O-Antigenic Lipopolysaccharides Isolated from a Marine *Vibrio*, Bio-serogroup 1875, Possessing an Antigenic Factor in Common with O1 *Vibrio cholerae*

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The chemical and serological characteristics of lipopolysaccharides (LPS) isolated from *Vibrio* bio-serogroup 1875 were compared with those of O1 *Vibrio cholerae* LPS. *Vibrio* bio-serogroup 1875 LPS contained all the component sugars which were found in O1 *V. cholerae* LPS, i.e. glucose, L-glycero-D-manno-heptose, fructose, glucosamine, perosamine and quinovosamine, though the amount of perosamine, a characteristic component of O1 *V. cholerae* LPS, was very low compared with that of O1 *V. cholerae* LPS. Their LPS additionally contained mannose and two unidentified neutral sugars which are not regular constituents of O1 *V. cholerae* LPS. Definite serological cross-reactivity in the passive haemolysis test between LPS from *Vibrio* bio-serogroup 1875 and LPS from O1 *V. cholerae* was demonstrated.

INTRODUCTION

O1 group *Vibrio cholerae* is divided into two major serological types, Ogawa and Inaba, on the basis of their heat-stable somatic antigen (or O-antigen). These two serotypes share group somatic antigen A, but can be separated by antigens B (Ogawa factor) and C (Inaba factor) (Sakazaki & Tamura, 1971; Finkelstein, 1973). In 1982, a marine vibrio which was agglutinated by O1 *V. cholerae* antisera and Ogawa factor antisera was isolated from estuarine sea-water in Osaka Bay. The biochemical characteristics of strains of this group, referred to as *Vibrio* bio-serogroup 1875, were reported by Shimada *et al.* (1987). The strains of this group are phenotypically different from O1 *V. cholerae* because, for example, they are halophilic and, in contrast to the O1 group, are both lysine decarboxylase and ornithine decarboxylase negative but arginine dehydroxylase positive. In addition, these authors showed that this group can be completely distinguished from the O1 group by DNA homology. Furthermore, they demonstrated by cross-agglutination and agglutinin-absorption tests that the group's somatic antigen is composed of at least three factors: its own antigenic factor (referred to as D), and B and C factors of O1 *V. cholerae*. An antigenic variation similar to that found in O1 *V. cholerae* was observed in bio-serogroup 1875. The original antigenic forms of strains of this group contained B factor and a small quantity of C factor, whereas the variant (referred to a bio-serogroup 1875 Variant) possessed only C factor (Shimada *et al.*, 1987). This was the first time such a group of marine vibrio possessing an antigenic factor in common with O1 *V. cholerae* had been isolated.

In the present study, the chemical and serological properties of O-antigenic lipopolysaccharides (LPS) of *Vibrio* bio-serogroup 1875 Original and Variant were compared with LPS isolated from O1 *V. cholerae* Ogawa and Inaba strains.

METHODS

Bacteria and growth conditions. Strains of *Vibrio* bio-serogroup 1875 were provided by Drs T. Shimada and R. Sakazaki, National Institute of Health, Tokyo, Japan. *V. cholerae* P1418 (Ogawa) and its Inaba mutant UV601

Abbreviations: DEGS, diethylene glycol aditate; DPS, degraded polysaccharide; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonic acid); PCP, phenol/chloroform/petroleum ether; SRBC, sheep red blood cells.

were obtained from Dr M. Ohashi, Tokyo Metropolitan Research Laboratory for Public Health. *V. cholerae* NIH41 and 569B were provided by Dr N. Ohtomo, Chemo-Sero-Therapeutic Institute, Kumamoto, Japan. The bacteria were cultured for 16 h at 28 °C in nutrient broth (Difco) (pH 7.4) containing 3% (w/v) NaCl. After being killed by heating at 120 °C for 20 min, the cells were harvested and washed with distilled water by centrifugation, and then acetone dried.

Preparation of LPS. The acetone-dried cells were first extracted by Galanos's phenol/chloroform/petroleum ether (PCP) method (Galanos *et al.*, 1969), which is generally used for extraction of LPS or glycolipid from R-form Gram-negative bacteria. The cells remaining after PCP extraction were then extracted by Westphal's phenol/water method (Westphal *et al.*, 1952) for extraction of LPS from S-form Gram-negative bacteria. LPS were purified by repeated ultracentrifugation and RNAase treatment (Westphal & Jann, 1966), and then lyophilized.

Analytical methods. Neutral and amino sugars were analysed by gas-liquid chromatography (GLC) as described by Hisatsune *et al.* (1985). Fatty acids released by heating in 4 M-HCl at 100 °C for 4 h and then in 1 M-NaOH at 100 °C for 2 h (Haeffner *et al.*, 1977) were esterified with diazomethane (Rietschel *et al.*, 1972) and then analysed by GLC (column: 25% DEGS, 3 mm × 2 m). Methyl-nonadecanoate was used as an internal standard. Neutral and amino sugars and fatty acids were identified by their retention times in GLC analysis and mass spectra in GLC/MS analysis compared with those of authentic standards. GLC/MS spectrometry was performed on a JEOL DX-300 instrument with the same column as described above.

Heptose was determined either by the method of Osborn (1963) or by GLC. Standard L-glycero- and D-glycero-D-mannoheptose were prepared as described by Bagdian *et al.* (1966). 2-Keto-3-deoxyoctonate (KDO) was estimated by the conventional Weissbach periodate/thiobarbituric acid reaction under conventional hydrolysis conditions (0.01 M-H₂SO₄ at 100 °C, 20 min) (Weissbach & Hurwitz, 1959). KDO (ammonium salt) used as a standard and alkaline phosphatase (type III-R) were purchased from Sigma.

High-voltage paper electrophoresis was done on Whatman no. 1 paper with a solvent system of pyridine/acetic acid/water (10:4:86, by vol.), pH 5.3, at 45 V cm⁻¹ for 40 min. On the paper electrophoregrams, KDO and 5-O-phosphoryl-KDO were detected with a thiobarbituric acid reagent (Warren, 1960).

Gel-chromatography of degraded polysaccharide (DPS) was done with a Sephadex G-50 column (1.6 × 100 cm) using pyridine/acetic acid/water (10:4:1000, by vol.) as eluent, at a flow rate of 13 ml h⁻¹.

Isolation of the unknown substance X, positive in the periodate/thiobarbituric acid reaction. LPS (50 mg) were heated in 4 M-HCl (20 ml) at 100 °C for 45 min, then hydrolysates were rotary-evaporated to dryness under reduced pressure. The residues were dissolved in distilled water and passed through a Dowex 50 (H⁺ form) column (1.5 × 20 cm), then the eluates together with the water washings were applied to a Dowex 1 (acetate form) column (1.5 × 20 cm). After being washed with distilled water, the column was eluted with 2 M-HCl and the eluates which were positive for periodate/thiobarbituric acid test were collected and rotary-evaporated to dryness under reduced pressure to isolate the unknown substance X.

Antisera and serological assay. Antisera against bio-serogroup 1875 Original and Variant, and O1 *V. cholerae* Ogawa and Inaba strains were prepared by immunizing rabbits with heat-killed whole cells of each strain. Factor A, B and C sera against the somatic antigen factors of O1 *V. cholerae*, and factor D serum against that of bio-serogroup 1875, were provided by Drs T. Shimada and R. Sakazaki. Monoclonal antibodies against factors A, B and C were purchased from Denka Seiken Co. Passive haemolysis and passive haemolysis inhibition tests were done as described by Hisatsune *et al.* (1978).

RESULTS AND DISCUSSION

Extraction of LPS

The yield of LPS obtained by Westphal's phenol/water method from either 1875 Original or 1875 Variant cells was 0.5% of the cell dry weight. The overall chemical composition of LPS isolated from bio-serogroup 1875 was not very different from that of the usual Gram-negative bacterial LPS. A marked difference between this group and O1 *V. cholerae* LPS was observed in the extraction product, however: a significant amount of an unknown substance was released from cells of 1875 by Galanos's PCP extraction, whereas no such product was obtained from O1 *V. cholerae*. This unknown fraction is referred to tentatively as NL fraction.

Neutral and amino sugar composition of LPS

The results of sugar analysis of LPS of bio-serogroup 1875 are presented in Table 1 in comparison with O1 *V. cholerae* LPS. Both Original and Variant LPS contained all the component sugars present in LPS of O1 *V. cholerae*, i.e. glucose, fructose, L-glycero-D-mannoheptose, glucosamine, perosamine and quinovosamine; the latter two amino sugars were shown by Kondo *et al.* (1988) to be characteristic sugar components of O1 *V. cholerae*. Mannose and

Table 1. *Sugar composition (molar ratios) of LPS isolated from V. cholerae and Vibrio bio-serogroup 1875*

<i>Vibrio</i> LPS	Glucose	Mannose	Fructose	Unknown NS*		L-Glycero-D-manno-heptose†	KDO	Glucos-amine	Peros-amine	Quinovos-amine
				I	II					
P1418 (Ogawa)	1.0	—	0.8	—	—	3.0	ND	1.6	8.4	0.6
P1418-UV601 (Inaba)	0.9	—	0.8	—	—	3.0	ND	1.4	12.0	1.1
1875 Original	1.6	0.1	1.9	+	+	3.0	ND	2.0	1.2	0.4
1875 Variant	1.9	0.2	2.1	+	+	3.0	ND	2.0	1.1	0.4

ND, not detectable by Weissbach's periodate/thiobarbituric acid test.

* Unknown NS, unknown neutral sugar.

† Molar ratios were calculated on the assumption that each fraction contained 3 moles of L-glycero-D-manno-heptose.

two unknown neutral sugars, NSI and NSII, were found as additional component sugars in bio-serogroup 1875. We speculate that these three sugars, which are not found in O1 *V. cholerae*, are associated with the common antigenic factor 'D' that was reported by Shimada *et al.* (1987) to be shared by the 1875 Original and 1875 Variant forms like the common antigenic factor A in O1 *V. cholerae*.

Apparent undetectability of KDO by conventional colour test under conventional hydrolysis conditions and the presence of O-phosphorylated KDO in strong-acid hydrolysates

KDO, a regular component sugar of the usual Gram-negative bacterial LPS, was not detected in LPS of bio-serogroup 1875 by the conventional Weissbach periodate/thiobarbituric acid test (Weissbach & Hurwitz, 1959) under conventional hydrolysis conditions. However, treatment (100 °C, 45 min) of LPS with strong acid (4 M-HCl) released a Weissbach-reaction-positive substance X, yielding a colour with maximal absorption at 549 nm, which is identical to that yielded by standard KDO. However, X was not identical to KDO with respect to stability in strong acid hydrolysis and behaviour in high-voltage paper electrophoresis. Free-form KDO was rapidly decomposed by this strong acid treatment, and the R_{KDO} (mobility relative to KDO) value of X was 1.54. Furthermore, in high-voltage paper electrophoresis, X was found to be identical to X1, one of the two Weissbach-reaction-positive substances, X1 and X2 (respective R_{KDO} values 1.54 and 1.80), isolated from O1 *V. cholerae* LPS after the same treatment with strong acid (Kondo *et al.*, 1988). X was also found to be identical, in the paper electrophoresis, to 5-O-phosphoryl-KDO isolated from *Bordetella pertussis* LPS after the same treatment according to Chaby & Szabo (1975). Conversely, no Weissbach-reaction-positive substance corresponding to X2 of O1 *V. cholerae* was found in the strong-acid hydrolysates of bio-serogroup 1875 LPS, although they contained the pair perosamine and quinovosamine that is characteristic of O1 *V. cholerae* LPS (Kondo *et al.*, 1988).

The strong-acid hydrolysates of these LPS were treated with alkaline phosphatase, and then subjected to high-voltage paper electrophoresis. The respective spots corresponding to X, X1 and X2, and 5-O-phosphoryl-KDO from *B. pertussis*, disappeared, and concomitantly a spot which had the same mobility as that of standard KDO appeared. These findings indicate that X, X1 and X2 are some kind of phosphorylated derivatives of KDO, and that, as far as the results of high-voltage paper electrophoresis show, either X or X1 is identical to 5-O-phosphorylated KDO.

Gel filtration of degraded polysaccharide

The distribution of the component sugars between the core and O-specific side-chain regions of LPS can often be determined by fractionating, by gel filtration, 'degraded polysaccharide (DPS)' isolated from LPS by mild acid hydrolysis (Wilkinson, 1977). As shown in Fig. 1, the elution profile for bio-serogroup 1875 DPS was almost the same as that of O1 *V. cholerae* DPS (Hisatsune *et al.*, 1985), where three distinct peaks were eluted. The first peak (fraction II)

Fig. 1. Sephadex G-50 gel-chromatography of DPS fraction isolated from *Vibrio* bio-serogroup 1875 LPS. Total carbohydrate (○) was measured by the method of Dubois *et al.* (1956). Total phosphorus (●) was determined as described by Hisatsune *et al.* (1967). ----, Refractive index detector recorder response.

Table 2. *Sugar composition (molar ratios) of DPS fractions separated by Sephadex G-50 gel-chromatography from O1 V. cholerae and Vibrio bio-serogroup 1875*

DPS fraction*	Glucose	Mannose	Fructose	Unknown NS†		L-Glycero- D-manno- heptose‡	Glucos- amine	Peros- amine	Quinovos- amine
				I	II				
Fraction II									
P1418 (Ogawa)	0.9	—	—	—	—	3.0	—	18.0	1.7
P1418-UV601 (Inaba)	0.7	—	—	—	—	3.0	—	18.0	1.7
1875 Original	1.2	0.7	—	+	+	3.0	1.0	3.2	1.3
1875 Variant	1.2	0.7	—	+	+	3.0	1.0	3.2	1.3
Fraction III									
P1418 (Ogawa)	0.5	—	—	—	—	3.0	0.1	—	—
P1418-UV601 (Inaba)	0.9	—	—	—	—	3.0	0.1	—	—
1875 Original	1.2	—	—	—	—	3.0	1.2	—	—
1875 Variant	1.2	—	—	—	—	3.0	1.2	—	—
Fraction IV									
P1418 (Ogawa)	—	—	+++	—	—	—	—	—	—
P1418-UV601 (Inaba)	—	—	+++	—	—	—	—	—	—
1875 Original	—	—	+++	—	—	—	—	—	—
1875 Variant	—	—	+++	—	—	—	—	—	—

* See Fig. 1.
† Unknown NS, unknown neutral sugar.
‡ See footnote † of Table 1.

corresponds to the polymeric O-specific side-chain with attached core, the second peak (fraction III) to core oligosaccharide and the third peak (fraction IV) to the monosaccharide fraction that contains fructose instead of KDO.

The results of compositional analysis of the three fractions obtained from bio-serogroup 1875 DPS are presented in Table 2 in comparison with those of O1 *V. cholerae* P1418 Ogawa and its Inaba mutant. The data represent the molar ratios of the component sugars found in each fraction, the values being calculated on the assumption that each fraction contains 3 moles of L-glycero-D-mannoheptose. Fraction III contained glucose, glucosamine and heptose in molar ratios 1:1:3, while fraction II contained the two unknown neutral sugars, NSI and NSII, and mannose, perosamine and quinovosamine in molar ratios of 1:3:1 as the additional components. These results indicate that the core region of bio-serogroup 1875 LPS consists of 1 mole of glucose, 1 mole of glucosamine and 3 moles of heptose, in contrast to P1418 LPS core,

Table 3. Fifty-percent inhibition doses ($\mu\text{g ml}^{-1}$) of LPS isolated from *V. cholerae* NIH41 (Ogawa), *V. cholerae* 569B (Inaba), and bio-serogroup 1875 Original and Variant in the passive haemolysis inhibition test

Inhibitor	Passive haemolysis system (antigen/antiserum)			
	NIH41 LPS/ anti-NIH41	569B LPS/ anti-569B	Original LPS/ anti-Original	Variant LPS/ anti-Variant
<i>V. cholerae</i> NIH41 (Ogawa) LPS	0.19	0.65	—	—
<i>V. cholerae</i> 569B (Inaba) LPS	730	0.18	—	—
<i>Vibrio</i> bio-serogroup 1875 Original LPS	0.09	280	0.03	1.60
<i>Vibrio</i> bio-serogroup 1875 Variant LPS	24.0	220	6.80	0.03

—, More than 1000 $\mu\text{g ml}^{-1}$.

where glucosamine is almost absent (Hisatsune *et al.*, 1985). It was also indicated that the O-specific side-chain of LPS of this group contains 1 mole of mannose and unknown sugars, NSI and NSII, in addition to 1 mole of quinovosamine and a perosamine-homopolymer consisting of three repeating units. On the other hand, the present results indicate that, although the perosamine-homopolymer found in O1 *V. cholerae* LPS is also present in the LPS of bio-serogroup 1875, its chain is very short compared with that of the same homopolymer present in O1 *V. cholerae* (P1418) LPS, which consists of 18 repeating units (Hisatsune *et al.*, 1985).

Serological properties

LPS from bio-serogroup 1875 and O1 group *V. cholerae* were examined for serological cross-reactivity by passive haemolysis and passive haemolysis inhibition analysis using LPS. The passive haemolysis systems consisted of sheep red blood cells (SRBC) sensitized with 1875 Original LPS, guinea-pig complement and various anti-whole-cell sera. High haemolytic titres were observed with anti-Original, anti-Variant and anti-NIH41 Ogawa sera, the 50% haemolysis titres being $\times 6100$, $\times 2400$ and $\times 11800$, respectively. A lower titre was observed with anti-569B Inaba serum ($< \times 200$). With SRBC sensitized with 1875 Variant LPS, high haemolytic titres were observed with anti-569B Inaba ($\times 12600$), anti-Variant ($\times 8900$) and anti-Original ($\times 8000$) sera, while a very low titre was observed with anti-Ogawa serum ($< \times 200$). Thus, definite serological cross-reactivity in passive haemolysis between LPS from 1875 and O1 *V. cholerae* was demonstrated.

Factor A serum exhibited a high titre in the passive haemolysis test against SRBC coated with either NIH41 Ogawa or 569B Inaba LPS (50% haemolysis titres $\times 5600$ and $\times 10300$, respectively). It was notable that factor A serum did not exhibit a significant titre against either 1875 Original or 1875 Variant LPS ($\times 300$ and $\times 280$, respectively). Factor B serum exhibited a high titre against both Ogawa LPS ($\times 2800$) and 1875 Original LPS ($\times 1700$) but not against Inaba LPS ($< \times 200$) or 1875 Variant LPS ($< \times 200$). On the other hand, factor D serum exhibited a high titre against both 1875 Original and 1875 Variant LPS ($\times 2000$ and $\times 2200$, respectively) but not against Ogawa LPS ($< \times 200$) or Inaba LPS ($< \times 200$). These results obtained by the passive haemolysis test using factor sera were consistent with the O-antigenic structure of O1 *V. cholerae* (Sakazaki & Tamura, 1971) and bio-serogroup 1875 (Shimada *et al.*, 1987). However, it was of particular interest that factor C serum did not exhibit a significant passive haemolysis titre against either Ogawa or Inaba LPS ($\times 280$ and $\times 220$, respectively). Furthermore, monoclonal antibody against Inaba factor C also failed to react with SRBC coated with LPS of Ogawa, Inaba, 1875 Original or 1875 Variant.

Table 3 presents the results of the passive haemolysis inhibition analysis. Four homologous passive haemolysis systems, Ogawa/anti-Ogawa, Inaba/anti-Inaba, Original/anti-Original and Variant/anti-Variant, were inhibited when either homologous or heterologous LPS was used as inhibitor. Ogawa LPS exerted strong inhibition in both Ogawa/anti-Ogawa and Inaba/anti-Inaba systems to the same extent, while Inaba LPS exerted strong inhibition in the homologous Inaba/anti-Ogawa system, but very weak inhibition in the heterologous Ogawa/anti-Ogawa

system. These results are consistent with our previous data on the serological activity of O1 *V. cholerae* LPS (Hisatsune & Kondo, 1980), which can be explained reasonably by the generally accepted ABC concept of the O-antigenic structure of O1 *V. cholerae* obtained by whole-cell cross-agglutination and agglutinin-absorption tests. On the other hand, both 1875 Original and 1875 Variant LPS exerted strong inhibition only in their homologous systems, Original/anti-Original and Variant/anti-Variant, although in their heterologous systems the inhibition was not very much less. Thus, in contrast to the passive haemolysis test, half cross-serological reactivity was demonstrated between group 1875 and O1 *V. cholerae* LPS in the passive haemolysis inhibition test.

It was observed that both Original and Variant cells evoked the production of high-titre vibriocidal antibody in rabbits.

In the present study, a significant amount of an unknown substance (NL) was separated from cells of bio-serogroup 1875 by Galanos's PCP extraction method. Although this fraction contained more than 50% lipid, it was entirely different from that of its homologous and Ogawa Lipid A; for example, only small amounts of 3-OH-C12:0 (2.0%) and 3-OH-C14:0 (1.3%) were present in the NL fraction. Analytical data indicated that NL is neither LPS nor glycolipid.

The only constituent present in group O1 LPS but absent from bio-serogroup 1875 LPS was X2. Therefore, it might be supposed to be related to common antigen factor A, if the assumption that O-antigenic factors A, B and C reside in the LPS of the cell-wall outer membrane was correct. It is unlikely, however, that X2 is factor A, because, as shown in our previous study (Kondo *et al.*, 1988), X2 is present in both O1 and non-O1 *V. cholerae*.

As described above, factor C serum did not have a significant antibody titre against either Ogawa or Inaba LPS. Furthermore, monoclonal antibody against Inaba factor C also failed to react with LPS of Ogawa, Inaba, 1875 Original and 1875 Variant. Further study is necessary to explain these unexpected serological results.

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