An immunochemical study of serological cross-reaction between lipopolysaccharides from *Vibrio cholerae* O22 and O139

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A comparative chemical and serological study of the LPS of *Vibrio cholerae* O139 and O22 was performed. Chemical analysis revealed that the sugar composition of the LPS of strain O22 was quite similar to that of O139 LPS. Each contained D-glucose, L-glycero-D-manno-heptose, colitose (3,6-dideoxy-L-galactose), D-fructose, D-glucosamine, D-quinovosamine and D-galacturonic acid. The O-antigenic relationship between the two strains was analysed by passive haemolysis (PH) and passive haemolysis inhibition (PHI) tests with the respective LPS being used as antigens to sensitize sheep red blood cells (SRBC) and, in the latter case, as inhibitors in a PH system that consisted of LPS-sensitized SRBC, guinea-pig complement and anti-O139 or anti-O22 antiserum, both unabsorbed and absorbed with the heterologous antigen. In the PH experiment, unabsorbed anti-O139 antiserum had haemolytic titres of 66 000 and 22 000 against O139 LPS- and O22 LPS-sensitized SRBC, respectively; unabsorbed anti-O22 antiserum had haemolytic titres of 900 and 13 000, respectively. Thus, the anti-O139 antiserum contained an antibody that reacted with a heterologous O22 antigen at a high titre (22 000) and this antibody was completely removed from anti-O139 antiserum with the O22 antigen. The anti-O22 antiserum contained an antibody that reacted with the heterologous O139 antigen at a low titre (900) and this antibody was completely removed from anti-O22 antiserum with the O139 antigen. In PHI tests O139 LPS and O22 LPS each strongly inhibited (the ID₅₀ of LPS ranged from 0.03 to 0.14 µg ml⁻¹) the heterologous haemolytic systems of both O139 LPS-sensitized SRBC/anti-O22 antiserum and O22 LPS-sensitized SRBC/anti-O139 antiserum, which are substantially equivalent to the common antigen factor in the O139 LPS-sensitized SRBC/anti-O22 antiserum system and the common antigen factor in the O22 LPS-sensitized SRBC/anti-O139 antiserum system, respectively. The results indicated that the O antigen of O139 is closely related to that of O22 in an a,b-a,c type of relationship where a is common antigenic factor, b is an O139-specific antigenic factor and c is an O22-specific antigenic factor.

Keywords: lipopolysaccharide, *Vibrio cholerae* O22, *Vibrio cholerae* O139

INTRODUCTION

The members of the species *Vibrio cholerae* are presently divided into two groups, namely O1 *V. cholerae* (cholera

vibrio) and non-O1 *V. cholerae* (NAG vibrio), on the basis of their respective O antigens (heat-stable somatic antigens). The first group is further subdivided into Ogawa and Inaba O forms, while the latter is divided into 144 or more O forms (Shimada *et al.*, 1994a). Historically, O1 *V. cholerae* has been regarded as the causative agent of the acute intestinal disease, cholera. However, the epidemic strain responsible for the recent fast-spreading cholera on the Indian subcontinent, which started in October 1992, was classified by Shimada *et al.* (1993) as *V. cholerae* O139

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Abbreviations: DPS, degraded polysaccharide; Kdo, 2-keto-3-deoxyoctonate; LOS, lipo-oligosaccharide; PH, passive haemolysis; PHI, passive haemolysis inhibition; SRBC, sheep red blood cells.

synonym Bengal. The O antigen of Gram-negative bacteria, including *V. cholerae*, resides in the LPS that is located in the outer membranes of cell walls. LPS is a heteropolysaccharide composed of three moieties: lipid A, which is the centre of its endotoxic activity; a core polysaccharide; and an O polysaccharide chain that determines the serological O-antigenic specificity of the parent cells from which the LPS is derived (Lüderitz *et al.*, 1971; Wilkinson, 1977). The chemical properties, in particular the sugar composition and molecular architecture, of LPS isolated from *V. cholerae* O139 were first reported by Hisatsune *et al.* (1993). It is of particular interest that the LPS contains colitose, which had not hitherto been found in *V. cholerae* or even in Vibrionaceae.

Recently, serological cross-reaction between *V. cholerae* O139 and O22 in agglutination and agglutinin absorption tests was reported (Shimada *et al.*, 1994b). In this study, a comparative chemical and serological analysis was performed with LPS isolated from *V. cholerae* O139 and O22. The results demonstrated that the major O antigen of the O139 strain is a common antigen shared by the two strains, while the major O antigen of the O22 strain is an O22-specific antigen.

METHODS

Bacterial strains and growth conditions. *V. cholerae* O22 (169-68, reference strain) and O139 (MO 45, reference strain) strains were used in this study. The microbes were cultured in nutrient broth (pH 7.4) at 37 °C for 16 h with aeration. Cells were harvested by centrifugation after they had been killed by heating at 120 °C for 20 min. They were then washed with water and acetone-dried. *V. cholerae* O1 NIH 41 (Ogawa) and 569B (Inaba) were provided by Dr N. Ohtomo, The Chemo-Sero Therapeutic Institute, Kumamoto, Japan, and *Salmonella typhimurium* LT2 was provided by Dr M. Yoshida, Department of Bacteriology, Iwate Medical School, Morioka, Japan. These strains were also cultured in nutrient broth and the cells were acetone-dried after being killed by heating as described above.

Preparation and chemical modification of LPS. LPS was extracted from the acetone-dried cells by the hot phenol/water method (Westphal *et al.*, 1952) and purified as described previously (Hisatsune *et al.*, 1993). LPS was hydrolysed by treatment with 5% (v/v) acetic acid at 100 °C for 2.5 h to yield a degraded polysaccharide (DPS) fraction that contained the polysaccharide portion of LPS. The DPS fraction was subjected to gel chromatography on a column (3.6 cm i.d. × 100 cm) of Sephadex G-50 (Pharmacia) which was eluted with distilled water and the effluent was monitored with a refractive index monitor (RID-6A, Shimadzu). The fractions corresponding to the polysaccharide portion of LPS were combined and lyophilized. Dephosphorylation and oxidation of LPS were carried out as described previously (Kondo *et al.*, 1991b) in 48% (v/v) HF at 4 °C for 48 h and in 0.025 M NaIO₄ at 0 °C for 120 h, respectively. Methylation analysis was performed by the method of Hakomori (1964) and the methylated material was purified on a Sep-Pak C18 cartridge (Waters).

Analytical methods. Neutral sugars with the exception of fructose and colitose were analysed by GC as alditol acetates after hydrolysis in 2 M trifluoroacetic acid at 120 °C for 1 h. Fructose and colitose were quantified by the previously reported method (Hisatsune *et al.*, 1993). Amino sugars were analysed by GC as *N*-acetylaminoalditol acetates after hydrolysis in 4 M HCl

at 100 °C for 16 h. 2-Keto-3-deoxyoctonate (Kdo) was estimated by Weissbach's periodate/thiobarbituric acid test (Weissbach & Hurwitz, 1959) and Kdo phosphate was detected by GC and GC/MS using the method of Kondo *et al.* (1991b). Galacturonic acid was identified by GC and GC/MS as galactose (6-2³H) that was prepared by methanolysis (1 M HCl in methanol at 86 °C, 2 h), carboxyl reduction (NaB²H₄ in H₂O at room temperature overnight), hydrolysis in 2 M trifluoroacetic acid at 120 °C for 1 h and *O*-acetylation. Uronic acid was quantified by the carbazole/sulfuric acid method (Bitter & Mürer, 1962). The absolute configuration of monosaccharides was determined by GC and GC/MS as their acetylated (S)-(+)- or (R)-(-)-2-butylglycosides as described by Gerwig *et al.* (1978). The GC retention times were compared with those of authentic standards. SDS-PAGE was performed as described by Tsai & Frasch (1982).

GC and GC/MS. GC was carried out on a model 14A gas chromatograph (Shimadzu) equipped with a fused silica capillary column that had been coated with HR52 (Shinwa Chemical Industries Ltd.) or DB210 (J&W Scientific). The temperature was programmed to remain at 180 °C for 3 min and then to increase to 240 °C at 4 °C min⁻¹ for the analysis of neutral and amino sugars (DB210 column), and to remain at 150 °C for 3 min and then to increase to 320 °C at 5 °C min⁻¹ for permethylated and partially methylated sugars (HR52 column). GC/MS was carried out on a model DX-300 system (JEOL) with an HR52 column. Electron impact mass spectra were recorded at 70 eV and isobutane was used as the reactant gas in chemical ionization MS.

Antisera and serological methods. Antisera against *V. cholerae* O139 and O22 were prepared by immunizing rabbits with heat-killed whole cells and antisera were absorbed with *V. cholerae* CA385 (R mutant of *V. cholerae* O1 as an R antigen) as reported previously (Shimada & Sakazaki, 1988). Passive haemolysis (PH) and passive haemolysis inhibition (PHI) tests were performed by the method of Hisatsune *et al.* (1978) using sheep red blood cells (SRBC) that had been sensitized with LPS. Anti-O139 antiserum absorbed with O22 cells and anti-O22 antiserum absorbed with O139 cells were used in absorption experiments. The absorbed antisera were obtained by the method of Sakazaki & Donovan (1984).

RESULTS AND DISCUSSION

Chemical properties of LPS

Sugar composition of LPS. Table 1 shows the sugar composition of LPS from *V. cholerae* O22 and its polysaccharide moieties that were fractionated by gel filtration chromatography on Sephadex G-50. For comparative purposes, as well as for convenience, the sugar composition of O139 LPS, published previously (Hisatsune *et al.*, 1993), is included in Table 1. The sugar composition of O22 LPS was quite similar to that of O139 LPS. Each LPS contained D-glucose, L-glycero-D-mannoheptose, colitose, D-fructose, D-glucosamine, D-quinovosamine and D-galacturonic acid. D-Galacturonic acid has not been formerly reported (Hisatsune *et al.*, 1993) as a constituent of O139 LPS. However, in this study, a uronic acid detected in O139 and O22 LPS was identified as D-galacturonic acid. Kdo was not detectable by the conventional periodate/thiobarbituric acid test under mild conditions for acid hydrolysis. Instead, Kdo phosphate was detected in methanolysates of O22 LPS,

Table 1. Sugar composition of *V. cholerae* O22 LPS and its polysaccharide portions after fractionation by gel filtration chromatography on Sephadex G-50

Sugar	Composition [$\mu\text{mol (mg sample dry wt)}^{-1}$]*				
	O139†	O22	Frc I	Frc II	Frc III
D-Glucose	0.35	0.40	0.86	0.93	—
D-Fructose	0.21	0.16	—	—	0.99
Colitose	0.26	0.28	—	—	1.51
L-glycero-D-manno-Heptose	0.51	0.29	0.89	1.02	—
D-Glucosamine	0.45	0.42	0.31	0.25	—
D-Quinovosamine	0.12	0.11	0.27	0.14	—
D-Galacturonic acid	0.17	0.36	1.32	0.94	0.16
Kdo	ND	ND	ND	ND	ND
Kdo-phosphate	+	+	NT	NT	NT

*ND, not detectable by the conventional periodate/thiobarbituric acid test; NT, not tested; —, less than $0.01 \mu\text{mol (mg sample dry wt)}^{-1}$.

†Data for O139 LPS are taken from a previous report (Hisatsune *et al.*, 1993).

Fig. 1. Elution of the degraded polysaccharide fraction isolated from *V. cholerae* O22 LPS from a column of Sephadex G-50.

suggesting that the structure of the Kdo region of O22 LPS was the same as that reported for O139 and hence for LPS of O1 *V. cholerae* (Kondo *et al.*, 1991a, 1992).

Polysaccharide portion of LPS. Gel filtration chromatography on Sephadex G-50 of the DPS fraction isolated from O22 LPS gave three fractions, as shown in Fig. 1, and the elution profile was almost identical to that obtained for the DPS fraction isolated from O139 LPS (Hisatsune *et al.*, 1993). As seen in the case of O139 LPS, a polysaccharide of high molecular mass corresponding to the O-specific polysaccharide attached to the core stubs that is, in the case of a typical smooth form (S-form) LPS,

eluted just after the void volume of the column, was not obtained from O22 LPS. The first (Frc I) and the second (Frc II) fractions that eluted close to each other corresponded to the core oligosaccharides and the third one (Frc III) corresponded to the monosaccharide fraction.

The sugar compositions of Frc I, II and III (Table 1) were also quite similar to those of the respective fractions obtained from O139 LPS (Hisatsune *et al.*, 1993). Frc I contained all of the constituent sugars of LPS with the exception of colitose and D-fructose; the latter two sugars were released by the mild acid hydrolysis used for preparation of the DPS fraction and were detected in Frc III in their free forms. Frc II also lacked colitose and D-fructose. The levels of D-quinovosamine, D-glucosamine and D-galacturonic acid in Frc II were considerably lower than those estimated for Frc I, indicating that Frc II might be an incomplete core polysaccharide portion of LPS. Frc III contained mainly colitose, D-fructose and a small amount of D-galacturonic acid in its free form.

Structural analysis. Methylation analysis, performed with mild hydrolysis conditions (0.01 M HCl, 100 °C, 30 min), revealed the presence of 1,5-di-O-acetyl-2,4-di-O-methyl-3,6-dideoxygalactitol and indicated that colitose was present at a non-reducing terminus in O22 LPS. GC/MS of the product obtained after methanolysis (1 M HCl in methanol, 86 °C, 2 h) and permethylation of O22 LPS and Frc I revealed a disaccharide that consisted of D-galacturonic acid and N-acetyl-D-quinovosamine. After partial purification on a Sep-Pak cartridge and carboxyl reduction with NaBH₄, the disaccharide was subjected to methylation analysis. GC/MS of the product revealed the presence of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgalactitol and 1,3,5-tri-O-acetyl-2-methylacetamido-2,6-dideoxy-4-O-methylglucitol, indicating that the disaccharide was D-galacturonic acid-(1 → 3)-N-acetyl-D-quinovosamine. The same disaccharide was also detected in O139 LPS. Thus, the chemical structure of the polysaccharide portions of O22 and O139 LPS were closely related.

Fig. 2. Silver-stained gel after SDS-PAGE of LPS isolated from: 1, *S. typhimurium* LT2; 2, *V. cholerae* NIH 41 (Ogawa); 3, *V. cholerae* 569B (Inaba); 4, *V. cholerae* O22; 5, *V. cholerae* O139.

SDS-PAGE analysis. The molecular architecture of O22 LPS was compared with that of O139 LPS by SDS-PAGE (Fig. 2). Only two bands, moving rapidly in the low-molecular-mass region toward the bottom of the gels, were produced with the LPS. No bands such as the doublet ladders obtained typically with high-molecular-mass O polysaccharide chains of the smooth type (S-type) LPS from enteric bacteria, for example *S. typhimurium* and *Escherichia coli*, were obtained. The banding pattern was quite similar to that for O139 LPS, showing that O22 LPS was devoid of a detectable long-chain O polysaccharide that consisted of repeating units. This result was entirely

consistent with that obtained from the analysis of the sugar composition of the three fractions (Frc I, II and III) prepared from the DPS by chromatography on Sephadex G-50. It is tempting to think that the more slowly migrating band might represent the polysaccharide portion of the LPS material contained in Frc I while the more rapidly migrating band might represent that contained in Frc II. Thus, despite the smooth colonies and serological O specificity, O22 LPS appeared to be an 'R-type' LPS, as was O139 LPS, on the basis of the elution profiles of the DPS after gel filtration on Sephadex G-50, the banding patterns on SDS-PAGE and the sugar composition. Recently, the term lipo-oligosaccharide (LOS) was proposed to describe the LPS of Gram-negative organisms that are devoid of the genetic ability to produce the O polysaccharide chain that consists of polymeric repeating units that is generally found in the Gram-negative S-type enteric bacteria (Wilkinson, 1977). O22 and O139 LPS are, in terms of molecular architecture, similar to the so-called LOS of non-enteric mucosal pathogens such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. However, as designations for these O22 and O139 LPS, 'R-type' LPS is considered to be preferable to the term LOS as argued by Hitchcock *et al.* (1986).

Serological relationship between O139 and O22 LPS

Cross-reactivity of O139 and O22 LPS. Table 2 shows the serological reactivity of O139 and O22 LPS with anti-O139 and anti-O22 antisera, unabsorbed and absorbed with the heterologous O22 and O139 antigens. In the homologous PH system, unabsorbed anti-O139 antiserum had a very high haemolytic titre against SRBC that had been sensitized with homologous O139 LPS. It is noted that the unabsorbed anti-O139 antiserum also had a high titre against SRBC that had been sensitized with heterologous O22 LPS. This high activity of anti-O139 antiserum against O22 LPS on SRBC was completely eliminated after the absorption with the O22 antigen. The haemolytic activity of anti-O22 antiserum was also tested, before and after absorption with O139 antigen, against

Table 2. Passive haemolysis titres of antisera against *V. cholerae* O139 and O22 with SRBC that had been sensitized with their LPS

LPS	Haemolysis titre*			
	Anti-O139		Anti-O22	
	No abs.†	Abs. O22‡	No abs.†	Abs. O139§
O139	66000	1000	900	—
O22	22000	—	13000	9700

* Results are expressed as reciprocals of the serum dilution that caused 50% haemolysis. —, 50% haemolysis was not observed at a dilution of 1:200 or greater.

† Antiserum against whole cells absorbed with *V. cholerae* CA385 as an R antigen.

‡ Antiserum against whole cells absorbed with *V. cholerae* CA385 as an R antigen and with *V. cholerae* O22.

§ Antiserum against whole cells absorbed with *V. cholerae* CA385 as an R antigen and with *V. cholerae* O139.

SRBC that had been sensitized with either O139 or O22 LPS. Unabsorbed anti-O22 antiserum had a very high haemolytic titre against SRBC that had been sensitized with O22 LPS. Furthermore, in the heterologous PH system, anti-O22 antiserum (unabsorbed) also reacted with heterologous O139 LPS on SRBC, albeit not to such a great extent, but still significantly. However, the absorption of anti-O22 antiserum with the heterologous O139 antigen entirely eliminated its activity against SRBC that had been sensitized with O139 LPS. Thus, the anti-O139 antiserum contained an antibody that reacted with heterologous O22 antigen at a high titre (22000) and this antibody was completely removed from the anti-O139 antiserum with the O22 antigen, while anti-O22 antiserum contained an antibody that reacted with the heterologous O139 antigen at a low titre (900) and was also completely removed from anti-O22 antiserum with the O139 antigen. These results lead to the conclusion that O139 and O22 strains share at least one common O-antigenic factor.

In PHI tests, O139 LPS and O22 LPS both strongly inhibited (the ID_{50} of LPS ranged from 0.03 to 0.14 $\mu\text{g ml}^{-1}$) the heterologous haemolytic systems of both O139 LPS-sensitized SRBC/anti-O22 antiserum and O22 LPS-sensitized SRBC/anti-O139 antiserum, which are substantially equivalent to the common antigenic factor in O139 LPS-sensitized SRBC/anti-O22 antiserum, and the common antigenic factor in O22 LPS-sensitized SRBC/anti-O139 antiserum systems, respectively. The results show that the O antigen of O139 is closely related to that of O22 in an a,b-a,c type of relationship, where a is a common antigenic factor, b is an O139-specific factor and c is an O22-specific factor.

Serological reactivity of chemically modified LPS. Treatment of O139 and O22 LPS with 48% HF resulted in total elimination of colitose and D-fructose from both LPS. Furthermore, the treatment of O139 and O22 LPS with NaIO_4 yielded oxidized LPS from which D-glucose, D-fructose and D-galacturonic acid were absent. However, even though their levels were considerably reduced, the other component sugars, such as L-glycero-D-manno-heptose, colitose, D-quinovosamine and D-glucosamine, remained unoxidized by NaIO_4 .

The serological reactivities of the chemically modified O139 and O22 LPS were examined in the PHI system with homologous anti-O139 and anti-O22 antiserum. The NaIO_4 -treated O139 LPS strongly inhibited the homologous O139 LPS-sensitized SRBC/anti-O139 antiserum system to the same extent as the intact O139 LPS (ID_{50} 0.03 $\mu\text{g ml}^{-1}$). Although the ID_{50} (0.65 $\mu\text{g ml}^{-1}$) of NaIO_4 -treated O22 LPS in the homologous O22 LPS-sensitized SRBC/anti-O22 antiserum system was higher than that of the intact O22 LPS (0.03 $\mu\text{g ml}^{-1}$), the serological reactivity of O22 LPS was fully retained by NaIO_4 -treated O22 LPS. In contrast, HF-treated O139 and O22 LPS did not inhibit the respective homologous PH systems. These data seem to suggest that, at least, the D-glucose, D-fructose and D-galacturonic acid residues are not immunodominant sugars while the colitose component is related, in some unknown fashion, to the immunodominant region of the LPS molecules.

This immunochemical study of LPS isolated from *V. cholerae* O139 and O22 revealed that the sugar composition and the chemical structure of O22 LPS were quite similar to those of O139 LPS. The results obtained appear to be compatible with the interpretation that the major O antigen of O139 is the common antigen factor a, while the major O antigen of O22 is the O22-specific antigen factor c.

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