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Monoclonal Antibodies Against *Vibrio cholerae* Lipopolysaccharide

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A cell line producing monoclonal antibodies directed against the core region of *Vibrio cholerae* lipopolysaccharide has been established. These antibodies were inhibited by lipopolysaccharide preparations of both O-group 1 vibrios and some non-O-group 1 vibrios as detected in enzyme-linked immunosorbent assay-inhibition experiments. Coagglutination experiments with monoclonal and polyclonal antibodies adsorbed to protein A-carrying staphylococci were performed. All *V. cholerae* strains tested, regardless of serotype, were agglutinated when mixed with staphylococci coated with the monoclonal antibodies, whereas staphylococci coated with group-specific (O1) polyclonal antibodies only agglutinated with O-group 1 vibrios.

Our structural studies on the cell wall lipopolysaccharide (LPS) of *Vibrio cholerae* aim at the characterization of the chemical structure of the antigenic determinants of the different serotypes. Studies of the O group have revealed a great similarity in the sugar composition of the Ogawa and Inaba serotypes (5, 7, 9a, 10, 16, 17). The sole difference demonstrated thus far is the presence of 4-amino-4-deoxy-L-arabinose in serotype Ogawa, which contains this sugar in addition to the sugars it has in common with serotype Inaba (17). The antigen common to the two serotypes has been designated A (2, 3, 9). Antigen A is shown to reside in the O-specific part of the LPS (5, 14, 21). R mutants of Ogawa and Inaba serotypes, as well as those from cholera vibrios of other O groups, have serologically identical R antigens (21). Most *V. cholerae* O antisera contain antibodies against the R antigen, but probably in concentrations insufficient to cause difficulties when used at dilutions suitable for slide or tube agglutination.

The fact that there was a concomitant decrease in the A antigen activity and release of fructose by mild acid hydrolysis made Redmond et al. (18) suggest that a fructofuranosyl residue may be involved in the type A determinant. Kenne et al. (9a, 10) have suggested that the dominating amino sugar of the O antigen, perosamine, with the amino group acylated with 3-deoxy-L-glycerotetronic acid, may represent the A antigen. Other than these speculations, no data have been presented as to the chemistry of the O antigenic determinants of the *V. cholerae* LPS.

Diagnostic reference sera for the determination of the serotypes of *V. cholerae* include a "group serum," which is unabsorbed, and absorbed type-specific antisera against types Ogawa (anti-B) and Inaba (anti-C). In addition, an X (antirough) serum is available (Vibrio Reference Laboratory, Jefferson Medical College, Philadelphia, Pa.) that usually does not react with serotypes other than rough strains of O-group 1. It has been claimed that serotype Ogawa contains the antigens A, B, and C and that the Inaba form is a mutant lacking antigen B (9, 20). These observations are in conflict with the fact that type-specific antisera to the Inaba serotype are produced and are usable in the differentiation of the *V. cholerae* serotypes.

For the determination of the serological specificity of different polysaccharide fractions from *V. cholerae* LPS, monospecific antisera are needed. It is obvious from the conflicting evidence concerning the antigens of *V. cholerae* that antisera with a high degree of specificity would be of great value for the characterization of antigenic determinants. Commercially available sera are produced only for diagnostic purposes and are not sufficiently well characterized for immunochemical work.

The present paper reports on the production of a monoclonal antibody against *V. cholerae* LPS. Such antibodies may provide improved opportunities for the characterization of antigenic determinants and, in addition, allow the separation of polysaccharide fractions by immunosorbent methods if such antibodies can be produced in large quantities. The availability of monoclonal antibodies may also prove valuable in improving serodiagnosis of *V. cholerae*.

TABLE 1. Bacterial strains[a]

Strain	Serotype	Source[b]
Vibrio cholerae		
34 Ogawa	O:1	a
35 Inaba	O:1	a
1824 Ogawa	O:1	a
NCTC 4711	O:2	a
NCTC 4716	O:4	a
B4202-64	O:5	a
10317-62	O:8	a
109-68	O:21	a
Escherichia coli		
611	O:6, K15, H16	b
617	O:8, K40, H9	b
614	O:78, H11	b
Neisseria gonorrhoeae 1465		b
Salmonella typhimurium		b
Salmonella kentucky IS 98		c
Salmonella paratyphi-A IS 1		c
Shigella sonnei		b
Yersinia enterocolitica type 3		b

[a] The *V. cholerae* serotypes are designated according to Shimada and Sakazaki (22).

[b] a, Strains provided by J. Holmgren, University of Gothenburg, Sweden; b, strains from our strain collection; c, strains obtained from the National Bacteriological Laboratory, Stockholm, Sweden.

MATERIALS AND METHODS

Bacterial strains and cultivation. The strains used in this study are listed in Table 1. All strains 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 (TY-2) (6) was used for all strains except *Neisseria gonorrhoeae*. Precultures were grown overnight in Erlenmeyer flasks on a rotary shaker in a modified TY-1 medium (6) at 37°C; the modification was a reduction in sugar content to 5 g/liter. *N. gonorrhoeae* was cultivated in a medium containing 15 g of proteose-peptone no. 3 (BBL Microbiology Systems, Cockeysville, Md.), 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch, and 10 ml of IsoVitaleX, made up to 1 liter with distilled water. During cultivation, the bacteria were aereated with a mixture of 5% CO_2 in air.

LPS preparation. The bacteria were suspended in distilled water at a concentration of 20 g (dry weight) per liter and extracted with an equal volume of 90% phenol at 68°C for 15 min (25). The crude LPS preparation was suspended in distilled water at a concentration of 2% (wt/vol) and centrifuged at 100,000 × *g* for 4 h at 4°C in a fixed-angle rotor. The gel-like pellet was suspended in distilled water and lyophilized.

Protein content. The protein content of LPS preparations was measured by the method of Lowry (13).

Immunization. Female BALB/c mice, 6 to 10 weeks of age, were immunized once a week for 9 weeks. For the first immunization, mice were given 0.05 ml of Inaba 35 LPS (1 mg/ml) emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) subcutaneously. The second immunization consisted of 0.05 ml of Inaba 35 LPS (1 mg/ml) emulsified with an equal volume of Freund incomplete adjuvant given intraperitoneally. For immunizations 3 to 9, the mice were given 0.1 ml of Inaba 35 LPS (1 mg/ml) without adjuvant intraperitoneally.

O-group 1 antiserum was prepared by collecting sera from mice immunized as described above.

Fusion and cloning. Hybridomas were established according to the protocol of Nowinski et al. (15). BALB/c mice were immunized with purified cholera LPS as described above. Three days after the last booster, the spleen was removed under sterile conditions and placed into RPMI 1640 medium (GIBCO) containing 10% fetal calf serum with 100 U of penicillin and 100 μg of streptomycin per ml, cut and minced through a metal net, and sieved through a sterile nylon net to obtain a fine suspension. The cells were washed three times in RPMI 1640 alkaline medium (pH 7.8) and counted in a Bürker chamber. The mouse myeloma (P3X-NS-1) variant was washed once in the same medium at 37°C. To 100×10^6 spleen cells, 25×10^6 myeloma cells were added. The cell mixture was centrifuged, and the supernatant was removed. A 1-ml amount of polyethylene glycol solution (PEG 1000; Merck, Darmstadt, West Germany), prepared by dissolving 3 g of PEG 1000 (autoclaved for 20 min at 120°C and cooled to 37°C) into 3 ml of RPMI 1640 medium without fetal calf serum (37°C), was added dropwise to the cell mixture for 1 min. After gentle shaking for 1 min plus 1 min of incubation, 1 volume of alkaline RPMI was added for 1 min, followed by 1 volume of RPMI for 1 min. Finally, 8 volumes of alkaline RPMI 1640 was added, and the cells were gently centrifuged for 10 min at 300 × *g*. The supernatant was removed, and the cells were diluted to 10 ml with RPMI–10% fetal calf serum medium. The cells were gently resuspended, added in 100-μl portions per well to microtiter plates (Falcon 3042; Falcon Plastics, Oxnard, Calif.), and incubated for 24 h in a tissue culture incubator at 37°C with 80% humidity and 5% CO_2. The medium was then changed to the selective HAT (12) medium. After 2 weeks on HAT medium, with changes twice a week, the cells were switched back to regular RPMI 1640–5% fetal calf serum. Three weeks after fusion, the supernatants were tested for antibody reactivity by the micro-enzyme-linked immunosorbent assay (ELISA) method specific for cholera LPS.

Antibody production. Monoclonal antibodies were produced in 6-ml sterile polystyrene cell culture tubes (Falcon 2058) by growing hybrid cells in RPMI 1640 medium supplemented with 5% fetal calf serum, 1 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.), and antibiotics as described above. The tubes were incubated in a tissue culture incubator for about 1 week, after which the antibody-containing supernatant was removed.

Detection of antibodies. Antibody production was measured by using the ELISA (4), performed in 96-well microtiter trays (Dynatech M 129 A; Flow Laboratories, Irvine, Scotland) (23, 24).

(i) Antigen coating. Each well was coated with 100 μl of Inaba LPS (25 μg/ml) in 10 mM phosphate-buffered

saline (PBS) (pH 7.2) and 0.02% NaN_3 overnight at 37°C. Remaining binding sites were blocked by the addition of 100 μl of 5% bovine serum albumin in PBS–0.05% Tween 20. After 15 min at 22°C, the wells were rinsed three times with 100 μl of PBS-Tween 20.

(ii) Immune reaction. Samples (50 μl each) were added to each well, and the trays were incubated for 1 h at 37°C. After three rinses with PBS-Tween 20, 50 μl of rabbit antimouse immunoglobulin (Dako, Copenhagen, Denmark), diluted 1/100 in PBS-Tween 20, was added to each well. The trays were incubated for 15 min at 37°C before rinsing as described above. A 50-μl amount of horseradish peroxidase-conjugated sheep antirabbit immunoglobulin, diluted 1/100 in PBS-Tween 20, was added to each well. The trays were incubated for 30 min at 37°C before rinsing as described above. Peroxidase-conjugated antibodies were prepared as described by Boorsma and Streefkerk (1).

(iii) Enzyme reaction. A 55-mg amount of 1,2-phenylenediaminedihydrochloride (Fluka AG) was dissolved in 100 ml of 40 mM Tris-hydrochloride buffer (pH 7.6) with 0.9% NaCl. Before use, 30 μl of 30% H_2O_2 was added, and 100 μl of this substrate solution was added to each well. The enzyme reaction proceeded for 5 min at 37°C and was terminated by the addition of 50 μl of 1 M H_2SO_4. The optical density at 450 nm was measured in a Titertec Multiscan (Flow Laboratories) spectrophotometer. An optical density value of 0.2 above background was considered a positive result. As a negative control, wells were incubated with RPMI 1640 medium.

ELISA-inhibition. Inhibition of monoclonal antibodies and polyvalent mouse serum was performed in polystyrene tubes (Heger Plastics, Stallarholmen, Sweden). The diluted antibodies, at a concentration four times the concentration yielding an approximate optical density at 450 nm of 1 after 5 min, were preincubated for 30 min at 22°C with an equal volume of LPS in different concentrations. After incubation, an equal volume of PBS-Tween 20 was added to each tube. The samples were tested by ELISA by using 96-well microtiter trays as described above. The 50% inhibitory value was recorded as the concentration of LPS needed to obtain a 50% decrease in the optical density as compared with control tubes with no inhibitor added.

Immunoglobulin class, subclass, and light chain. Immunoglobulin class, subclass, and light chain were determined by immunodiffusion according to the method of Ouchterlony by using 1% agarose (Marine Colloids, Rockland, Me.) in 10 mM phosphate buffer (pH 7.2) and specific rabbit antiserum to mouse immunoglobulin M (IgM), IgG (7S), IgG1 IgG2a, IgG2b, and IgG3 and κ and λ light chains (Bionetics, Kensington, Md.).

Coagglutination. Coagglutination was performed by using protein A-carrying *Staphylococcus aureus* (11).

Isoelectric focusing. Isoelectric focusing was performed in 0.5-mm thin-layer agarose gels containing 1% agarose IEF (Pharmacia Fine Chemicals, Uppsala, Sweden), dissolved in double-distilled water and 0.8% ampholytes (pH 3.5 to 9.5) (LKB-Produkter AB, Bromma, Sweden). The gel was prepared by molding the gel on top of an agarose-coated plastic sheet (Gel-Bond; LKB-Produkter) placed between two glass slides, which were kept apart with a spacer. The gel was allowed to solidify in a humidity chamber at 4°C for 1 h before use. Acetic acid (0.5 M) and 0.5 M NaOH were used as anode and cathode solutions, respectively. The gel was placed on a Multiphor cooling plate (LKB-Produkter), and 20-μl samples were applied to sample application pieces placed on the surface of the gel. The samples were run for 30 min at a constant power of 4 W. After focusing, the pH gradient was measured by means of a surface pH electrode (Radiometer, Copenhagen, Denmark).

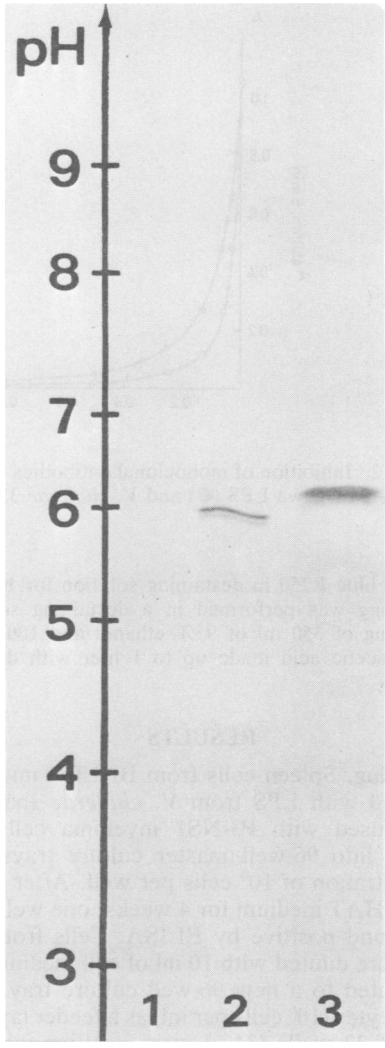

FIG. 1. Isoelectric focusing followed by immunofixation of culture supernatant from NS-1 myeloma cell line (1), monoclonal antibodies directed against pneumococcal C-substance (2), and monoclonal antibodies directed against *V. cholerae* LPS (3).

Fixation. The mouse immunoglobulin bands were immobilized by immunofixation. The gel was soaked with rabbit antimouse immunoglobulin (Dako, Copenhagen, Denmark) diluted 1:5 in PBS for 30 min, followed by washing overnight in a 0.9% NaCl solution. The next day the gel was washed in 95% ethanol for 10 min, after which the gel was dried.

Staining. The gel was stained in 0.5% Coomassie

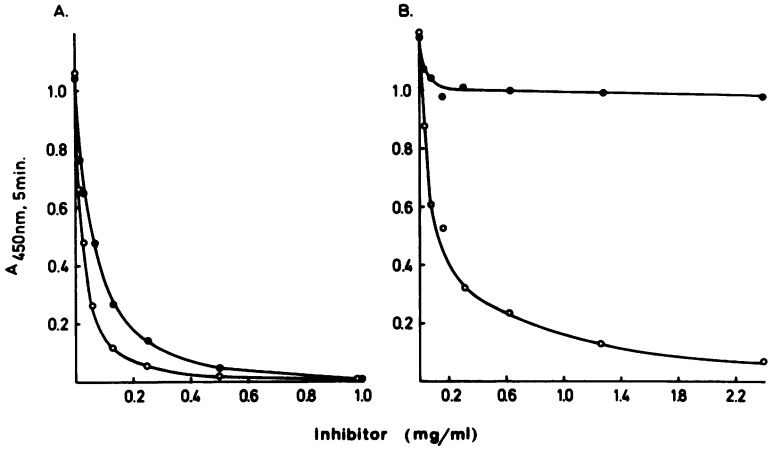

FIG. 2. Inhibition of monoclonal antibodies (A) and polyvalent mouse anti-Inaba LPS antiserum (B) with *V. cholerae* 34 Ogawa LPS (●) and *V. cholerae* 35 Inaba LPS (○) as inhibitors. A, Absorbance.

brilliant blue R250 in destaining solution for 10 min. Destaining was performed in a destaining solution consisting of 350 ml of 95% ethanol and 100 ml of glacial acetic acid made up to 1 liter with distilled water.

RESULTS

Cloning. Spleen cells from BALB/c mice immunized with LPS from *V. cholerae* Inaba 35 were fused with P3-NS1 myeloma cells and seeded into 96-well master culture trays at a concentration of 10^6 cells per well. After selection in HAT medium for 4 weeks, one well (1%) was found positive by ELISA. Cells from this well were diluted with 10 ml of cell medium and distributed to a new 96-well culture tray, with thymocytes (10^6 cells per ml) as a feeder layer. A total of 33 wells (34%) were positive within 3 weeks. Cells from four wells of this tray were each diluted with 10 ml of cell medium with thymocytes and distributed to four new 96-well trays. Within 3 weeks, all wells (100%) of two trays were positive by ELISA, 30 wells (31%) were positive in the third tray, and the fourth tray was negative. Cells of 12 wells from the three trays were cultivated in cell culture tubes for about 2 weeks before freezing.

Isoelectric focusing. Culture supernatants from 12 clonal isolates were tested by isoelectric focusing with agarose gel (19). All samples yielded one single band at pH 6.2 after immunofixation and were subsequently considered identical monoclonal antibodies. Antibodies from the clonal isolate, E8, were used for all further studies.

Isoelectric focusing was also performed on monoclonal antibodies (E8), culture supernatant from the P3-NS1 myeloma cell line, and monoclonal antibodies against pneumococcal C-substance polysaccharide (kindly provided by A. M. Sjögren, Department of Bacteriology, Karolinska Institute, Stockholm, Sweden) (Fig. 1).

Antibodies produced by clone E8 showed only one single band (sample 3) at pH 6.2, whereas monoclonal antibodies directed against pneumococcal C-substance showed one single band at pH 6.0 (sample 2). The culture supernatant of the parent myeloma cell line showed no band (sample 1).

Class and subclass. Double-diffusion experiments with Ouchterlony techniques indicated that the antibodies from clone E8 were of the IgG type, subclass IgG1, with κ light chains.

ELISA-inhibition. The monoclonal antibodies and a polyvalent unabsorbed mouse antiserum directed against Inaba LPS were tested by ELISA-inhibition, with Ogawa LPS and Inaba LPS as inhibitors. Inhibition of the monoclonal antibodies with Ogawa LPS and Inaba LPS gave almost identical results (Fig. 2A). When the inhibition experiment was performed on an unabsorbed polyvalent anti-Inaba serum, total inhibition was obtained only when Inaba LPS was used as inhibitor. Ogawa LPS showed only a minor inhibitory effect due to its A antigen content (Fig. 2B).

The protein content of Ogawa and Inaba LPS preparations were 124 and 173 μg/ml, respectively. To avoid misleading results due to the protein content of the LPS preparations, ELISA-inhibition was also performed on heat-treated (30 min at 100°C) Ogawa and Inaba LPS. This experiment resulted in inhibition curves almost identical to those in Fig. 2.

The specificity of the antibodies produced by the clone E8 were further studied by estimating the amount of LPS needed to inhibit the ELISA

TABLE 2. ELISA-inhibition of clone E8 antibodies with LPS from different strains as inhibitors

Inhibitor (LPS)	50% Inhibitory value[a] (μg/ml)
Vibrio cholerae	
34 Ogawa	6.5
35 Inaba	21
1824 Ogawa	240
4711 (O:2)	280
4716 (O:4)	>1,000
B 402-64 (O:5)	260
10317-62 (O:8)	>1,000
109-68 (O:21)	46
Escherichia coli	
611	>1,000
617	>1,000
614	>1,000
Neisseria gonorrhoeae 1465	>1,000
Salmonella kentucky	>1,000
Salmonella paratyphi-A	>1,000

[a] Concentration of LPS needed to reduce the optical density at 450 nm by 50%.

system to 50%. LPS from *V. cholerae* 34, 35, and 109-68 inhibited the monoclonal antibodies in concentrations of 6.5 to 46 μg/ml. LPS from *V. cholerae* 1824, 4711, and B 402-64 inhibited the monoclonal antibodies in concentrations varying from 240 to 280 μg/ml. All other LPS preparations tested were needed in concentrations greater than 1 mg/ml (Table 2).

Coagglutination. Antibodies produced by the clone E8 as well as O-group 1 antiserum were adsorbed to protein A-carrying *S. aureus* Cowan 1 and used in a slide agglutination test (Table 3). Anti-O-group 1 coagglutinated strongly with all of the three *V. cholerae* O-group 1 strains tested. No coagglutination was observed in tests against non-O-group 1 vibrios or noncholera species. Monoclonal antibodies adsorbed to staphylococci coagglutination with both O-group 1 and non-O-group 1 *V. cholerae*, whereas strains from noncholera species were negative.

DISCUSSION

Earlier immunological studies of serotypes Ogawa and Inaba were based on the use of polyvalent rabbit antisera. By cross-absorption, it is possible to produce sera that distinguish at least three antigens among the O-group 1 vibrios. Antigen A is common to both Ogawa and Inaba. Antigen B is found only in Ogawa, and antigen C is found in Inaba. The monoclonal antibody described in this paper was completely inhibited by both Ogawa and Inaba LPS, as shown in the ELISA-inhibition experiment (Fig. 2A). This shows that the monoclonal antibodies are directed against either antigen A or another antigen shared by the serotypes. Inhibition was also obtained when LPS preparations from *V. cholerae* 109-68, B402-64, and 4711 were used (Table 2). These strains represent non-O-group 1 vibrios and lack the group- and type-specific antigens found in O-group 1 vibrios. This indicates that the specificity of the monoclonal antibodies is directed against a determinant of the core structure. Shimada and Sakazaki (21) found cross-reacting antigens in R mutants from *V. cholerae* O1 as well as a number of R mutants from non-O1 vibrios. LPS from *V. cholerae* 4716 and 10317-62 did not inhibit the monoclonal antibodies. This may be due to either steric hindrance by the O-antigens of these serogroups or to variations in the core region within the cholera species. Such variations have been observed for other bacteria, e.g., *Escherichia coli* (8).

Conventional antisera may contain antibodies directed against contaminating antigens or against several antigenic determinants on the same antigen. Both types of antibodies can often be removed by absorption, giving a relatively monospecific antiserum, but often with concomitant loss in titer. Monoclonal antibodies are directed against one single antigenic determinant and are thus highly specific. Nevertheless, cross-reactions may occur if the monoclonal antibody recognizes related antigenic determinants on different antigens. Such cross-reactions

TABLE 3. Coagglutination of different strains with antibodies coated to *S. aureus*

Strain	Coagglutination[a] with		
	O-group 1 antiserum	Clone E8 antibodies	NaCl
Vibrio cholerae			
34	++	+	−
35	++	++	−
1824	++	++	−
4711 (O:2)	−	++	−
4716 (O:4)	−	+	−
B 402-64 (O:5)	−	++	−
10317-62 (O:8)	−	++	−
109-68 (O:21)	−	+	−
Escherichia coli			
611	−	−	−
617	−	−	−
614	−	−	−
Salmonella typhimurium	−	−	−
Salmonella kentucky	−	−	−
Salmonella paratyphi-A	−	−	−
Shigella sonnei	−	−	−
Yersinia enterocolitica	−	−	−

[a] ++, Strong positive reaction; +, weak positive reaction; −, no agglutination.

cannot be eliminated by absorption, since antibodies derived from one clone are identical.

The fact that LPS from unrelated bacterial species was unable to inhibit the monoclonal antibodies indicates that the specificity of the monoclonal antibodies is directed against an antigen present in *V. cholerae* only. However, the presence of this antigen in other vibrio species was not investigated.

The monoclonal antibodies produced by the clone E8 were also used in a rapid slide agglutination test (Table 3). Since the antibodies are of the IgG type, they exhibit poor agglutinating capacity. However, by adsorbing the antibodies onto protein A-carrying staphylococci, the Fc part of the antibodies binds to protein A, whereas the combining sites are free and are oriented outward. This makes it possible to perform slide agglutination (11). The fact that the monoclonal antibodies could coagglutinate both O-group 1 virbrios and non-O-group 1 vibrios but not bacteria of other species indicates a specificity of the monoclonal antibodies for a structure common to all *V. cholerae* regardless of serotype, e.g., the core. The lack of inhibitory activity by the O-group 4 and O-group 8 LPS antigens (Table 2) may be explained by a decrease in core antigens exposed on the LPS micelles in comparison to the intact bacterial cells.

In conclusion, in this paper we described a monoclonal antibody directed against the core structure of LPS from *V. cholerae*. Such antibodies are of great value for studies of the chemical structure and immunological properties of LPS from *V. cholerae*.

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