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Immunological Characterization of *Vibrio cholerae* O:1 Lipopolysaccharide, O-Side Chain, and Core with Monoclonal Antibodies

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Lipopolysaccharide (LPS) was extracted from *Vibrio cholerae* O:1 strains of the serotypes Ogawa, Inaba, and Hikojima and delipidated by mild-acid hydrolysis. Two polysaccharide fragments with the molecular weights of approximately 9,000 and 900, respectively, were isolated by gel permeation chromatography. The LPS preparations and the polysaccharide fragments were studied in enzyme-linked immunosorbent assay inhibition, rocket immunoelectrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroblotting with monoclonal antibodies directed against the group-specific antigen A, the type-specific antigens B (Ogawa) and C (Inaba), and the core region. Antigen A was demonstrated in all LPS preparations and all 9,000-molecular-weight fragments tested. The type-specific antigens B and C were demonstrated in LPSs and 9,000-molecular-weight fragments from Ogawa and Inaba, respectively. Furthermore, antigens B and C were both demonstrated in LPSs and 9,000-molecular-weight fragments from two of four Hikojima strains tested. Core antigen was demonstrated in the LPS and in the 9,000- and 900-molecular-weight fragments. The results indicate that the 9,000-molecular-weight fragment represents the complete polysaccharide chain, including group- and type-specific antigens as well as core antigens, whereas the 900-molecular-weight fragment constitutes the main part of the core region.

The disease cholera is caused by *Vibrio cholerae* strains belonging to the serogroup O:1, characterized by the group-specific antigen A. This serogroup is subdivided into two main serotypes, Ogawa and Inaba, owing to the presence of type-specific antigens B and C, respectively. In addition, a third serotype, Hikojima, containing all three antigens has been described (16).

The dominant part of the O-antigenic side chain of *V. cholerae* O:1 is composed of a (1-2)-linked homopolymer of D-perosamine (4-amino-4,6-dideoxy-D-mannose) in which the amino groups are acylated by 3-deoxy-L-glycero-tetronic acid (13, 14, 22). It has been suggested that the sites representing the A antigen are present in this part of the lipopolysaccharide (LPS) (13, 14). The Ogawa- and Inaba-specific antigens B and C have not been localized, but Redmond has tentatively identified 4-amino-4-deoxy-L-arabinose as a constituent of the Ogawa LPS (21). D-quinovosamine has also been demonstrated in smooth strains of *V. cholerae* O:1 (11), whereas rough mutants lacked all amino sugars except glucosamine (9). The last workers could not demonstrate the presence of 4-amino-4-deoxy-L-arabinose in the LPS from three Ogawa serotype strains.

As shown by Kenne et al. (14) and Raziuddin (20), the LPS of *V. cholerae* O:1 is split into lipid A and two polysaccharide fractions upon mild-acid hydrolysis. Kenne et al. (13) found that the major polysaccharide fraction had an approximate molecular weight of 9,000. A minor fraction with a molecular weight of 900 was also demonstrated. From comparative analysis of sugars of the two fractions, Raziuddin (20) proposed that they represented the O-specific side chain and the core region, respectively.

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In this paper, we report immunological studies of the LPS and the two separate polysaccharide fractions. Specific O-antigens as well as core antigens were detected in the purified 9,000-molecular-weight fragment. Only core antigens were detectable in the 900-molecular-weight fragment. These results offer an improved basis for the further chemical characterization of the *V. cholerae* O-antigens as well as the core antigens.

MATERIALS AND METHODS

Bacterial strains and cultivations. *V. cholerae* O:1 strains Ogawa 34, 1451, Inaba 35, and 569B were obtained from our strain collection. The *V. cholerae* O:1 strains Hikojima 433, 1602, 1603, and 11689 were obtained from T. Donovan, Public Health Laboratory, Maidstone, United Kingdom. All strains were recultivated at daily intervals by serial plating of a single colony on blood agar for 7 days. The bacteria were cultivated in an aerated, stirred 12-liter fermentor at 37°C and at a constant pH of 7.2. A tryptone-yeast extract medium (10) was used for all strains. Precultures were grown overnight in Erlenmeyer flasks on a rotary shaker at 37°C in the same medium with a sugar content reduced to 5 g/liter.

LPS preparation. LPS was extracted from the bacteria by the hot phenol-water method (30) and purified by high-speed centrifugation as described earlier (8).

Hydrolysis and chromatography of LPS. LPS was hydrolyzed into polysaccharide and lipid A by a modification of the method described by Redmond et al. (23). Briefly, LPS was dissolved in 2% acetic acid (pH 3.1) at a final concentration of 20 mg/ml and incubated at 100°C for 7 h (13). After incubation, the solution was allowed to cool to room temperature and was washed with anhydrous ether to remove released lipid A. The polysaccharide moiety was fractionated by gel filtration on a Sephadex G50 column (Pharmacia

TABLE 1. Characteristics of monoclonal antibodies and their ELISA titers

Monoclonal antibody	Class, subclass, light chain	Specificity	Titer[a]	Reference no.
H4	IgM, κ	A	8,000	7
112-3-2G	IgG3, κ	A	16,000	6
H8	IgG3, λ	B	16,000	7
C6	IgG3, κ	C	8,000	7
E8	IgG1, κ	Core	128,000	8

[a] Endpoint titer of ascites fluid in LPS ELISA. An endpoint titer was defined as the highest dilution in a twofold serial dilution still giving an optical density at 450 nm of >0.2 (8).

Fine Chemicals, Uppsala, Sweden) connected to a refractometer (R403; Waters Associates, Millford, Mass.) with distilled water as eluant. Two polysaccharide fragments with molecular weights of 9,000 and 900 (13) were recovered from all strains, with the exception of strain 433, from which no 9,000-molecular-weight fragment was recovered. Upon refractionation of the 900-molecular-weight fragment on a Sephadex G25 column (Pharmacia), two peaks containing the 900-molecular-weight fragment and D-fructose, respectively, were obtained (13).

Monoclonal antibodies. Hybridomas were produced by fusing SP2/0 mouse myeloma cells (25) with B-lymphocytes from BALB/c mice previously immunized with purified LPS or with formalized, heat-inactivated whole bacteria. The production and characterization of the monoclonal antibodies used in this study have been described earlier (6–8). The antibodies were prepared from ascitic fluid. The characteristics and specificity of each antibody are summarized in Table 1.

ELISA-inhibition. Inhibition of monoclonal antibodies with LPS and polysaccharide fragments was performed as described earlier (8). Briefly, monoclonal antibodies were incubated with LPS or polysaccharide fragments in different concentrations in polystyrene tubes (Hegerplastics, Stallarholmen, Sweden). Remaining antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (4) performed in 96-well microtiter trays (Dynatech M 129 A; Flow Laboratories, Irwine, Scotland) (29). Samples (50 μl) were added to wells previously coated with LPS from the *V. cholerae* Ogawa 34 or Inaba 35. The immune reaction was performed by adding 50 μl of rabbit anti-mouse immunoglobulin (Dako, Copenhagen, Denmark) followed by incubation with 50 μl of horseradish-peroxidase-conjugated sheep anti-rabbit immunoglobulin. As substrate, 100 μl of 3 mM 1,2-phenylenediaminedihydrochloride (Fluka, Buchs, Switzerland) in 40 mM Tris-hydrochloride buffer (pH 7.6) was used. The optical density at 450 nm was measured in a Titertek Multiscan spectrophotometer (Flow Laboratories). The 50% inhibitory value was recorded as the concentration of LPS or polysaccharide fragments needed to obtain a 50% decrease in the optical density as compared with control tubes with no inhibitor added.

SDS-PAGE. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), polyacrylamide (14% [wt/vol]) slab gels containing 1% SDS and 4 M urea were prepared as described by Tsai and Frasch (27). Stacking gels (3.15% [wt/vol]) contained 0.01% SDS. Samples were prepared by mixing LPS dissolved in distilled water with an equal volume of 0.1 M Tris-hydrochloride buffer (pH 6.8) containing 2% (wt/vol) SDS, 20% (wt/vol) sucrose, 1% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. The samples were incubated at 100°C for 5 min, and 20-μl amounts were applied to the sample wells. The gels were run in a double-slab electrophoresis cell (Protean; Bio-Rad Laboratories, Richmond, Calif.) at a constant current of 35 mA until the bromophenol blue dye reached 1 cm from the bottom of the gel. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Silver stain. Gels were stained as described by Tsai and Frasch (27). Briefly, the gels were incubated overnight in 40% ethanol–5% acetic acid, followed by incubation with 0.7% periodic acid in ethanol-acetic acid for 5 min. The gels were washed in distilled water and then incubated for 10 min with a staining solution consisting of 28 ml of 0.1 M sodium hydroxide, 2 ml of 25% (wt/vol) ammonium hydroxide, 5 ml of 20% (wt/vol) silver nitrate, and 115 ml of distilled water. The gels were washed again in distilled water and then incubated with 200 ml of formaldehyde developer containing 50 mg of citric acid per liter and 0.5 ml of 37% formaldehyde per liter.

Electroblotting. The polyacrylamide slab gels were blotted onto nitrocellulose sheets (26) with a transblot electrophoretic transfer cell (Bio-Rad). Briefly, transfer of LPS was performed in a buffer solution containing 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (pH 8.3) overnight at 30 V and 0.1 A. The nitrocellulose sheets were blocked with 3% gelatin in Tris-hydrochloride (20 mM)-buffered saline (0.5 M), pH 7.5 (TBS), for 45 min at 22°C before incubation with the monoclonal antibodies, diluted 1/50 in TBS, for 2 h at 22°C. Unbound antibodies were removed by washing the sheets twice for 10 min with TBS–0.5% Tween 20 before incubation with horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin for 1 h at 22°C. The nitrocellulose sheets were washed twice for 10 min with TBS-Tween and incubated with a substrate solution containing 60 mg of 4-chloro-1-naphthol (Sigma) dissolved in 100 ml of TBS with 20 ml of methanol. H_2O_2 (60 μl; 30%) was added immediately before use.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed by a modification of the method described by Laurell (15). Three gels were molded onto two glass plates each. This was performed by pouring 3 ml of 1% agarose (IEF; Pharmacia) dissolved in 24 mM diemal buffer (pH 8.6) on glass plates (5 by 5 cm) placed on a horizontal table. After congelation, two-thirds of each gel was removed, and 2 ml of agarose containing 150 μl of H8 (anti-B) antibodies (plate A) or 150 μl of C6 (anti-C) antibodies (plate B) was poured onto each glass plate (intermediate gel). After congelation, half of the second gels were removed, and 1 ml of agarose with 75 μl of C6 antibodies (plate A) or 75 μl of H8 antibodies (plate B) was poured on the corresponding glass plate (top gel). Sample wells were punched out from the bottom gel containing no antibodies. The glass plates were placed on a Multiphor cooling plate (LKB Products, Bromma, Sweden), and 15-μl samples containing 0.5 mg/ml were added to each well. The samples were run towards the anode at 4 V/cm for 6 h. After electrophoresis, the gels were washed in 0.9% NaCl overnight, dried, and stained in 0.5% Coomassie brilliant blue R250 (15).

RESULTS

SDS-PAGE. LPS preparations were subjected to SDS-PAGE followed by silver staining (27). A silver-stained major band containing several finely resolved bands was obtained (Fig. 1, lanes 1 through 6). This pattern is characteristic of O-chains with a monosaccharide repeating unit (2)

and not as distinct as the step ladder-like banding pattern obtained with LPS containing oligomeric repeating units, e.g., *Salmonella typhimurium* (Fig. 1, lane 7). A second major band was obtained that comigrated with the front. All *V. cholerae* O:1 LPS preparations tested showed similar profiles after SDS-PAGE, without respect to serotype.

Electroblotting. Figure 2 shows the immunoblot reactions obtained when SDS-PAGE-separated LPSs from *V. cholerae* O:1 and *S. typhimurium* were incubated with monoclonal antibodies directed against group-, type-, and core-specific antigens. The LPS from *V. cholerae* O:1 reacted with the group-specific antibody 112-3-2G (anti-A) (Fig. 2A, lanes 1 through 6). H8 (anti-B) antibodies reacted with LPS from serotypes Ogawa and Hikojima (Fig. 2B, lanes 1, 2, 5, and 6) whereas C6 (anti-C) antibodies reacted with LPS from the serotypes Inaba and Hikojima (Fig. 2C, lanes 3 through 6). No staining was obtained with the core-specific antibody E8 and 5 to 40 μg of LPS. However, when the gel was loaded with higher amounts of LPS (50 to 100 μg), staining was distributed over the whole lane, indicating that the LPS did not separate in these concentrations. No staining of *S. typhimurium* LPS was obtained with any of the monoclonal antibodies (Fig. 2A through C, lanes 7).

Rocket immunoelectrophoresis. The presence of both B and C antigens in LPS from the Hikojima strains 1602 and 1603 was demonstrated by rocket immunoelectrophoresis with monoclonal antibodies directed against the antigens B and C. Ogawa LPS (Fig. 3, lane 1) formed a rocket only in the gel containing antibodies against antigen B, whereas Inaba LPS (lanes 2) showed a rocket only in the gel containing antibodies against antigen C. This result was obtained also when the positions of the top and intermediate gels were switched. Hikojima 1602 (Fig. 3, lane 3) and 1603 (lane 4) LPSs always formed rockets in the intermediate gel irrespective of whether antibodies against B or C were present (Fig. 3). LPS from strains 433 and 11689 formed rockets only in gels containing antibodies directed against antigen C. No rockets were formed when 9,000-molecular-weight fragments were used as antigens with antibodies against the antigens B and C. However, in gels containing antibodies to antigen A, rockets were formed with both complete LPS as well as with 9,000-molecular-weight fragments (data not shown).

ELISA-inhibition. ELISA-inhibition was performed with LPS and polysaccharide fragments from *V. cholerae* O:1 as inhibitors (Table 2). LPS from all strains tested inhibited the H4 (anti-A) antibody. LPS from the strains 34 and 1451 inhibited the H8 (anti-B) monoclonal antibody, whereas LPS from strains 35, 569B, 433, and 11689 inhibited the C6 (anti-C) antibody only. LPS from 1602 and 1603 inhibited both H8 and C6 antibodies. The amounts of LPS needed to obtain 50% inhibition of these antibodies were much higher as compared with LPS from strains of Ogawa and Inaba (Table 2). Group- and type-specific antigens were also demonstrated in 9,000-molecular-weight fragments, except strain 433, from which no 9,000-molecular-weight fragment was recovered upon acid hydrolysis and gel chromatography. However, only 9,000-molecular-weight fragments from the strains 1602 and 1603 contained both B and C antigens. Furthermore, the amount of polysaccharide needed to obtain 50% inhibition of the antibodies was substantially higher as compared with polysaccharide fragments from strains of Ogawa and Inaba type (Table 2). Core antigen was demonstrated in all LPS and 9,000- and 900-molecular-weight fragments tested (Table 2).

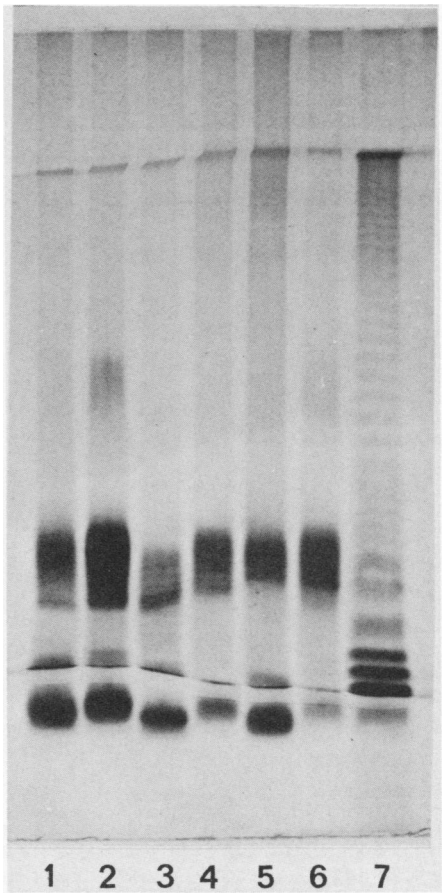

FIG. 1. Silver staining of SDS-polyacrylamide gel. Wells were loaded with 20 μl per well. Lanes contained LPSs from *V. cholerae* strains Ogawa 34 (lane 1, 10 μg), Ogawa 1451 (lane 2, 10 μg), Inaba 35 (lane 3, 5 μg), Inaba 569B (lane 4, 5 μg), Hikojima 1602 (lane 5, 10 μg), Hikojima 1603 (lane 6, 5 μg), and *S. typhimurium* (lane 7, 10 μg).

From these data, it seems that only strains 1602 and 1603 are true Hikojima strains, whereas strain 11689 belongs to the Inaba type. Strain 433 seems to be a strain with a very weak expression of its O-antigens and more like an Inaba strain than like an Ogawa. Furthermore, the results indicate that the 9,000-molecular-weight fragment represents the complete polysaccharide chain, carrying the group- and type-specific antigens together with the core region, whereas the 900-molecular-weight fragment represents the core region only.

DISCUSSION

It has been shown by Brade et al. (1) that *V. cholerae* O:1 LPS contains 3-deoxy-D-manno-octulosonic acid, in contrast to the previously accepted view of the absence of this compound in LPSs from these bacteria. The splitting of the LPS into lipid A and a polysaccharide fraction by mild-acid hydrolysis is then in accordance with the findings with LPS from other gram-negative bacteria that 3-deoxy-D-manno-octulosonic acid is the link between the lipid A and the core region of the LPS. However, in contrast to the finding with, e.g., smooth *Salmonella* bacteria that the polysaccharide moiety was fairly homogeneous and dominated by the sugars constituting the O-side chain, the polysaccharide of *V.*

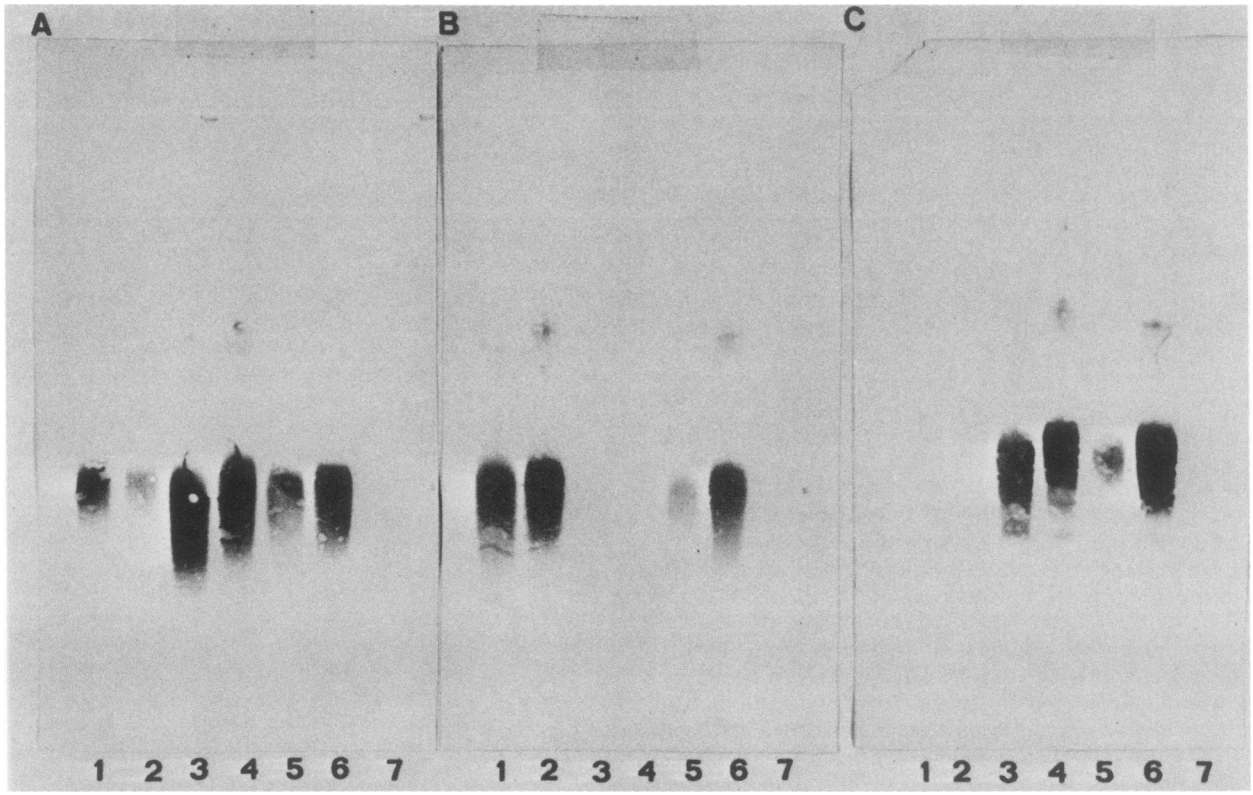

FIG. 2. Immunoblots of SDS-polyacrylamide gel-separated LPS with monoclonal antibodies. (A) Blotting with the monoclonal antibody 112-3-2G. Lanes contained LPSs from *V. cholerae* strains Ogawa 34 (lane 1; 5 μg), Ogawa 1451 (lane 2; 5 μg), Inaba 35 (lane 3; 20 μg), Inaba 569B (lane 4; 20 μg), Hikojima 1602 (lane 5; 20 μg), Hikojima 1603 (lane 6; 10 μg), and *S. typhimurium* (lane 7; 10 μg). (B and C) Blotting with the monoclonal antibodies H8 (B) and C6 (C), with 50 μg of LPS per well.

cholerae LPS could be further separated into two fractions with different molecular weights.

The fragment with a molecular weight of 900 reacted with the anti-core monoclonal antibody and did not react with any of the antibodies against the O-specific antigens. These findings indicate that the 900-molecular-weight fragment constitutes the core region.

The polysaccharide fraction with a molecular weight of approximately 9,000 reacted with monoclonal antibodies against the O-specific antigens. In addition, the presence of the core antigen was also demonstrated by ELISA-inhibition with monoclonal antibodies against the core. This finding indicates that the 9,000-molecular-weight fragment constitutes the complete polysaccharide moiety of the LPS, including the O-antigenic side chain and the core.

These results indicate that the cleavage of the glycosidic linkages of the 3-deoxy-D-manno-octulosonic acid on mild acid hydrolysis is the only reaction needed to explain the splitting of the *V. cholerae* LPS into lipid A and two polysaccharide fragments. It is reasonable to assume that the 900-molecular-weight fragment consists of unsubstituted core stubs, which apparently are more frequent in LPSs from smooth *V. cholerae* O:1 strains than in LPSs from *Salmonella* strains. However, as shown by Palva and Mäkelä (19), two-thirds of the LPS molecules of an *S. typhimurium* mutant with a smooth phenotype were devoid of O-side chains. A similar heterogeneity in number of repeating units has also been demonstrated in *Escherichia coli* LPS (5, 28) and for *Rhizobium* LPS (3).

LPS preparations from the different *V. cholerae* O:1 serotypes were further analyzed by SDS-PAGE (Fig. 1). The banding pattern of the *S. typhimurium* LPS is in good agreement with that reported by other workers (17). *V. cholerae* LPS displays narrow bands, indicating the small difference in molecular weight between the LPS fractions with different numbers of monosaccharide repeating units in their O-side chains. In addition, a low-molecular-weight fraction, apparently present in various amounts in different strains, was clearly demonstrated.

Electroblotting with monoclonal antibodies against the different O-antigens confirmed that the major bands observed in the silver-stained gels consisted of LPS molecules carrying O-specific side chains. Also, with this method of visualization, banding was observed in some of the preparations. The low-molecular-weight fraction, comigrating with the front, most probably represents LPS with unsubstituted core stubs, devoid of O-side chains. The variable staining of these bands indicates a difference between different strains in the numbers of unsubstituted core stubs in their LPS.

The *V. cholerae* O:1 strain 433 used in the present study was initially classified as a serotype Hikojima on the basis of slide agglutination (6). However, the facts that no 9,000-molecular-weight fragment could be isolated from this strain and that the expression of the O-antigens A and C was very weak, as shown in ELISA-inhibition, might be explained by the assumption that the number of LPS molecules carrying an O-side chain was very small in this strain.

Earlier immunological studies of the *V. cholerae* O:1 serotypes have been concentrated to the Ogawa and Inaba types, and very little attention has been paid to Hikojima strains (18). The occurrence of this serotype has been questioned by Kauffmann (12). Citing data from conven-

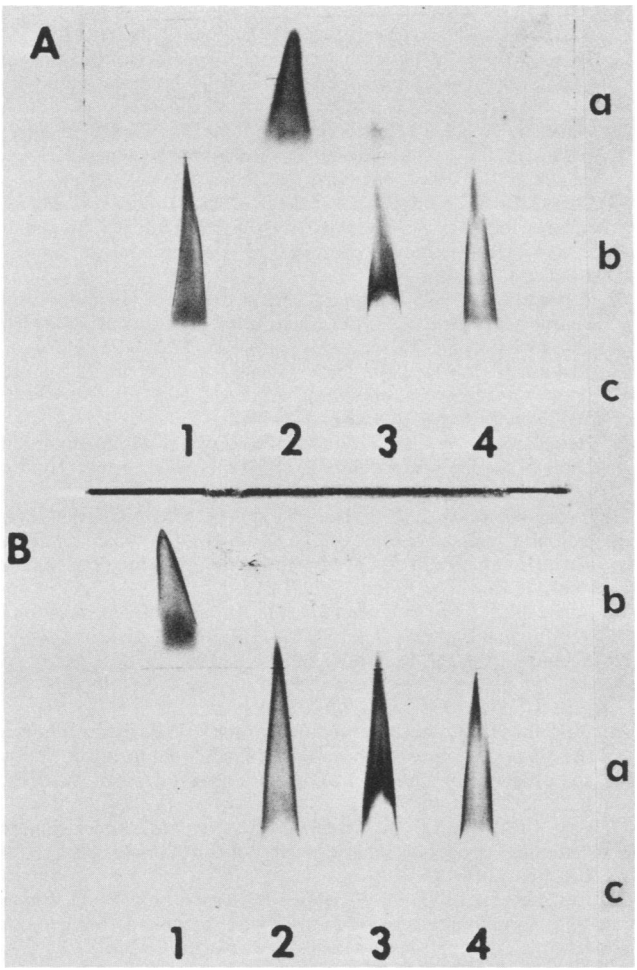

FIG. 3. Rocket immunoelectrophoresis of LPS from different serotypes of *V. cholerae* O:1. (A) Glass plate with 1% agarose containing monoclonal antibody C6 (a), monoclonal antibody H8 (b), and no antibody (c). (B) Antibody-containing gels as in (A) but with gels (a) and (b) in switched positions. Samples consisted of LPSs (0.5 mg/ml) from strains Ogawa 34 (lane 1), Inaba 35 (lane 2), Hikojima 1602 (lane 3), and Hikojima 1603 (lane 4).

tional serological analysis with absorbed antisera, he suggested that the Ogawa type exhibited the antigens A, B, and C and that Inaba was deficient in the B antigen. The variable results of serotyping obtained by different groups has been suggested to depend on a variation in the expression of the C antigen in the Ogawa strains (24). By using monoclonal antibodies, it was clearly shown that Ogawa strains contain only the antigens A and B and that Inaba strains contain the antigens A and C (7). In a recently published study, only two of four Hikojima strains were found to be true Hikojimas when tested by monoclonal antibody-based ELISA techniques (6).

The results of the rocket immunoelectrophoresis confirm the conclusions drawn from the ELISA-inhibition and electroblotting experiments that two of four Hikojima strains tested carried both B and C antigens. Rockets were also formed when 9,000-molecular-weight fragments were run against the anti-A antibody. This indicates that the A antigen is present as multiple determinants, supporting the view that the perosamine polymer is the structural basis for this antigen. The fact that no rockets were formed with anti-B and anti-C antibodies indicates that the B and C antigens may be present as single determinants on each polysaccharide chain.

TABLE 2. ELISA-inhibition of monoclonal antibodies with LPS and polysaccharide fragments from strains of *V. cholerae* O:1

Strain, inhibitor (molecular weight)[a]	Concn (mg/ml) 50% inhibitory to the following[a]:			
	H4	H8	C6	E8
Ogawa 34				
LPS	0.15	0.13	>5	0.03
PS				
9,000	2.10	0.50	>5	0.15
900	>5	>5	>5	0.02
Ogawa 1451				
LPS	0.20	0.13	>5	0.25
PS				
9,000	4.40	0.44	>5	0.36
900	>5	>5	>5	0.06
Inaba 35				
LPS	0.10	>5	0.32	0.01
PS				
9,000	3.80	>5	3.20	0.07
900	>5	>5	>5	0.03
Inaba 569B				
LPS	0.32	>5	0.14	0.07
PS				
9,000	4.10	>5	0.38	0.05
900	>5	>5	>5	0.02
Hikojima 1602				
LPS	0.44	4.41	3.10	0.30
PS				
9,000	4.95	2.50	5.00	0.06
900	>10	>10	>10	0.01
Hikojima 1603				
LPS	0.31	3.60	5.20	0.02
PS				
9,000	5.00	5.50	5.40	0.09
900	>10	>10	>10	0.01
Hikojima 433				
LPS	2.45	>10	4.00	0.62
PS				
9,000	NT[b]	NT	NT	NT
900	>10	>10	>10	0.25
Hikojima 11689				
LPS	0.65	>10	2.92	0.06
PS				
9,000	1.50	>10	4.68	0.10
900	>10	>10	>10	0.02

[a] Inhibition was performed in wells coated with LPS from strains Ogawa 34 for H4 (anti-A) and H8 (anti-B) and Inaba 35 for C6 (anti-C) and E8 (anticore). PS, Polysaccharide.
[b] NT, Not tested.

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