Immunochemical characterization of an Ogawa-Inaba common antigenic determinant of *Vibrio cholerae* O1

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Cholera remains an important public health problem in many parts of the world and the availability of an effective cholera vaccine is important for the prevention of cholera in the countries affected by this disease. Despite the appearance in 1992 of a new serogroup, O139, of *Vibrio cholerae*, most of the cholera outbreaks are still caused by *V. cholerae* O1 biotype El Tor. Vaccine trials in Asia from 1968 to 1971, and studies of the production of serotype-specific antiserum in rabbits and of the protective activity of monoclonal antibodies against diarrhoeal disease in neonatal mice, have led to the conclusion that the Ogawa serotype contains a specific antigenic determinant whereas the Inaba serotype contains a different antigenic determinant that cross-reacts with the Ogawa serotype. By studying the binding of anti-Ogawa monoclonal antibodies to synthetic oligosaccharide fragments mimicking the Ogawa O-specific polysaccharide, it has been shown that the terminal monosaccharide, bearing the 2-O-methyl group in the O-specific polysaccharide, is most probably the serotype-specific determinant for the Ogawa strain. However, study of the binding of a monoclonal antibody recognizing both Ogawa and Inaba serotypes suggested partial recognition of the core as well as of the O-specific polysaccharide of the LPS of *V. cholerae* O1. To further characterize this antigenic determinant that is common to the Ogawa and Inaba serotypes, the core and the O-specific polysaccharide linked to the core of *V. cholerae* O1 LPS were purified by preparative electrophoresis. The O-specific polysaccharide linked to the core was subjected to periodate oxidation to destroy sugars from the core. Binding studies of these purified saccharide fragments to a monoclonal antibody which is protective in mice and specific to the antigenic determinant common to Ogawa and Inaba serotypes showed that both the core and the O-specific polysaccharide are involved in this common antigenic determinant. This explains how the presence or the absence of the Ogawa-specific antigenic determinant would lead to the expression of two independent antigenic determinants of *V. cholerae* O1, one specific to the Ogawa serotype and the other common to both Ogawa and Inaba serotypes.

Keywords: *Vibrio cholerae* O1, cholera, LPS, core, preparative electrophoresis

INTRODUCTION

Cholera remains an important public health problem. The long-term control of cholera depends on good

personal hygiene, an uncontaminated water supply and appropriate sewage disposal. However, the improvement of hygiene is a distant goal for many countries. Thus, the availability of an effective cholera vaccine is important for the prevention of cholera in these countries. Cholera is caused by *Vibrio cholerae* strains belonging to two serogroups, O1 and O139, based on

Abbreviations: LAL, *Limulus* amoebocyte lysate; O-SP, O-specific polysaccharide; O/R, oxidized and reduced.

their LPS structure. Serogroup O1, which is responsible for the seventh cholera pandemic, is divided into three serotypes, Inaba, Ogawa and Hikojima (a variant of the Ogawa serotype) (Stroehner *et al.*, 1992). Vaccine trials in Asia from 1968 to 1971 showed that killed whole-cell Inaba vaccines protected against both Inaba and Ogawa cholera, whereas Ogawa vaccine protected only against Ogawa cholera (Mosley *et al.*, 1970, 1973; Philippines Cholera Committee, 1973; Levine & Pierce, 1992). A conjugate vaccine composed of the detoxified LPS of *V. cholerae* O1 serotype Inaba bound to cholera toxin elicits vibriocidal antibodies against both Ogawa and Inaba serotypes (Gupta *et al.*, 1992, 1998). The results obtained with mAbs tested for their protective activity against diarrhoeal disease in neonatal mice are consistent with the findings with vaccines. Several mAbs directed against *V. cholerae* O1 have been prepared (Gustafsson *et al.*, 1982; Gustafsson & Holme, 1983, 1985a; Gustafsson, 1984; Brayton *et al.*, 1987; Sugiyama *et al.*, 1987; Adams *et al.*, 1988; Winner *et al.*, 1991; Colwell *et al.*, 1992; Apter *et al.*, 1993; Bougoudogo *et al.*, 1995; Ramamurthy *et al.*, 1995; Lüllau *et al.*, 1996). Most of these mAbs were studied for diagnostic purposes and only three mAbs have been tested for their protective activity in mice (Winner *et al.*, 1991; Apter *et al.*, 1993; Bougoudogo *et al.*, 1995). A secretory IgA (clone 2D6) and an IgG (clone S-20-3/S-20-4), both obtained by immunization with an Ogawa strain and directed against the Ogawa serotype, were associated with protection against the homologous serotype (Winner *et al.*, 1991; Apter *et al.*, 1993; Bougoudogo *et al.*, 1995). An IgG (clone I-24-2) obtained by immunization with an Inaba strain and directed against both the Ogawa and Inaba serotypes protected neonatal mice against both serotypes (Bougoudogo *et al.*, 1995). Therefore, observations made during cholera vaccine trials and experimental cholera in mice suggest that the Ogawa serotype contains a specific antigenic determinant whereas the Inaba serotype contains a different antigenic determinant that cross-reacts with the Ogawa serotype.

The Ogawa and Inaba serotypes of *V. cholerae* O1 differ only by a 2-O-methyl group that is present in the non-reducing terminal sugar of the Ogawa O-specific polysaccharide (O-SP) of the LPS, but is absent from the Inaba O-SP (Hisatsune *et al.*, 1993; Ito *et al.*, 1994; Wang *et al.*, 1998). Studies with mAb S-20-3/S-20-4 directed against Ogawa LPS and synthetic methyl α -glycosides of oligosaccharide fragments mimicking the Ogawa O-SP show that the terminal monosaccharide defines, as its major component, the serotype-specific antigenic determinant for the Ogawa strain (Wang *et al.*, 1998). It is thus most likely that the protection induced in vaccine trials by Ogawa whole-cell vaccines is due to the induction of antibodies recognizing only this serotype-specific antigenic determinant. This would explain why the vaccine does not protect against the Inaba serotype. In contrast, the binding of mAb I-24-2 (which recognizes both serotypes) to Ogawa LPS is somewhat puzzling (Wang *et al.*, 1998). MAb I-24-2, like

whole-cell Inaba vaccine, induces protection in mice against both the Ogawa and Inaba serotypes, and it is therefore likely that the antigenic determinant recognized by this mAb would be useful to induce protective antibodies against both serotypes. To further characterize this antigenic determinant, we investigated the binding of purified saccharide fragments from *V. cholerae* O1 LPS to mAbs I-24-2 and S-20-3/S-20-4 by immunoblotting and ELISA inhibition. We found that the core and the O-SP are both involved in the antigenic determinant common to the Ogawa and Inaba serotypes. This would explain how the presence or the absence of a single 2-O-methyl group in the non-reducing terminal residue of the O-SP chains of the LPS could lead to the expression of two independent antigenic determinants, one specific to the Ogawa serotype and the other common to both the Ogawa and Inaba serotypes.

METHODS

Bacterial strains and growth conditions. The *V. cholerae* serogroup O1 strains used in this study were of the El Tor biotype. The serotype Ogawa strain (CNRVC 920172) was isolated in 1987 in France from a case of cholera imported from Algeria. The serotype Inaba strain (CNRVC 950707) was isolated in 1995 from a patient in Mali. Cells were grown in Tryptic soy agar (Difco) in Roux flasks at 37 °C for 18 h.

Monoclonal antibodies. mAb I-24-2 is an IgG₃ recognizing LPS from both Ogawa and Inaba serotypes. mAb S-20-3/S-20-4 is an IgG₁ specific for serotype Ogawa LPS. Production, biological activity and the cDNA-derived amino acid sequence of both mAbs have been described elsewhere (Bougoudogo *et al.*, 1995; Wang *et al.*, 1998).

Preparation of LPSS and derived fragments. Cultures were suspended in distilled water and LPS was obtained by hot phenol/water extraction (Westphal & Jann, 1965), followed by enzymic treatment (DNase, RNase and protease) and ultracentrifugation (100 000 g for 3 h). The pellet containing LPS was dialysed against distilled water and freeze-dried. The LPS preparations from each serotype contained 1% (w/v) protein and less than 0.2% (w/v) nucleic acid. Inaba LPS was detoxified by acid hydrolysis (Hancock & Poxton, 1988): LPS (10 mg ml⁻¹ in 1% aqueous acetic acid) was heated at 100 °C for 60 min. The samples were subjected to low-speed centrifugation (350 g for 10 min) to precipitate lipid A, and the supernatant containing the polysaccharide moiety of the LPS collected. It was extracted with an equal volume of chloroform/ethanol (2:1), shaken vigorously and centrifuged (10 000 g for 30 min). The aqueous phase was dialysed against distilled water to remove ethanol and freeze-dried.

The oxidized and reduced (O/R) polysaccharide moiety of the LPS was isolated by Smith-type degradation of the polysaccharide moiety of the LPS (Goldstein *et al.*, 1970; Le Blay *et al.*, 1994), involving oxidation in 1% (w/v) aqueous sodium periodate at 20 °C for 2 d in the dark, dialysis and freeze-drying followed by reduction in 0.6% (w/v) sodium borohydride solution at 20 °C for 6 h, acid hydrolysis (2%, v/v, acetic acid) at 100 °C for 2 h and purification by Sephadex G-25 chromatography. Several sugars of the core, containing vicinal diols, are susceptible to this treatment, whereas the O/R polysaccharide moiety of the LPS [consisting of (1 → 2)-linked linear 4,6-dideoxy-4-(3-deoxy-L-glycero-tetronamido)-

Fig. 1. ^{13}C NMR spectrum of the polysaccharide moiety of the LPS from *V. cholerae* O1 serotype Inaba (40 mg in 0.5 ml D_2O , external reference Dioxan $\delta_0 = 67.4$ p.p.m.). The signals are identical to those previously reported (Kenne *et al.*, 1982; Gupta *et al.*, 1992).

α -D-mannopyranosyl residues] is resistant (Kenne *et al.*, 1982; Vinogradov *et al.*, 1995; Wang *et al.*, 1998).

The polysaccharide moiety of the LPS was subjected to preparative PAGE (prep-PAGE) on a model 491 Prep Cell apparatus (Bio-Rad), using deoxycholic acid (DOC) buffer as described by Krauss *et al.* (1988). A 6 cm, 12% polyacrylamide resolving gel (12% acrylamide, 0.35% bisacrylamide, 0.5% DOC, 375 mM Tris base, pH 8.8) was cast according to the manufacturer's recommendations. The resolving gel was then overlaid with a 0.5 cm stacking gel (4% acrylamide, 0.1% bisacrylamide, 0.5% DOC, 250 mM Tris base, pH 8.8). Before electrophoresis, the polysaccharide moiety of the LPS sample [0.75% (w/v) polysaccharide moiety of the LPS in 0.25% DOC, 10% (v/v) glycerol, 8% β -mercaptoethanol, 0.05% bromophenol blue, pH 6.8] was heated at 100 °C for 10 min. The sample was loaded onto the gel and subjected to electrophoresis at 30 mA. Fractions (2 ml) were collected for up to 10 h after the tracking dye had eluted. Three bands were obtained. Fractions corresponding to bands I and II were pooled (pool I) and fractions corresponding to band III were pooled (pool II). These pools were separately dialysed against 10% ethanol at room temperature to remove buffer salts and DOC as previously described (Reuhs *et al.*, 1993), and freeze-dried.

SDS-PAGE analysis. LPS, the polysaccharide moiety of the LPS and the prep-PAGE fractions were analysed by Tricine SDS-PAGE (Schägger & Von Jagow, 1987; Lesse *et al.*, 1990), using a 16.5% running gel and a 4% stacking gel. The cathode buffer (pH 8.25) consisted of 0.1 M Tris base, 0.1 M Tricine and 0.1% SDS and the anode buffer (pH 8.9) consisted of 0.2 M Tris base. Gels were fixed overnight at 4 °C in 25% (v/v) propan-2-ol, 7% acetic acid and silver stained as described by Tsai & Frasch (1982).

Immunoblot analysis. Polysaccharides separated by Tricine SDS-PAGE were electrophoretically transferred to nitrocellulose (0.2 μm pore size; Bio-Rad) at constant voltage (100 V)

for 1.5 h in 25 mM Tris base, 192 mM glycine, 20% ethanol, pH 8.3. The nitrocellulose was then soaked overnight at 4 °C in 3% BSA in PBS. The membrane was washed three times by shaking gently for 5 min in Tween (0.1%) in PBS (PBS-Tween). It was then gently shaken at room temperature for 2 h in 3 μg mAb I-24-2 ml^{-1} in PBS-Tween containing 0.5% gelatin. The membrane was washed three times for 5 min each in PBS-Tween and incubated with a 1/800 dilution of alkaline-phosphatase-conjugated goat anti-mouse IgG (heavy- and light-chain specific; Biosys) in PBS-Tween with 0.5% gelatin for 60 min at room temperature with gentle shaking. The membrane was washed and the blot developed by gently shaking for 10 min at room temperature in 1 g 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium I^{-1} (Sigma Fast; Sigma) in distilled water.

ELISA inhibition. The amount of antigen giving 50% inhibition of antibody binding was determined by a two-step ELISA. In step 1, mAb was incubated with solutions of polysaccharides and in step 2, the resulting mixtures of free and bound antibody were added to microtitre plates coated with serotype Ogawa or Inaba LPSs. The experiment was carried out as follows. Flat-bottom microplates (Immuno Microwell; Nunc) were blocked by incubation with 0.5% gelatin (Prolabo) in PBS for 1 h at 37 °C and washed with PBS containing 0.1% Tween-20. Polysaccharide dilutions (100 μl) and 100 μl mAb in PBS-Tween-gelatin were added to the wells, at a dilution, determined by direct ELISA titration, giving an A_{492} of 0.5. The mixture was incubated for 1 h at 37 °C and 100 μl samples from each well were transferred to a second plate, that had been previously coated by incubation for 1 h at 37 °C with purified serotype Ogawa or Inaba LPSs (5 μg ml^{-1}) in carbonate/bicarbonate buffer (0.1 M, pH 9.5), blocked with gelatin and washed with PBS-Tween. This plate was incubated at 37 °C for 1 h and washed with PBS-Tween. An anti-mouse peroxidase-conjugated IgG (heavy- and light-chain specific) (Sanofi Diagnostic Pasteur) diluted 1 in 1000 in PBS-Tween containing 0.5% gelatin was added to the wells. The plate was incubated at 37 °C for 45 min and washed with PBS-Tween. The enzyme substrate (*o*-phenylenediamine dihydrochloride; Sigma; 100 μl at 0.4 mg ml^{-1}) in 0.1 M sodium citrate (pH 5.2), containing 0.02% hydrogen peroxide, was added to each well and the plate incubated for 10 min at room temperature. The reaction was stopped by adding 3 M HCl (50 μl per well) and the A_{492} was read in an EL 800 spectrophotometer (Bio-Tek Instruments). The degree of inhibition was calculated as percentage inhibition = $[(A_{\text{inhibitor}} - A_{\text{inhibitor}})/A_{\text{inhibitor}}] \times 100$.

Analytical methods. Protein was determined by the Lowry method with BSA as the standard. Double immunodiffusion was performed in 1% agarose in 0.5 M NaCl for 18 h at 4 °C. The LPS concentration, assayed by the *Limulus* amoebocyte lysate (LAL) assay (Bio-Whittaker), is expressed in endotoxin units relative to the US standard (Hochstein, 1990).

NMR data were recorded on a BRUKER AC 300P spectrometer. The ^{13}C NMR spectrum was acquired with composite pulse decoupling, ^1H decoupling, 64K data points, 60 kHz spectral window (1.7 s acquisition time) and 3.0 s delay between pulse cycles. Prior to Fourier transformation, each free-induction-decay signal was exponentially multiplied so as to result in an additional 3 Hz line, broadening the frequency domain spectrum. Forty milligrams of the Inaba polysaccharide moiety of the LPS was exchanged twice with D_2O and dissolved in 0.5 ml D_2O . The spectrum was recorded at ambient probe temperature (23 °C), using dioxan as an external reference ($\delta_0 = 67.4$ p.p.m.).

Fig. 2. SDS-PAGE (a) and immunoblot (b) analysis of the polysaccharide moiety of the Inaba LPS and derived fragments. Lanes: 1, molecular mass markers; 2, polysaccharide moiety of the LPS (15 µg); 3, prep-PAGE pool II (15 µg); 4, prep-PAGE pool I (15 µg). The gel was silver stained and the immunoblot was probed with mAb I-24-2.

Samples for electrospray mass spectrometry were dissolved in water/methanol/formic acid (50:50:0.1, by vol.). They were introduced into an API 365 triple-quadrupole mass spectrometer (Perkin Elmer-Sciex) at a rate of 5 µl min⁻¹ with a syringe pump (Harvard Apparatus). The mass spectrometer was equipped with an atmospheric pressure ion source which was used to sample positive ions produced from a pneumatically assisted electrospray interface. The ionspray probe tip was held at 4.5 kV and the orifice voltage was set at 60 V. The mass spectrometer scanned continuously from m/z 400 to 1400 with a scan step of 0.1 and a dwell time per step of 2.0 ms, resulting in a scan duration of 20.0 s. Ten scans were averaged for each analysis. Mass calibration of the instrument was

carried out by matching ions of polypropylene glycol to known reference masses stored in the calibration table of the spectrometer.

RESULTS

Characterization of LPSs and derived fragments

A yield of 38 mg polysaccharide moiety was obtained from 100 mg Inaba LPS. The serotype Inaba LPS had 5×10^3 endotoxin units µg⁻¹ and the corresponding polysaccharide moiety of the LPS had 5 endotoxin units µg⁻¹ as assessed by the LAL assay; this 1000-fold reduction was consistent with previous data (Gupta *et al.*, 1992). The ¹³C NMR spectrum of the polysaccharide moiety of the LPS (Fig. 1) has 9 major signals with chemical shifts identical to, or slightly shifted in position from, those reported for detoxified *V. cholerae* O1 LPS (Kenne *et al.*, 1982; Gupta *et al.*, 1992).

As described for the Inaba LPS (Bougoudogo *et al.*, 1995), two main bands were detected by silver staining of SDS-PAGE gels containing the purified Inaba polysaccharide moiety of the LPS (Fig. 2a, lane 2). Fig. 3 shows the profile of the LPS polysaccharide moiety subjected to prep-PAGE. Three bands were obtained, with M_r values of 2500, 4000 and 14000. Pool I (60–160 ml), corresponding to bands I and II, had a M_r of 2000 (Fig. 2a, lane 4). These two bands were pooled because they have been previously described to compose the core polysaccharide (Gustafsson & Holme, 1985b). The difference between the observed M_r of pool I (compare Fig. 2 and Fig. 3) was probably due to a difference in the amount of loaded product. Pool II (280–580 ml), corresponding to band III, had a M_r of 14000 (Fig. 2a, lane 3).

The polysaccharide moiety of the LPS (Fig. 2b, lane 2) and of the corresponding purified polysaccharides [Fig. 2b, lanes 3 (pool II) and 4 (pool I)] were analysed by

Fig. 3. SDS-PAGE analysis and silver staining of ten 2 ml fractions following the dye front, eluted from a 12% prep-PAGE gel for separation of the core and the O-SP linked to the core from the polysaccharide moiety of the LPS. Lane T: polysaccharide moiety of the LPS (5 µg). Molecular mass markers are shown on the left and elution volume (in ml) along the top.

Fig. 4. Double immunodiffusion analysis of Inaba LPS and derived fragments. Wells: 1, mAb I-24-2, 3 mg ml⁻¹; 2, LPS, 10 mg ml⁻¹; 3, polysaccharide moiety of the LPS, 10 mg ml⁻¹; 4, prep-PAGE pool II, 10 mg ml⁻¹; 5, prep-PAGE pool I, 10 mg ml⁻¹.

immunoblotting. mAb I-24-2 recognized only pool II. Pool I contained less than 0.05% (w/v) of the polysaccharide present in pool II because the detection threshold of pool II, determined in a separate experiment, was 10 ng.

Double immunodiffusion detected a single precipitate between mAb I-24-2 and the Inaba LPS (Fig. 4, wells 1

and 2). A single precipitate was also observed when mAb I-24-2 was tested against the polysaccharide moiety of the LPS (Fig. 4, wells 1 and 3) and against the pool II (Fig. 4, wells 1 and 4). The fusion of immunoprecipitation lines indicates immunochemical identity of Inaba LPS, the polysaccharide moiety of the LPS and pool II. However, no precipitate was observed when mAb I-24-2 was tested against pool I (Fig. 4, wells 1 and 5).

Electrospray mass spectrometry of pool I gave a molecular ion at $m/z = 1650$, consistent with the structure proposed for the core of *V. cholerae* O1 LPS (Vinogradov *et al.*, 1995) (Fig. 5). No higher-molecular-mass component was observed in pool I, indicating that pool I was free of the species making up pool II or that any such contamination was only minor. Electrospray mass spectrometry of pool II (data not shown) gave a heterogeneous spectrum consistent with the O-SP of *V. cholerae* O1 being made up of 12–18 residues resulting in (1 → 2)-linked linear 4,6-dideoxy-4-(3-deoxy-L-glycero-tetronamido)- α -D-mannopyranosyl homopolymers (Kenne *et al.*, 1982). Thus the Inaba polysaccharide moiety of the LPS is a mixture of O-SP linked to the core and of free core. The O-SP linked to the core was isolated by prep-PAGE as pool II and the free core as pool I.

Fig. 5. Electrospray mass spectrum of prep-PAGE pool I. Peaks are shown as relative intensities of their mass in arbitrary mass units (a.m.u.).

Table 1. Amount of polysaccharide ($\mu\text{g ml}^{-1}$) yielding 50% inhibition in ELISA inhibition tests

The data shown are from Fig. 6.

Inhibitor	ELISA using:		
	mAb I-24-2 and Inaba LPS	mAb I-24-2 and Ogawa LPS	mAb S-20-3/S-20-4 and Ogawa LPS
Inaba LPS	0.35	0.35	> 100
Ogawa LPS	3.00	3.00	50.00
Inaba O-SP linked to the core	0.40	0.30	> 100
Inaba O/R polysaccharide moiety of the LPS	4.00	5.00	> 100
Inaba core	6.00	8.00	> 100
<i>E. coli</i> LPS	> 100	> 100	> 100

Fig. 6. ELISA inhibition of mAbs I-24-2 and S-20-3/S-20-4 by various LPS samples and derived fragments. Ascites fluid samples containing mAbs, diluted to a predetermined concentration, were mixed with various concentrations of polysaccharides: Inaba LPS (□), Ogawa LPS (○), Inaba O-SP linked to the core (△), Inaba O/R polysaccharide moiety of the LPS (●), Inaba core (■) and *E. coli*-LPS (▲). The mixture was incubated for 1 h. Reactions of mAb I-24-2 with Inaba LPS (a), mAb I-24-2 with Ogawa LPS (b) and mAb S-20-3/S-20-4 with Ogawa LPS (c) were analysed. The percentage inhibition was calculated for each concentration of inhibitor. Data are the means of five independent experiments.

Reactivity of mAbs I-24-2 and S-20-3/S-20-4 with Ogawa and Inaba LPSs and derived fragments

We investigated the fine specificity of recognition of these two mAbs by studying their interaction with the polysaccharides described above by ELISA inhibition. Controls were run to check the specificity of the binding. Only Ogawa LPS inhibited mAb S-20-3/S-20-4 binding to Ogawa LPS itself (Table 1 and Fig. 6c). *Escherichia coli* LPS did not inhibit binding of the mAb S-20-3/S-20-4 or I-24-2 to either Ogawa or Inaba LPSs (Table 1 and Fig. 6).

Both Ogawa and Inaba LPSs inhibited the binding of mAb I-24-2 to LPSs from the two serotypes (Fig. 6a, b). However, for 50% inhibition of binding, almost ten times more Ogawa LPS than Inaba LPS was required (Table 1, $3.00 \mu\text{g ml}^{-1}$ and $0.35 \mu\text{g ml}^{-1}$, respectively). This shows that the antigenic determinant recognized by

mAb I-24-2 is associated with the LPS from both serotypes although to a lesser degree with Ogawa LPS.

Binding of mAb I-24-2 to Ogawa and Inaba LPSs was also inhibited by the Inaba O-SP linked to the core (Fig. 6a, b). This preparation was as effective as the native Inaba LPS at inhibiting binding of Ogawa and Inaba LPSs to mAb I-24-2 (Table 1, $0.3\text{--}0.4 \mu\text{g ml}^{-1}$ and $0.35 \mu\text{g ml}^{-1}$). Thus the O-SP linked to the core contains an antigenic determinant common to both Ogawa and Inaba serotypes.

In contrast, the O/R polysaccharide moiety of the Inaba LPS was less effective than the native Inaba LPS at inhibiting binding of Ogawa and Inaba LPSs to mAb I-24-2 (Table 1, $4\text{--}5 \mu\text{g ml}^{-1}$ and $0.35 \mu\text{g ml}^{-1}$). This is consistent with one or more sugar residues in the core that are sensitive to the periodate being involved in the antigenic determinant recognized by mAb I-24-2. As could be predicted from this observation, the core inhibited the binding of both Ogawa LPS and Inaba LPS to mAb I-24-2 (Table 1, $6\text{--}8 \mu\text{g ml}^{-1}$), indicating that the core is involved in the antigenic determinant common to Ogawa and Inaba serotypes and recognized by mAb I-24-2. These results show that both the core and the O-SP are involved in the Ogawa-Inaba common antigenic determinant.

DISCUSSION

IgG₃ I-24-2 protects neonatal mice against *Vibrio cholerae* O1 serotypes Ogawa and Inaba (Bougoudogo *et al.*, 1995). The reactivity of this mAb against Ogawa LPS and Inaba LPS has been studied by measuring the inhibitory effects of synthetic oligosaccharide fragments mimicking the Inaba O-SP and of sugars from the core (Wang *et al.*, 1998). These experiments indicated partial specificity for the core as well as for a single residue of the O-SP of the LPS. Our findings with polysaccharide fragments purified from *V. cholerae* O1 LPS are entirely consistent with the antigenic determinant common to Ogawa and Inaba serotypes and recognized by mAb I-24-2 being mediated by the O-SP and the core of the LPS. This location for an antigenic determinant is unusual but is similar to that identified in LPS from *Bordetella bronchiseptica* (Le Blay *et al.*, 1994). The antigenic determinant recognized by mAb I-24-2 is common to Ogawa and Inaba LPSs, and must therefore be either the A or the C antigen described in *V. cholerae* O1 LPS (Sakazaki & Tamura, 1971). Iredell *et al.* (1998) used immunogold electron microscopy to determine the relative amounts of the A, B and C antigens on Ogawa and Inaba cells. The A and C antigens were present on both Ogawa and Inaba cells. However, the amount of C antigen present on Ogawa cells was about half that detected on Inaba cells, and the A antigen appeared more abundant on Ogawa cells. In our experiments, the binding of IgG₃ I-24-2 to Ogawa and Inaba LPSs was more strongly inhibited by the Inaba LPS than by the Ogawa LPS. It is therefore possible that the antigenic determinant recognized by mAb I-24-2 is the C antigen.

Iredell *et al.* (1998) have suggested that the lack of methylation of the O-SP exposes the C antigen resulting in the Inaba serotype. Our results are completely consistent with this suggestion because the 2-O-methyl group in the non-reducing terminal residue of the O-SP on Ogawa cells may hamper recognition by the immune system of an antigenic determinant expressed by the O-SP linked to the core of the LPS.

Inaba strains are *rfbT* mutants of an Ogawa strain in which B antigen synthesis (i.e. methylation of the O-SP at its non-reducing end) is inactivated (Stroehrer *et al.*, 1992; Hisatsune *et al.*, 1993). Ogawa O-SP is defined as bearing a 2-O-methylated non-reducing terminal residue, but not all Ogawa O-SP chains are methylated (Iredell *et al.*, 1998). Thus, if 2-O-methylated O-SP chains express the B antigen and non-methylated O-SP chains express the C antigen, it is possible to account for the presence or absence of a 2-O-methyl group in the non-reducing terminal residue of the O-SP chains, leading to either serotype Ogawa or Inaba. In this case, as reported, Inaba cells, containing only non-2-O-methylated O-SP, would express the C antigen only, whereas Ogawa cells, containing both 2-O-methylated-O-SP chains and, to a lesser extent, non-2-O-methylated chains, would express both B and C antigens.

The identification of the Ogawa and Inaba serotypes of *V. cholerae* O1 is based on the use of specific agglutinating antisera produced by rabbits immunized with heat-inactivated whole bacterial cells of either Ogawa or Inaba serotype. However, cross-absorption of these sera is needed to improve their specificity. Ogawa serotype-specific antiserum is easy to obtain by absorption with bacteria of the Inaba serotype (Donovan & Furniss, 1982). In contrast, Inaba serotype-specific antiserum is difficult to produce since there is often a concomitant loss of Inaba serotype-specific antibodies during the absorption with bacteria of the Ogawa serotype (Donovan & Furniss, 1982). The presence of small amounts of the C antigen in Ogawa cells renders the absorption step difficult because antibodies directed against the C antigen are also adsorbed. As Inaba cells do not express the B antigen, they are the cells of choice in the adsorption step for producing a specific anti-Ogawa serum.

In conclusion, our results explain how the presence or the absence of the Ogawa-specific antigenic determinant would lead to the expression of two independent antigenic determinants of *V. cholerae* O1, one specific to the Ogawa serotype and the other common to both Ogawa and Inaba serotypes.

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