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Identification of *Vibrio vulnificus* O Serovars with Antilipopolysaccharide Monoclonal Antibody

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A serotyping scheme for *Vibrio vulnificus* predicated on the detection of lipopolysaccharide (LPS) antigens is proposed. The serovar O typing scheme used to type *V. vulnificus* employs polyclonal antisera raised in rabbits immunized with heat-killed whole-cell vaccines. Polyclonal typing sera produced in this manner cross-react with heterologous strains. Affinity purification of polyclonal antisera with LPS affinity columns resolved some of these cross-reactions; however, affinity-purified polyclonal antisera still showed cross-reactions that were nonreciprocal. On the basis of the serological patterns that were obtained with affinity-purified polyclonal antisera, *V. vulnificus* strains were selected as vaccine strains for production of monoclonal antibody. Spleen cells harvested from BALB/c mice immunized with formalin-killed *V. vulnificus* cells were fused with SP2/O-Ag 14 myeloma cells. Hybridomas were screened by using LPS and whole-cell enzyme-linked immunosorbent assay to identify clones secreting LPS-specific antibodies. Monoclonal antibodies identified five LPS serological varieties of *V. vulnificus* and a single serovar each for *Vibrio damsela* and *Vibrio hollisae*. No cross-reactions between *V. vulnificus* and *V. hollisae* or *V. damsela* were observed.

Infections caused by *Vibrio vulnificus* have been reported in most parts of the world (4, 5, 7, 13, 14). Environmental surveys show that *V. vulnificus* is found in virtually all coastal areas in the United States during the warmer months (9, 10, 16–18). Because of the widespread distribution and virulence of *V. vulnificus*, rapid detection of this pathogen is becoming increasingly important.

A species-specific flagellar (H) antigen, which groups members of *V. vulnificus* into a single serotype, has been identified (21) and exploited in the rapid identification of this pathogen (21–23). Shimada and Sakazaki (19) proposed a serovar O serological typing scheme based on direct agglutination of heat-killed *V. vulnificus* cells by polyclonal rabbit sera. This typing scheme recognized seven O serovars; the identity of the serovar-specific antigen, however, remained to be defined. Because heat treatment destroys the H antigen, the agglutination patterns observed were assumed to represent reactions between the cell wall serovar O determinants and antibody. However, the surface of the bacterial cell presents a diverse menu of potential antigens to the rabbit immune system. As a consequence, antibodies directed against heat-stable outer membrane protein and lipopolysaccharide (LPS) antigens may be present in polyclonal antiserum.

In addition, virulent *V. vulnificus* strains produce a capsule that blankets the outer leaflet of the cell wall (3, 11, 12, 25, 26). Encapsulated organisms can be recognized by the opacity they exhibit on agar surfaces, while nonencapsulated organisms produce smaller, translucent colonies (25, 26). The presence of a capsule is associated with a decrease in the 50% lethal dose and sensitivity to the bactericidal action of serum, while there is an increased capacity for subcutaneous tissue invasion (25). The capsule may present a barrier to serological reagents designed to detect cell wall-associated determinants and may contribute antigens (11, 12) which do not align with serovar-specific O determinants.

Because the current typing scheme has not defined the serovar-specific antigen, uses polyclonal serum that is not uniformly serovar specific, and requires *V. vulnificus* cells be heat treated prior to testing, the serological typing scheme described by Sakazaki and Shimada (19) was reassessed. In the investigation reported here, five *V. vulnificus* serovars were identified with monoclonal antibody (MAb) specific for epitopes in the LPS portion of the cell wall.

MATERIALS AND METHODS

Bacterial strains studied. The following *Vibrio* isolates were used in this study: *V. vulnificus* ATCC 27526; CDC E4125; Louisiana Department of Health and Human Resources strains recovered from wound infections, 846, 1003, and 1015; stool cultures, 1000, 1004, and 1008; and blood cultures, 1001, 1002, 1005, 1006, 1007, 1009, 1010, 1012, 1013 and 1014, and 1015; *Vibrio hollisae* ATCC 33564; and *Vibrio damsela* ATCC 35083. The opaque (O) and 1005 translucent (T) colonial phenotypic variants were derived from Louisiana Department of Health and Human Resources strain 1005.

Production of polyclonal antisera. Outbred New Zealand White rabbits were immunized with formalin-killed and heat-killed whole-cell vaccines prepared from selected clinical *V. vulnificus* strains by methods described elsewhere (2, 19, 20).

LPS extraction. LPS was extracted from acetone-dried cells of selected *V. vulnificus* strains by the phenol-water extraction procedure described by Westphal and Jann (24). *Vibrio* cells were grown on alkaline peptone agar at 30°C for 48 h and harvested in 0.15 M NaCl. Each LPS (1 mg/ml) was detoxified by treatment with 0.25 N NaOH at 37°C for 4 h and 1 h at 56°C and dialyzed against 0.067 M phosphate-buffered saline (PBS) at 4°C overnight (2). Detoxified LPS, which served as O antigen for the LPS enzyme-linked immunosorbent assay (ELISA), was suspended (50 μg/ml) in 0.067 M PBS and stored at −20°C. The polysaccharide, which contains the O-antigen determinants and which was used for affinity chromatography, was separated from the lipid by centrifugation of detoxified LPS at 100,000 × *g*. The

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resulting supernatant, which contained polysaccharide O antigen, was lyophilized.

Affinity purification of anti-LPS immunoglobulin. Polysaccharide obtained from LPS was coupled to an amino-hexyl Sepharose matrix (Sigma Chemical Co., St. Louis, Mo.) through a benzoquinone bridge by the method described by Girard and Goichot (8). To a column (1 by 20 cm) containing the matrix and equilibrated with 0.067 M PBS (pH 7.3), 1 ml of clarified anti-*V. vulnificus* serum was applied, allowed to enter the bed, and incubated in the matrix for 1 h at room temperature. Antipolysaccharide immunoglobulin was eluted with 0.2 M glycine buffer (pH 2.8), dialyzed against PBS, and concentrated to the starting volume.

MAb production. Anti-LPS-secreting hybridomas were generated by fusing immune splenocytes collected from female BALB/c mice, which were immunized with whole-cell vaccines (1), with nonimmunoglobulin-secreting SP2/O-Ag 14 cells (American Type Culture Collection, Rockville, Md.). The fusion protocol used was a modification of the method used by Oi and Herzenberg (15). Cells were fused in a ratio of four splenocytes per myeloma cell in 50% polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 75 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Culture supernatant from wells which contained hybridomas were tested for anti-LPS activity by both whole-cell and LPS ELISA by methods described previously (1). Hybridomas which tested positive were cloned by limiting dilution until a stable cell line was generated. MAbs were isotyped, purified (1), and standardized to 200 μg of protein per ml.

Whole-cell ELISA. The *Vibrio* strains tested were grown on alkaline peptone agar at 30°C for 24 h, harvested in 0.05 M $NaHCO_3$-$NaCO_3$ coupling buffer (pH 9.6), washed one time in coupling buffer, and suspended to 3×10^8 cells per ml. A 50-μl aliquot of the vibrio cell suspension was added to each well of a 96-well polystyrene ELISA plate (Immunlon II; Dynatech Laboratories, Inc., Alexandria, Va.). The uncovered plates were incubated overnight at 37°C to dehydrate the cell suspension. Each well was blocked with 200 μl of 2% bovine serum albumin in TEN buffer (0.05 M TRIS hydrochloride, 0.001 M EDTA, 0.15 M NaCl; pH 7.2) for 30 min at 37°C and washed one time with TEN buffer. Fifty microliters of hybridoma supernatant or control serum was added to each well and incubated at 37°C for 30 min. Following a single washing step, to remove unbound MAb, 50 μl of anti-mouse immunoglobulin-alkaline phosphatase conjugate (Sigma) diluted 1:200 in TEN buffer supplemented with 2% bovine serum albumin was added to each well. Following incubation at 37°C for 30 min with the conjugate, the plates were washed five times with TEN buffer and dried, and 200 μl of 1 mg of phosphatase substrate (*o*-nitrophenyl phosphate; Sigma) per ml diluted in diethanolamine buffer (97 ml of diethanolamine, 100 mg of $MgCl_2 \cdot 6H2_2O$, 0.2 mg of NaN_3 per liter [pH 9.8]) was added to each well. After a 30-min incubation period at 37°C, the A_{405} was read by using an ELISA reader (Bio-Tek, Inc.). Culture supernatants which produced an A_{405} of 0.2 or greater were considered positive.

LPS ELISA. The LPS ELISA was done by following the whole-cell ELISA protocol described previously (1). Briefly, detoxified LPS diluted to 50 μg/ml in PBS was used as antigen. Polyvinyl chloride microtiter plates (Dynatech) were coated with 50 μl of LPS (2.5 μg of LPS per well) and incubated at 37°C overnight to dry the plates.

TABLE 1. Slide agglutination reactions for five anti-*V. vulnificus* serum specimens produced in rabbits immunized with formalin-killed *V* whole-cell vaccines tested against the vaccine strains

V. vulnificus strain tested[a]	Anti-*V. vulnificus* sera produced against strain[b]:				
	1002	1003	1005	1006	1008
1002	++	+	+	+	+
1003	+	++	+	0	++
1005	0	++	++	++	++
1006	0	++	++	++	++
1008	0	0	0	++	++
27562	0	++	++	++	++

[a] Live cell suspensions.

[b] Reactions were scored as follows: ++, when discernible agglutination occurred within 1 min; +, when agglutination occurred in 1 to 2 min; 0, no agglutination.

RESULTS AND DISCUSSION

The O antigens of *Vibrio cholerae* proved to be valuable markers in both ecological and epidemiological studies. O-antigen typing of *V. cholerae* into O1 and non-O1 serogroups (6) provided the basis to distinguish serologically between the O1 Asiatic cholera agent and the usually less severe infections caused by non-O1 serogroups.

The success of O serotyping, when applied to *V. cholerae*, gave impetus for us to use this approach with *V. vulnificus*. In 1984, an O serological typing scheme which identified seven *V. vulnificus* serovars was proposed by Shimada and Sakazaki (19). They immunized rabbits with heat-killed whole-cell vaccines to produce serovar-specific antiserum, which was absorbed with heterologous serovars to abolish nonspecific reactions. High mortality rates in those who contract *V. vulnificus* infections, often following ingestion of raw oysters, has attracted considerable attention in the public health sector and regulatory agencies. It was of interest to assess the distribution of O serovars among the *V. vulnificus* isolates recovered from victims in Louisiana and to determine whether specific serovars segregated with each of the two infection modes.

Antisera raised in rabbits immunized with whole-cell vaccines prepared with formalin-killed strains 1002, 1003, 1005, 1006, and 1008, when they were tested by slide agglutination, produced nonreciprocal, nonspecific agglutination patterns (Table 1). For example, anti-1008 serum reacted with each of six *V. vulnificus* strains tested, whereas antiserum raised to strains 1002, 1003, and 1005 did not agglutinate 1008 cells. These results were consistent with the findings of others (19). Similar nonspecific agglutination patterns were observed when sera from rabbits immunized with heat-killed whole-cell vaccines were tested (data not shown).

Historically, polyclonal typing serum has been rendered specific for the target antigen through dilution or absorption to eliminate or remove heterologous serological activity. Unlike the experience with *V. cholerae* (1, 2), rabbits immunized with formalin-killed or heat-killed *V. vulnificus* whole-cell vaccines produced low-level tube agglutination titers, which ranged from 20 to 160. As a consequence, a 1:5 dilution of each anti-*V. vulnificus* serum specimen abolished discernible slide agglutination reactions to both the heterologous and homologous vaccine strains. It is possible that the presence of an extracellular, nonimmunogenic capsular polysaccharide interfered with or masked optimal immunization to the cell wall-associated O antigens (19). Alterna-

TABLE 2. Slide agglutination reactions for five affinity-purified anti-LPS preparations obtained by affinity chromatography from polyclonal antiserum tested against live *V. vulnificus* cell suspensions

Strain tested[a]	Affinity-purified anti-LPS prepared from antiserum to strain[b]:				
	1002	1003	1005	1006	1008
1002	++	0	0	0	0
1003	0	++	0	0	0
1005	+	++	+	++	0
1006	0	0	0	++	0
1008	+	0	0	0	++
27562	0	0	+	0	0

[a] Live cell suspensions.
[b] Reactions were scored as follows: ++, when discernible agglutination occurred within 1 min; +, when agglutination occurred in 1 to 2 min; 0, no agglutination.

tively, antibody production to an immunogenic capsule, expressing non-serovar-specific antigens, may have obscured the desired specificity (11, 12).

In an effort to render the anti-*V. vulnificus* sera (Table 1) serovar specific, anti-LPS activity from each antiserum was immobilized onto and eluted from AH-Sepharose beads armed with LPS by affinity chromatography. Each of the five anti-LPS preparations was retested by slide agglutination with live cells (Table 2) and with heat-killed and formalin-fixed cells (data not shown). Heat-killed and formalin-fixed cells were more prone to autoagglutination in control serum or to produce nonreciprocal agglutination patterns in anti-LPS reagents.

Although cross-reactions were reduced and, in some instances, eliminated, anti-LPS agglutination reactions were not sufficiently specific to warrant the time expended to prepare these reagents. The agglutination profiles (Table 2) were used as the basis to identify potential *V. vulnificus* serovars and candidate strains for MAb production. Strains 1001, 1002, 1004, 1005, and 1009 were selected for the first round of fusion experiments.

Hybridomas were screened by LPS ELISA and whole-cell ELISA, in the event that during the extraction process the Westphal and Jann (24) phenol-water procedure altered or degraded LPS epitopes. None of the MAbs screened exhibited the extensive serological cross-reactions that polyclonal sera exhibited.

Table 3 compares the ELISA reactions produced by polyclonal anti-1002 and anti-1005, which were tested against 17 LPS preparations, with those produced by anti-1002 and anti-1005 MAbs. The results revealed that *V. vulnificus* expresses unique or serovar-specific LPS-associated epitopes. The nonspecific cross-reactions which polyclonal antisera exhibited did not occur with anti-*V. vulnificus* LPS MAb. For example, polyclonal anti-1005 reacted with each of 17 LPS preparations tested, whereas anti-1005 MAb (clone SM-11) reacted exclusively with LPSs prepared from strain 1005, 1006, and 27562 LPSs (Table 3). These findings suggest that these three strains represent a single serovar, while strains 1002, 1007, and E4125 constitute members of a second serovar. The vaccine strains 1002 and 1005 were recovered from blood cultures taken from two victims with primary septicemia, both of whom had eaten raw oysters.

Five *V. vulnificus* O serovars were identified with MAb (Table 4), and a possible sixth serovar was identified with affinity-purified anti-1008 LPS immunoglobulin (Table 2). In addition, MAbs produced to LPS extracted from *V. damsela* (clone SM-15) and *V. hollisae* (clone SM-19) did not react with the 17 *V. vulnificus* LPS preparations (Table 4), nor did anti-*V. vulnificus* LPS MAb react with the LPS prepared

TABLE 3. Serovar specificity of MAbs versus polyclonal antisera for LPS-associated antigens extracted from 17 clinical *V. vulnificus* isolates

LPS extracted from strain:	ELISA reaction for[a]:			
	Anti-1002		Anti-1005	
	Poly Ab	MAb	Poly Ab	MAb
1000	0	0	++	0
1001	+	0	++	0
1002	++	++	++	0
1003	++	0	+	0
1004	++	0	+	0
1005	+	0	++	++
1006	0	0	++	++
1007	+	++	++	0
1008	+	0	++	0
1009	0	0	+	0
1010	+	0	++	0
1012	+	0	+	0
1013	+	0	+	0
1014	+	0	+	0
1015	+	0	+	0
E4125	+	++	++	0
ATCC 27562	+	0	+	++

[a] ELISA reactions were scored as follows: +, optical densities at 405 nm of 0.2 to 0.9; ++, optical densities at 405 nm of 1.0 and greater; 0, optical densities at 405 nm below 0.2. Polyclonal antiserum (Poly Ab) was raised in rabbits immunized with formalin-killed strain 1002 and 1005 whole-cell vaccines. Clones SM-1 and SM-11 were used for anti-1002 and anti-1005 MAbs, respectively.

TABLE 4. Anti-LPS MAb tested by LPS ELISA against LPS prepared from 17 *V. vulnificus* strains and *V. damsela* and *V. hollisae*

LPS[a]	Anti-LPS secreting clone[b]:						
	SM-3	SM-1	SM-29	SM-11	SM-39	SM-15	SM-19
846	0[b]	0	0	0	0	0	0
1000	0	0	0	0	0	0	0
1001	++	0	0	0	0	0	0
1002	0	++	0	0	0	0	0
1003	0	0	0	0	0	0	0
1004	0	0	++	0	0	0	0
1005	0	0	0	++	0	0	0
1006	0	0	0	++	0	0	0
1007	0	++	0	0	0	0	0
1008	0	0	0	0	0	0	0
1009	0	0	0	0	++	0	0
1010	0	0	0	0	0	0	0
1012	0	0	0	0	0	0	0
1013	0	++	0	0	0	0	0
1014	0	0	0	0	0	0	0
1015	0	0	0	0	0	0	0
E4125	0	++	0	0	0	0	0
27562	0	0	0	++	0	0	0
V. damsela	0	0	0	0	0	++	0
V. hollisae	0	0	0	0	0	0	++

[a] LPS was extracted from the indicated *V. vulnificus* strain numbers and from *V. damsela* and *V. hollisae*.
[b] ELISA reactions were scored as follows: +, optical densities at 405 nm of 0.2 to 0.9; ++, optical densities at 405 nm of 1.0 and greater; 0, optical densities at 405 nm of below 0.2.

TABLE 5. Porposed serological varieties (serovars) of *V. vulnificus* LPS-associated O antigens detected with MAbs

Serovar no.	Vaccine strain		MAb clone designation	No. of clinical isolates reacted[a]
	No.	Source		
1	1005	Primary septicemia	SM-11	3
2	1001	Primary septicemia	SM-3	1
3	1002	Primary septicemia	SM-1	4
4	1004	Diarrhea	SM-29	1
5	1009	Primary septicemia	SM-39	1
6[b]	1008	Diarrhea	NA[c]	2

[a] Number of clinical *V. vulnificus* isolates which reacted with MAb by ELISA and slide agglutination of a total of 16 strains tested.
[b] Tentative serovar, on the basis of studies with affinity-purified anti-LPS polyclonal antibody.
[c] NA, not applicable.

from these two vibrios. The *V. vulnificus* serovars identified thus far were designated 1 through 6 (Table 5). Serovar 1 in our scheme is apparently the same as serogroup 1 in the Sakazaki and Shimada scheme (19), since the anti-serovar 1 reagent in both schemes agglutinated *V. vulnificus* ATCC 27562.

Vaccine strains for serovars 1, 2, 3, and 5 were prepared from isolates recovered from victims with primary septicemia. These four MAbs reacted by ELISA with 9 of the 11 *V. vulnificus* isolates recovered from primary septicemia victims but did not react with the 6 strains isolated from stool specimens or wound exudates. Anti-1004 MAb, serovar 4, reacted with the homologous vaccine strain only. A 16-fold reduction in the ELISA titer was observed when anti-1005 MAb was tested against opaque versus translucent strain 1005 cells (Table 6). By contrast, there was no obvious difference in titer when LPS from opaque and translucent 1005 cells served as the antigen in ELISA. The difference was not as obvious when polyclonal anti-1005 serum was tested under the same conditions (Table 6). The whole-cell ELISA titers suggest that LPS epitopes are more accessible on the surfaces of translucent cells than they are on the surfaces of opaque cells. This finding implies that the extracellular capsule or slime layer which shrouds opaque cells may obstruct access of the MAb to LPS-associated epitopes (3, 25, 26).

TABLE 6. ELISA titers for polyclonal antibody and MAb tested against LPS extracted from opaque encapsulated and translucent nonencapsulated colony phenotypes of *V. vulnificus* 1005 and against whole-cell preparations of these two colony types

V. vulnificus 1005		ELISA titer for[a]:		
		Polyclonal antibody raised to[b]:		Anti-1005 MAb (clone SM11)
Colony type	Antigen[c]	1005 (O)	1005 (T)	
Opaque	LPS	64,000	256,000	4,000
Translucent	LPS	64,000	256,000	4,000
Opaque	Whole cell	8,000	8,000	4,000
Translucent	Whole cell	16,000	16,000	64,000

[a] Values are reciprocals of the greatest dilution of polyclonal serum or MAb which produced an ELISA reaction with an optical density at 405 nm of 0.2 or greater.
[b] Produced in rabbits immunized with formalin-killed whole-cell vaccines prepared from the opaque (O) and translucent (T) colony phenotypes.
[c] Antigen preparation used in the ELISA.

Polyclonal antisera, produced in rabbits immunized with whole-cell vaccines, failed to discriminate O serovars among the 17 clinical *V. vulnificus* isolates examined. The use of anti-LPS immunoglobulin, which was affinity purified from polyclonal sera, eliminated much of the heterologous agglutination observed with the whole sera (Tables 1 and 2), but some preparations retained nonspecific activity possibly attributable to anti-rough core activity. Serological discrimination of *V. vulnificus* serovars predicated on detection of LPS antigens is possible by use of MAbs. The MAb, when purified and concentrated from culture or ascitic fluid, agglutinates cell suspensions of the homologous *V. vulnificus* serovar by the slide test within 1 to 2 min. The five serovar-specific MAbs (Tables 4 and 5) reacted with 10 of the 17 clinical *V. vulnificus* isolates tested, which suggests there is greater O serological heterogeneity in *V. vulnificus* LPS than was previously suspected (19). Because the majority of isolates used in this study were collected in Louisiana, there may be a greater universal diversity of LPS antigens than was suggested in previous reports (19).

To be of value in epidemiological and environmental studies, the O typing scheme will have to be expanded to accommodate all possible LPS serovars exhibited by this species. The proposed typing scheme identified serovar-specific antigens associated with the LPS, to which the scheme used in this study only alluded (19). In addition, MAb exhibits the desired serological specificity which polyclonal serum fails to display. It would save much confusion if the two schemes were amalgamated relative to serovar nomenclature.

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