of S-form lipopolysaccharides, i.e. quinovosamine and perosamine. This elimination resulted in the loss of O-specificity of S-form lipopolysaccharides and concomitant appearance of strong serological cross-reactivity, in the passive-haemolysis-inhibition test, among R-form lipopolysaccharides regardless of the serotypes (Inaba and Ogawa) of their S parent strains.

Despite extensive investigation on the endotoxic lipopolysaccharides of the Enterobacteriaceae, there have been relatively few chemical studies on *Vibrio cholerae* (Wilkinson, 1977).

Serological properties of R antigen (somatic antigen of R mutants) of *Vibrio cholerae* were investigated by Shimada & Sakazaki (1973) by using agglutination and agglutinin-absorption tests.

In the present work, a comparative study of the chemical composition of lipopolysaccharides isolated from the R mutants and from the S parent strains of *V. cholerae* was carried out. In addition, passive-haemolysis-inhibition analysis with both R-form and S-form lipopolysaccharides was performed in order to study the serological relationship among these lipopolysaccharides.

It was found that the mutation of the S form of *V. cholerae* to the R form involves total elimination of the two component amino sugars of S-form lipopolysaccharides, i.e. quinovosamine (2-amino-2,6-dideoxy-D-glucose) (Jann *et al.*, 1973) and perosamine (4-amino-4,6-dideoxy-D-mannose) (Redmond, 1975). This elimination resulted in the loss of O-specificity of S-form lipopolysaccharides and concomitant appearance of strong cross-reactivity among R-form lipopolysaccharides regardless of the serotypes (Inaba and Ogawa) of their S parent strains.

Materials and Methods

The R mutants (35A3-R, NIH 41-R and P1418-R), isolated from their S parent strains *V. cholerae* 35A3 (Inaba), NIH 41 (Ogawa) and P1418 (Ogawa) respectively, were provided by courtesy of Dr. T. Shimada and Dr. R. Sakazaki (Shimada & Sakazaki, 1973), the First Department of Bacteriology, National Institute of Health, Tokyo, Japan.

The vibrios were grown in broth medium (pH 8.0) under vigorous shaking at 37°C for 16h, and killed, before being harvested, with phenol at a concentration of 0.5% (w/v) at room temperature for 2h. S-form lipopolysaccharides were isolated from the acetone-dried cells by means of the phenol/water technique of Westphal *et al.* (1952). For the isolation of R-form lipopolysaccharides from the R mutants, the phenol/chloroform/light petroleum method of Galanos *et al.* (1969) was employed. Both S-form and R-form lipopolysaccharide preparations were highly purified by repeated ultracentrifugation (at 105000g) and treatment with ribonuclease (20μg/ml) in 25mM-Tris/HCl buffer, pH 7.4, by the method of Fensom & Gray (1969), with a minor modification.

Neutral sugars except fructose were determined, after hydrolysis in 2M-trifluoroacetic acid at 120°C for 1h, as alditol acetates by g.l.c. on a column (3mm×2m) of 3% ECNSS-M coated on Chromosorb W by the procedure of Laine *et al.* (1974). Fructose was determined, after hydrolysis in 0.2M-acetic acid at 100°C for 8h, as described by Jann *et al.* (1973). Amino sugars were determined either with a JEOL JLC 6AS amino acid autoanalyser after hydrolysis in 4M-HCl at 100°C for 8h or by g.l.c. on a column (3mm×2m) of TABSORB (Regis Chemical Co., Chicago, IL, U.S.A.) as *N*-acetylhexosaminitol acetates after fractionation of the hydrolysates on a Dowex 50 (X8) column by the procedure of Wheat (1966). Quinovosamine present in lipopolysaccharides from *Pseudomonas aeruginosa* P14 (Suzuki, 1974) and perosamine from *V. cholerae* 569B (Inaba) (Redmond, 1975) were used as standards. In the determination of perosamine, samples were hydrolysed in 10M-HCl at 90°C for 15min by the method of Redmond (1978). 2-Keto-3-deoxyoctonate and heptose were determined by the

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method of Osborn (1963), reducing-sugar activity, amino sugars and total P were determined as described by Hisatsune *et al.* (1967), carbohydrate was measured with phenol/H_2SO_4 reagent by the method of Dubois *et al.* (1956), and protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. 4-Aminoarabinose was isolated from *Salmonella minnesota* R595 as described by Redmond (1979) and purified by using Sephadex G-10 gel filtration and Dowex 50 and Dowex 1 ion-exchange chromatography. This amino sugar was also prepared chemically from mannosamine as described by Volk *et al.* (1970).

Antisera against the S and R forms of *V. cholerae* were obtained from rabbits essentially as described by Shimada & Sakazaki (1973). The passive-haemolysis-inhibition tests were carried out as described by Hisatsune *et al.* (1978).

Results and Discussion

The overall chemical compositions of S-form and R-form lipopolysaccharides of *V. cholerae* 35A3 (Inaba) were respectively as follows (% of total dry wt.): protein, 2.6 and 0.9%; total carbohydrate, 31.3 and 34.2%; reducing sugar (including amino sugar), 17.5 and 23.0%; amino sugar, 12.0 and 11.7%; total P, 1.9 and 3.9%; total lipid, 22.5 and 33.0%, total recovery, 70.3 and 72.9%. Comparable results were also obtained for S-form and R-form lipopolysaccharides of *V. cholerae* NIH 41 (Ogawa). R-form lipopolysaccharides of both cholera vibrios contained markedly higher amounts of total lipid (33.0–38.5%) than were found (19.9–22.5%) in S-form lipopolysaccharides.

The results of sugar composition analysis are presented in Table 1. It was noticed that both 2-keto-3-deoxyoctonate and galactose, which are usually present in the core region of Gram-negative bacterial lipopolysaccharides (Lüderitz *et al.*, 1968), were totally absent from all the lipopolysaccharides tested. The lack of 2-keto-3-deoxyoctonate and galactose, also found in the present study with lipopolysaccharides from *V. cholerae* HIN 90 (Ogawa) and P1418 (Ogawa), confirmed the earlier report by Jackson & Redmond (1971). Quantitatively analogous results were obtained for the neutral-sugar compositions of S-form and R-form lipopolysaccharides of *V. cholerae* 35A3 (Inaba) and NIH 41 (Ogawa). However, a striking difference was observed in the amino sugar compositions of S-form and R-form lipopolysaccharides. Glucosamine, quinovosamine and perosamine were present in S-form lipopolysaccharides of both *V. cholerae* 35A3 (Inaba) and NIH 41 (Ogawa); in contrast, both quinovosamine and perosamine were totally missing from R-form lipopolysaccharides.

Jann *et al.* (1973) have already demonstrated the occurrence of quinovosamine in lipopolysaccharides from *V. cholerae* 569B (Inaba) and NIH 41 (Ogawa). We have further confirmed that quinovosamine is also present in the lipopolysaccharides of *V. cholerae* 35A3 (Inaba), NIH 90 (Ogawa) and P1418 (Ogawa).

The occurrence of perosamine in *V. cholerae* lipopolysaccharides was reported by Redmond (1978) with *V. cholerae* 569B (Inaba). We have observed that perosamine is a component amino sugar in S-form lipopolysaccharides of not only *V. cholerae* 569B (Inaba) but also *V. cholerae* NIH 90 (Ogawa) and P1418 (Ogawa). Neither quinovosamine nor perosamine could be detected in lipopolysaccharides isolated from the 'NAG vibrio' (Hisatsune *et al.*, 1979). These strains, which are not agglutinable with diagnostic antisera capable of recognizing the classical and El Tor cholera vibrios, are nevertheless now classified as *V. cholerae* (Sakazaki *et al.*, 1967, 1970). The results were compatible with the interpretation that both quinovosamine and perosamine are ubiquitous sugar components of lipopolysaccharides isolated from the S form of the cholera vibrios (classical and El Tor), i.e. *V. cholerae*

Table 1. *Sugar compositions of Vibrio cholerae lipopolysaccharides*
Experimental details are given in the text.

	Composition (%, w/w)			
Strain ...	35A3 (Inaba)	NIH 41 (Ogawa)	35A3-R	NIH 41-R
Glucose	5.4	3.5	8.0	9.6
Mannose	–	–	–	–
Galactose	–	–	–	–
Fructose	4.0	4.4	3.8	7.0
Heptose (L-*glycero*-D-*manno*-heptose)	10.0	8.5	15.5	15.8
Glucosamine	5.4	4.9	7.3	8.7
Quinovosamine	+	+	–	–
Perosamine	+	+	–	–

of the O1 group described by Gardner & Venkatraman (1935) and Sakazaki *et al.* (1970).

Perosamine was clearly distinguished from both quinovosamine and glucosamine by its R_F in t.l.c. and colour reaction with ninhydrin on the t.l.c. chromatogram (Table 2). In the present work the behaviour of quinovosamine and perosamine in g.l.c. was studied for the first time (Table 2). Hydrolysis in 4M-HCl at 100°C for 8h released quinovosamine and also considerable amounts of perosamine, whereas only a trace amount of quinovosamine was released after hydrolysis in 10M-HCl at 90°C for 15min, the condition of analysis for perosamine.

Redmond (1978) reported that lipopolysaccharides from representatives of Inaba serotype strains contain D-perosamine, whereas these of the Ogawa strains have 4-amino-4-deoxy-L-arabinose as an additional component. This was based on results obtained in t.l.c. of the hydrolysates (10M-HCl, 90°C, 15min) of the lipopolysaccharides. In the present study, 4-amino-4-deoxy-L-arabinose was isolated from *S. minnesota* R595 and also prepared chemically from mannosamine to study its behaviour in chromatography and paper electrophoresis (Table 2). Our results were rather different from those obtained by Redmond (1979); for instance, the R_F value (0.29) of the spot of 4-amino-4-deoxy-L-arabinose in t.l.c. [on Avicel (Funakoshi Chemical Co., Tokyo, Japan) with formic acid/butanone/2-methylpropan-2-ol/water (3:5:7:5, by vol.) as solvent] was low compared with that (0.76) reported by Redmond (1979), and in contrast the spot of perosamine moved much faster than that of 4-amino-4-deoxy-L-arabinose in our experiment. Both t.l.c. and g.l.c. failed to demonstrate the presence of 4-amino-4-deoxy-L-arabinose in lipopolysaccharides from not only the Inaba serotype strains (*V. cholerae* 569B and 35A3) but also the Ogawa serotype strains (*V. cholerae* NIH 41, NIH 90 and P1418).

The results of the passive-haemolysis-inhibition analysis are shown in Table 3. Ogawa S-form lipopolysaccharides exerted a strong inhibition in the Ogawa (homologous) system; the concentration producing 50% inhibition (ID_{50}) was 0.16μg/ml.

Table 2. *Chromatographic behaviour of component amino sugars of Vibrio cholerae lipopolysaccharides*
Experimental details are given in the text. Key to systems: A, formic acid/butan-2-one/2-methylpropan-2-ol/water (3:5:7:5, by vol.) solvent; B, butanol/acetic acid/water (5:1:2, by vol.) solvent; C, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.) solvent; D, pyridine/acetic acid/water (5:2:43, by vol.) solvent; E, pyridine/formic acid/acetic acid/water (2:3:20:180, by vol.) solvent; F, TABSORB column, at 160–210°C, 2°C/min (acetylated glucosaminitol = 1.00); G, 3% ECNSS-M column, at 190°C (acetylated sorbitol = 1.00). N.T., Not tested.

	T.l.c. (R_{GlcN})			High-voltage paper electrophoresis (R_{GlcN})		G.l.c.		
						(R_{GlcN})	(R_{Sor})	Ninhydrin
System ...	A	B	C	D	E	F	G	reaction
Glucosamine	1.00	1.00	1.00	1.00	1.00	1.00	N.T.	+
Quinovosamine	1.43	1.70	1.83	1.04	1.06	0.59	1.41	+
Perosamine	1.13	1.37	1.46	1.05	1.10	0.57	1.37	−
4-Amino-4-deoxy-L-arabinose from *S. minnesota* R595 lipopolysaccharide	0.98	0.85	0.98	1.09	1.16	0.74	2.89	−
4-Amino-4-deoxy-L-arabinose from mannosamine	0.98	0.85	0.98	1.09	1.16	0.74	2.83	−

Table 3. *ID_{50} values for Vibrio cholerae lipopolysaccharides in the passive-haemolysis inhibition system*
Experimental details are given in the text. Alkali-treated lipopolysaccharides were tested.

	ID_{50} (μg/ml)			
	NIH 41 (Ogawa) lipopolysaccharide/anti-(NIH 41) serum	35A3 (Inaba) lipopolysaccharide/anti-(35A3) serum	NIH 41-R lipopolysaccharide/anti-(NIH 41-R) serum	35A3-R lipopolysaccharide/anti-(35A3-R) serum
S form				
NIH 41 (Ogawa)	0.16	0.50	45	62
35A3 (Inaba)	>1000	0.33	250	150
R form				
NIH 41-R	10	—	0.46	0.23
35A3-R	—	—	0.40	0.26

Ogawa S-form lipopolysaccharide was also found to exert a strong inhibition in the Inaba (heterologous) system (ID_{50} 0.50 μg/ml) to almost the same extent as did it in the Ogawa (homologous) system, supporting the presence of the group-specific determinant factor A and a small quantity of the Inaba serotype factor (c) in the O-antigenic structure of Ogawa lipopolysaccharides.

In contrast with the inhibition by Ogawa S-form lipopolysaccharides in both the homologous and heterologous systems, the type-specificity to Inaba serotype was clearly observed in the inhibition with Inaba S-form lipopolysaccharides in the immune system. Inaba S-form lipopolysaccharides exerted a strong inhibition in the Inaba (homologous) system (ID_{50} 0.33 μg/ml), but Inaba S-form lipopolysaccharides exerted such a weak inhibition in the Ogawa (heterologous) system ($ID_{50} > 1000$ μg/ml) that more than 3000-fold larger quantities of the Inaba S-form lipopolysaccharides were needed in the Ogawa system than in the Inaba (homologous) system for 50% inhibition. These observations support the conventional concept for the O-antigenic structure of *V. cholerae*, in which the Inaba serotype determinants do not share the antigenic determinant factor B, which is thought to be present in the O-antigenic structure of the Ogawa serotype (Finkelstein, 1973; Shimada & Sakazaki, 1973).

A strong and equivalent inhibition (50%-inhibition doses ranged from 0.23 to 0.46 μg/ml) was exerted by either R-form lipopolysaccharides from strains 35A3-R or NIH 41-R in both homologous and heterologous systems, i.e. 35A3-R lipopolysaccharide/anti-(35A3-R) system and NIH 41-R lipopolysaccharide/anti-(NIH 41-R) system. Thus a strong serological cross-reactivity was observed between R form lipopolysaccharides from strains 35A3-R and NIH 41-R. This strong cross-reactivity is observed widely among R-form lipopolysaccharides from not only these two strains but also from strains P1418 and CA385 (the latter an R mutant isolated from an Ogawa serotype *V. cholerae* by R. F. Finkelstein) (Shimada & Sakazaki, 1973). These results are in good agreement with those obtained by both agglutination and agglutinin-absorption analysis carried out by Sakazaki *et al.* (1970) of the R antigen (heat-killed whole cells) of *V. cholerae*, where it was found that the R antigens of all 46 strains tested were identical. In contrast, however, very weak inhibition was exerted by R-form lipopolysaccharides in the S-form lipopolysaccharide/anti-S-form system regardless of whether the systems were homologous or heterologous. This observation was also made in the reversed systems, i.e. the inhibition by S-form lipopolysaccharides in the R-form lipopolysaccharide/anti-R-form systems. This strongly suggests that there is no cross-reactivity between R-form lipopolysaccharides from the R form and S-form lipopolysaccharides from the parental S form of *V. cholerae*, at least in this passive-haemolysis-inhibition system.

Two recent publications threw new light on the problem of the structure of *V. cholerae* lipopolysaccharides (Kenne *et al.*, 1979; Redmond, 1979). On the basis of an n.m.r. study it was proposed that the O-antigenic polysaccharide part of *V. cholerae* lipopolysaccharides consists of a linear α-(1→2)-linked homopolymer of D-perosamine with *N*-acylation. We have shown in the present study that S–R mutation of cholera vibrios involves the total elimination of the amino sugar components quinovosamine and perosamine from S-form lipopolysaccharides regardless of the serotype of their S parent strains. Our results are compatible with the interpretation that perosamine present in S-form lipopolysaccharides is involved in the O1 specificity of S-form lipopolysaccharides of cholera vibrios, thus supporting the above proposal by Kenne *et al.* (1979) and Redmond (1979) for the chemical structure of the O-antigenic polysaccharide of *V. cholerae* lipopolysaccharides.

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