

Vibriocidal Antibody Responses in North American Volunteers Exposed to Wild-Type or Vaccine *Vibrio cholerae* O139: Specificity and Relevance to Immunity

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The emergence of a new agent of cholera, *Vibrio cholerae* O139, has prompted a reevaluation of the vibriocidal antibody assay. This assay, primarily directed to lipopolysaccharide, is an important correlate of O1 immunity. *V. cholerae* O139 strains are encapsulated, rendering them relatively resistant to killing by serum. Recent reports suggest that there is strain-to-strain variability in the sensitivity of the vibriocidal assay to fully encapsulated O139 strains. We have assessed a modified vibriocidal assay for fully encapsulated O139 strain AI-1837 and its unencapsulated mutant 2L in sera from 53 volunteers given wild-type AI-1837 or its attenuated derivative CVD 112 and from 48 controls challenged with *V. cholerae* O1 or strains of the family *Enterobacteriaceae*. Vibriocidal responses to the AI-1837 and 2L strains were seen in 67 and 89% of volunteers, respectively, following a single exposure to the wild-type strain. However, >50% of all controls had low-level vibriocidal responses to both strains. These nonspecific responses were transient and of the immunoglobulin G isotype. No binding activity against purified O139 lipopolysaccharide (LPS) by immunoblotting was seen in control sera. In contrast, vibriocidal assay and strain 2L LPS responses by immunoblotting were detectable in 91% of tested volunteers following a single exposure to O139. The presence of vibriocidal antibody to AI-1837 or 2L was not associated with protection in rechallenge studies with O139 strain AI-1837. The vibriocidal assay with unencapsulated strain 2L may be used to detect exposure to O139 strain AI-1837 in controlled research trials. However, its lack of specificity does not make it useful for determining exposure to *V. cholerae* O139 in the field.

Until 1992, only *Vibrio cholerae* strains of O group 1 (O1 strains) were associated with cholera. However, in late 1992, a previously unidentified toxigenic non-O1 strain of *V. cholerae*, O139 Bengal, was recognized as the etiologic agent of epidemic cholera disease. It appeared in Bangladesh and southern and eastern India and spread to other parts of Asia (2). Although these O139 strains have since been shown to be genetically closely related to epidemic *V. cholerae* O1 El Tor strains, prior immunity to O1 El Tor did not seem to be protective since adults as well as children were vulnerable to severe disease from this agent (1). These O139 strains contain a distinct semirough lipopolysaccharide O side chain and a polysaccharide capsule (10, 16).

Serum vibriocidal activity has long been recognized as a correlate of protective immunity in *V. cholerae* O1 infections (8, 18, 19). The serum vibriocidal response requires the fixation of complement by antibody specifically bound to vibrios resulting in bactericidal activity. Vibriocidal titers have been shown to rise with age in areas where O1 cholera is endemic, whereas the incidence of cholera declines with increasing age from early childhood. Extensive studies with North American volunteers challenged with wild-type *V. cholerae* O1 and with promising O1 vaccine strains have demonstrated a rapid induction in serum vibriocidal activity following primary exposure; vibriocidal activity can be detected as early as day 7,

peaking by day 10, with a marked decline by 6 months (4). In those studies we have shown that, following primary exposure in naive North American volunteers, the immunoglobulin isotype responsible for this activity is immunoglobulin M (IgM) (15).

Recent studies evaluating the usefulness of the vibriocidal assay for O139 infections have produced conflicting results. In challenge studies of naive North American volunteers with wild-type O139 strain AI-1837 and vaccine candidate strain CVD 112, we were unable to detect any vibriocidal activity in these subjects when standard vibriocidal assay parameters were applied by using AI-1837 as a target strain (17, 25). Variation in the susceptibilities of several O139 strains to vibriocidal activity was found in a study evaluating convalescent-phase sera from Bangladeshi patients infected with *V. cholerae* O139 (21). However, that study did suggest that a modified vibriocidal assay could be developed, although questions of its specificity were not fully explored.

Because of the possibility that the polysaccharide capsule may interfere with complement-mediated killing, we have established a modified vibriocidal assay using another O139 target strain, strain 2L, an unencapsulated insertion mutant of parent strain AI-1837 which retains the truncated O side chain. In addition, we attempted to further characterize the site of activity of the 2L vibriocidal response using strain 6a, a *TnphoA* insertion mutant of the encapsulated AI-1837 strain which retains only core oligosaccharide (5). We evaluated the usefulness of these assays in detecting serologic responses following exposure and the specificity of the responses using sera collected from studies conducted with our North American volunteer population challenged with *V. cholerae* O139 and O1

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TABLE 1. Vibriocidal responses to two O139 *V. cholerae* strains, the fully encapsulated strain AI-1837, or its capsule deficient mutant, 2L, in North American volunteers given wild-type O139 *V. cholerae* strain AI-1837 or its vaccine derivative, CVD 112

Study challenge strain, conditions ^a	AI-1837		2L	
	No. positive/total no. tested (%) ^b	Reciprocal peak vibriocidal GMT (range)	No. positive/total no. tested (%) ^b	Reciprocal peak vibriocidal GMT (range)
O139 AI-1837, first exposure	21/41 (51)	36 (10–80)	35/41 (85)	128 (20–320) ^c
O139 AI-1837, second exposure	7/9 (78)	98 (20–1280)	9/9 (100)	118 (20–640)
O139 CVD 112, first exposure	10/12 (83)	40 (20–80)	11/12 (92)	90 (40–160)
O139 AI-1837, second exposure	8/8 (100)	57 (10–80)	8/8 (100)	112 (40–320) ^c

^a Some volunteers were challenged twice, with first exposures being to wild-type strain AI-1837 or vaccine candidate strain CVD 112. Second exposure studies were all with wild-type strain AI-1837.

^b A fourfold rise in titer from pre- to postchallenge was considered significant.

^c Levels of vibriocidal antibody activity determined in the assay with strain 2L are significantly higher than those determined with strain AI-1837 ($P < 0.05$; paired t test).

and with organisms of the family *Enterobacteriaceae*. Finally, we also evaluated the correlation between O139 vibriocidal activity and protection with our secondary challenge studies with wild-type *V. cholerae* O139.

MATERIALS AND METHODS

Subjects. Sera from 53 subjects participating in four *V. cholerae* O139 vaccine candidate or wild-type challenge studies conducted at the Center for Vaccine Development were evaluated (17, 25). Nine subjects who received either 10^4 or 10^6 CFU of the wild-type O139 Bengal AI-1837 strain (isolated from a patient with diarrhea at the International Center for Diarrheal Disease Research, Bangladesh, and supplied for these challenge studies by John Albert) were rechallenged with 10^6 CFU of *V. cholerae* O139 AI-1837 3 months later. In separate studies, 8 of 12 volunteers who ingested 10^8 CFU of an attenuated O139 vaccine strain, strain CVD 112, derived from O139 strain AI-1837 were subsequently challenged 1 month later with 10^6 CFU of the wild-type parent strain. In addition, 32 volunteers who served as controls in these studies and who were challenged only with the wild-type O139 strain at 10^6 CFU were included. We assessed the specificity of the modified vibriocidal assay with sera from 12 subjects who received wild-type *V. cholerae* El Tor O1 Ogawa or Inaba strains and 48 volunteers who participated in challenge studies with various members of the family *Enterobacteriaceae*: 16 received wild-type *Shigella flexneri* 2a, 5 received wild-type enteropathogenic *Escherichia coli* (EPEC) E2348/69 or an EPEC vaccine candidate (CVD 206), 5 received wild-type enteroaggregative *E. coli* (EAggEC) 042, 34b, or 17-2 strains, and 5 received vaccine strain *Salmonella typhi* CVD 906. The study design and description of the volunteers have been detailed in previous reports (7, 9, 12, 17, 20, 25, 26).

Serum collection. For the O139 studies, sera were collected prior to each challenge and then at 7 or 10, 21, and 28 days following exposure. Prechallenge serum samples were available for all volunteers. For volunteers participating in *V. cholerae* El Tor studies, serum samples were available on days 7 or 10 and days 21 and 28; for volunteers challenged with EPEC, EAggEC, *Salmonella*, and *Shigella* strains, sera were available on day 7 or days 14, 21, and 28.

Immunology. Titers of vibriocidal antibody were determined against two fully encapsulated O139 strains, wild-type strain AI-1837 and strain CVD 112, and two unencapsulated derivatives of AI-1837, designated 2L and 6a. Strain 6a is a *TnphoA* insertion mutant of AI-1837 which lost all ability to express capsule and to produce lipopolysaccharide (LPS) beyond the core oligosaccharide (5). Strain 2L is a *TnphoA* mutant of AI-1837 that was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting (immunoblotting) to produce the low-molecular-weight LPS of O139 but not the higher-molecular-weight capsule.

Standard vibriocidal assay methodology was optimized from previously described methods (4). For the fully encapsulated strains AI-1837 and CVD 112, the modification entailed decreasing the bacterial inoculum used in the assay from 2.5×10^7 CFU/ml, which is the standard inoculum concentration used for O1 strains, to 2.5×10^6 CFU/ml and maintaining the guinea pig complement at a 1:10 dilution. Only opaque, fully encapsulated bacteria were used. The vibriocidal assay was further modified for the 2L strain to control for this strain's increased sensitivity to complement. By using a checkerboard titration of guinea pig complement and bacterial concentration, the nonspecific killing effects of complement were abrogated by using a 1:50 final dilution of complement. The titer of vibriocidal activity was determined by measuring the highest dilution of serum producing a $\geq 60\%$ reduction in the concentration of bacteria. A fourfold or greater increase between prechallenge and postchallenge titers signified a significant immune response.

To evaluate the immunoglobulin isotype involved in serum samples in which we detected vibriocidal activity, sera were separated into IgG and IgM-IgA isotype fractions by ion-exchange chromatographic extraction as described pre-

viously (15). The recovery of IgG and IgM in both fractions was determined by quantitative radial immunodiffusion assay (Accraassay; ICN Immunobiologics, Costa Mesa, Calif.), and the result was compared to that for whole serum to assess isotype purity. Every IgM and IgG fraction was then lyophilized and reconstituted back to the original immunoglobulin isotype concentration in whole serum by using sterile water; modified vibriocidal titers were determined for each fraction. In cases in which IgG fractions contained vibriocidal activity, protein A adsorption of whole sera was performed with insoluble protein A-bearing staphylococcal beads (Sigma, St. Louis, Mo.) as described previously (13). Levels of total IgG prior to and after adsorption were determined by an enzyme-linked immunosorbent assay to detect low levels of IgG; the assay was modified from one that was developed for IgA (14). In this assay, a standard curve was generated by using purified human IgG (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) and mouse monoclonal antibody generated against the Fc portion of IgG. The limit of detection of IgG in this assay is 100 ng/ml. Protein A-adsorbed fractions containing less than 5% total IgG were then run in the modified vibriocidal assay.

The specificity of the responses in the vibriocidal assay was further evaluated by performing IgG and IgM immunoblot analysis against LPS extracted from strain 2L by using sera from volunteers participating in O139 AI-1837 or CVD 112 studies or from volunteers who received strains of *V. cholerae* O1 or strains of the family *Enterobacteriaceae*. Sera collected prior to challenge and at the time of peak vibriocidal activity following exposure were tested by standard immunoblot methodology (5).

RESULTS

Serum vibriocidal responses to *V. cholerae* O139 strains AI-1837, 2L, and 6a. The serum vibriocidal responses generated by our volunteers to the encapsulated O139 strain AI-1837 and mutant strains 2L by the modified vibriocidal assay protocols are presented in Table 1. When the O139 test strain inoculum was decreased relative to the amount of complement present in the system, we detected vibriocidal activity in the majority of volunteers after oral challenge with either wild-type or vaccine candidate strains. This vibriocidal activity to AI-1837 was extremely low, however, with peak reciprocal geometric mean titers (GMTs) of less than 80. No significant differences were seen in peak GMT when CVD 112 was substituted as the test strain in the assay (data not shown). Almost all detectable vibriocidal activity was short-lived, peaking at 7 to 10 days after exposure and with all titers being $\leq 1:20$ by days 21 to 28. A secondary vibriocidal response to the fully encapsulated strain AI-1837 was in detected in 78 and 100% of volunteers following either wild-type or vaccine exposure, respectively, 3 months or 1 month previously ($P > 0.05$; chi-square test).

In contrast, when unencapsulated strain 2L was used as the target, exposure to wild-type *V. cholerae* O139 or to vaccine strain CVD 112 elicited detectable vibriocidal responses to 2L in 85 and 92% of volunteers, respectively. The vibriocidal assay against the 2L strain detected responses in 88% of the volunteers with primary exposures to O139 strains, whereas it detected responses in only 67% of volunteers with primary expo-

TABLE 2. Vibriocidal responses to two O139 *V. cholerae* strains, the fully encapsulated strain AI-1837, or its capsule-deficient mutant, strain 2L, in North American volunteers who ingested strains of *V. cholerae* O1 El Tor, EPEC, EAggEC, *S. typhi*, and *S. flexneri* 2a

Study challenge strain	AI-1837		2L	
	No. positive/total no. tested (%) ^a	Reciprocal peak vibriocidal GMT (range)	No. positive/total no. tested (%) ^a	Reciprocal peak vibriocidal GMT (range)
<i>V. cholerae</i> O1 El Tor Inaba or Ogawa	4/6 (66)	32 (20–40)	3/12 (25)	64 (20–80)
<i>S. flexneri</i> 2a	5/6 (83)	71 (10–160)	4/16 (25)	84 (40–160)
EPEC E2348-69 (O127:H6)	5/5 (100)	92 (80–160)	5/5 (100)	80 (80)
EAggEC 17-2, 34b, JM221	NT ^b		5/5 (100)	160 (80–320)
<i>S. typhi</i> CVD 906	NT		0/5 (0)	10

^a A fourfold rise in titer from pre- to postchallenge was considered significant.

^b NT, not tested.

responses to the fully encapsulated parent ($P < 0.05$; chi-square test). These responses against 2L peaked at days 7 to 10 after exposure and were still detectable at day 28 after exposure in 91% of the volunteers. In addition, the level of vibriocidal activity generated with the 2L strain as the test organism significantly surpassed those seen to the fully encapsulated O139 strain AI-1837 in several studies (see paired *t* test results in Table 1). After secondary wild-type O139 exposures vibriocidal responses against strain 2L were detected in all volunteers. Vibriocidal responses against strain 6a, a strain containing no specific O139 side chain, were detected in only 3 of the 41 (7%) individuals who had primary exposure to wild-type strain AI-1837 (reciprocal GMT, 42; range, 10 to 160).

Specificity of the serum vibriocidal responses to strains AI-1837 and 2L. We detected vibriocidal activity against the fully encapsulated strain AI-1837 and its unencapsulated derivative, strain 2L, in volunteers who had not been exposed to O139 strains but who had been challenged with EPEC, EAggEC, *S. flexneri* 2a, or *V. cholerae* O1 El Tor (Table 2). The observed vibriocidal activity was of low magnitude (reciprocal GMT, 58; range, 10 to 160). In contrast to the responses generated in *V. cholerae* O139-exposed individuals, these nonspecific responses were short-lived and were detectable only at 7 days after challenge.

This transient nature of the O139 vibriocidal activity seen in control sera prompted us to verify that antibody was responsible for this phenomenon. IgG and IgM were purified by chromatography from paired sera from 20 volunteers challenged with *Shigella*, EPEC, or EAggEC or with one or two

exposures to wild-type *V. cholerae* O139 AI-1837. All vibriocidal activity was contained in the IgG fractions of all control sera from volunteers or in the sera of volunteers undergoing secondary challenges with wild-type O139 (Table 3). Vibriocidal IgM antibody activity was detected in only six of eight (75%) volunteers following primary exposure to *V. cholerae* O139. In order to verify that the vibriocidal activity in the IgG fraction was due to antibody, the whole-serum samples containing detectable vibriocidal activity were then absorbed with protein A. Protein A absorption removed all vibriocidal activity in all samples but one, in which the activity decreased eightfold after absorption.

Immunoblot studies. Figure 1 demonstrates the migration patterns of the LPSs extracted from parent strain AI-1837 and its unencapsulated derivative, strain 2L. The LPS extracted from the opaque O139 strain, AI-1837, lacks the typical LPS ladder of O1 strains but retains the rapidly migrating low-molecular-weight truncated LPS moiety. Strain 2L, the translucent derivative of AI-1837, had the same migration pattern. To evaluate whether the control and O139-challenged sera with vibriocidal activity against strain 2L had antibody directed against the LPS moiety of the O139 strains, IgG and IgM immunoblots against 2L LPS were evaluated by using pre- and postchallenge sera. Results for a representative sample of paired sera binding to 2L LPS are presented in Fig. 2. Of the eight volunteers with primary exposure to wild-type O139, IgM responses to 2L LPS were seen in seven volunteers and IgG responses were detectable in four volunteers. All three volunteers who had two exposures to the wild type had both IgG and

TABLE 3. *V. cholerae* O139 AI-1837 vibriocidal activity in whole serum and serum immunoglobulin fractions of volunteers challenged with *V. cholerae* O139 strain AI-1837 or with EPEC or *S. flexneri* 2a

Challenge strain (no. of subjects tested)	<i>V. cholerae</i> O139 strain used as target	Peak reciprocal vibriocidal activity (GMT)			
		Whole serum	IgG fraction	IgM-IgA fraction	Protein A absorbed whole serum
EPEC (3)	AI-1837	80	110	20	20
	2L	160	160	20	20
<i>S. flexneri</i> 2a (3)	AI-1837	120	200	20	20
	2L	160	160	20	20
EAggEC (3)	2L	160	320	10	20
<i>V. cholerae</i> O139 AI-1837 (8), first exposure	2L	256	40	260 ^b	NT ^c
<i>V. cholerae</i> O139 AI-1837 (3), second exposure	AI-1837	560	320	20	50

^a Titers are represented as reciprocal GMTs for individual subjects for each challenge study evaluated.

^b Six of eight subjects had detectable IgM vibriocidal activity.

^c NT, not tested.

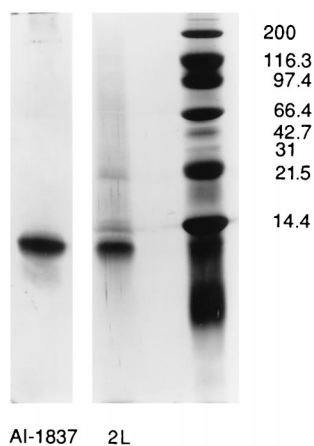


FIG. 1. Silver-stained polyacrylamide gel of LPS from *V. cholerae* O139 strain AI-1837 (parent) and acapsular mutant strain 2L. Molecular mass markers (in kilodaltons) are given in the far right (unlabeled) lane.

IgM antibodies that bound to the strain 2L LPS following the second exposure. We were unable to detect any IgG or IgM binding to the 2L LPS in any of the volunteers challenged with *V. cholerae* O1 or a strain of the family *Enterobacteriaceae*. Absorption of these sera with strain 2L, however, did result in complete removal of vibriocidal activity against strain 2L. Absorption of the sera with strain 6a did not influence the vibriocidal activity against strain 2L.

Lack of correlation between vibriocidal antibody and protection following rechallenge with the wild type. The presence of vibriocidal activity against the fully encapsulated strain AI-1837 following primary challenge with wild-type strain AI-1837 or vaccination with strain CVD 112 was not associated with protection on subsequent rechallenge with the wild-type strain ($P = 0.9$; chi-square test). Nine volunteers who initially received AI-1837 and eight volunteers who received the O139 vaccine candidate CVD 112 were rechallenged with AI-1837 1 month later. Of the 8 of 17 (47%) volunteers who generated detectable vibriocidal antibodies following primary exposure, 7 of 8 (88%) were fully protected from clinical disease following rechallenge. However, seven of nine (78%) volunteers who had no vibriocidal antibody to the encapsulated strain were also fully protected on rechallenge.

Likewise, the presence of vibriocidal activity against unencapsulated strain 2L following primary challenge with the wild-type strain or vaccination with CVD 112 was also not associated with protection on subsequent rechallenge with the wild-type strain ($P = 0.8$; chi-square test). Of the 15 of 17 volunteers (88%) who developed vibriocidal antibody against strain 2L after primary exposure, clinical disease occurred in two (13%) of these subjects after rechallenge with the wild-type strain. Of the two volunteers with no evidence of vibriocidal antibody against strain 2L, one was protected after rechallenge and one was not.

DISCUSSION

The vibriocidal assay has been found to be an extremely sensitive assay for determining exposure to *V. cholerae* O1 and, more importantly, has been found to be a correlate of immunity to O1 infections (8, 11, 18, 19). The challenge studies with the O139 vaccine strain and the wild-type strain conducted at our center and with the banked serum samples obtained from a variety of challenge studies with *V. cholerae* O1 and members

of the family *Enterobacteriaceae* offered a unique opportunity to extensively assess these new assays.

Recent studies by investigators in Bangladesh evaluating encapsulated strain AI-1837 using assay conditions similar to those used in the present study were unable to detect any vibriocidal antibody with sera from Bangladeshi patients recovering from O139 cholera (21). In contrast, other encapsulated strains were fully functional in eliciting vibriocidal activity in this population. In our hands, this assay detected exposure to the homologous O139 strain AI-1837 in 51 to 83% of volunteers who received a primary challenge with the wild-type O139 strain AI-1837 or vaccine strain CVD 112. It is impossible to know whether the discrepancies in the sensitivities of the assays using the encapsulated AI-1837 strain are due to differences in laboratory methodology or to differences in the O139 strains to which individuals were exposed. The answer to this question is particularly important since it has been proposed that the vibriocidal assay utilize encapsulated O139 strains to detect exposures to *V. cholerae* O139 (21). Our studies used completely homologous strains both for clinical and for laboratory studies. In contrast, the Bangladeshi sera came from patients whose infecting O139 strains were not characterized (21).

It is also possible that strain-specific differences in the degree of expression of capsule may account for the differences in susceptibility of our target strain AI-1837 to complement in the vibriocidal assay compared to those for other strains that reportedly had more activity in the vibriocidal assay. Inactivating the ability of expression of the capsule in AI-1837 by specific gene insertion resulting in strain 2L led to a significant increase in the sensitivity of this assay in detecting an initial exposure to *V. cholerae* O139. However, even these vibriocidal responses against strain 2L were of a low magnitude, never exceeding a titer of 1:640. This low level of vibriocidal activity may not be strain specific since similar vibriocidal antibody levels have been described for another unencapsulated O139 strain (6).

Peak vibriocidal activity to our O139 strains occurred primarily at 7 to 10 days following challenge and was fourfold lower or nondetectable by 28 days. Following exposure of naive hosts to *V. cholerae* O1, vibriocidal responses are composed almost entirely of IgM antibody, have a reciprocal GMT peak of $\geq 1,280$, peak at days 7 to 10, and remain detectable for as long as 6 months (4). Secondary vibriocidal responses to homologous O1 strains in North American volunteers are rarely

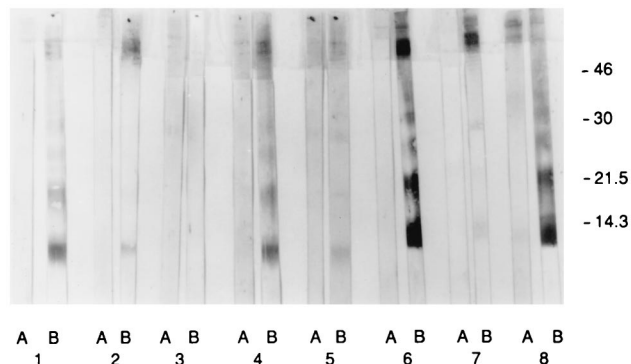


FIG. 2. IgM Western blot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated LPS extracted from strain 2L, the unencapsulated derivative of AI-1837 and prechallenge (A) and postchallenge (B) sera from eight volunteers (pairs of lanes labeled 1 to 8) given a primary challenge with wild-type *V. cholerae* O139 strain AI-1837. Molecular masses (in kilodaltons) are indicated to the right.

elicited within 4 months of primary exposure. When they occur, they are still predominately contained within the IgM fraction (15). In contrast, vibriocidal responses against our fully encapsulated O139 strain and its unencapsulated derivative were seen in most if not all volunteers following a second wild-type exposure. In addition, these secondary O139 responses and the vibriocidal activity demonstrated in the control sera were found only in the IgG fraction. The most likely IgG subclass responsible for this activity would be IgG3, since it has an extremely short half-life and readily aggregates and fixes complement. The lowered complement-fixing capacity of IgG compared to that of IgM probably also contributes to the modest magnitude of the vibriocidal response generated after O139 exposure and reexposure in our volunteers (22). Whether continued or more intensive exposure to O139 strains would generate vibriocidal activity of a higher magnitude cannot be answered by our studies, although secondary exposure tended to produce slightly higher titers in our volunteers.

We carefully evaluated our vibriocidal assays for their specificities. Using a range of sera from multiple time intervals following exposure, we have detected modest vibriocidal responses to our encapsulated and unencapsulated O139 strains in subjects participating in studies in which they were challenged with *V. cholerae* O1 and members of the family *Enterobacteriaceae*. We detected no cross-reactivity in the sera of subjects who ingested a live oral typhoid vaccine strain, CVD 906, but we detected low-level vibriocidal activity in more than half of the subjects exposed to various strains of *V. cholerae* O1, *S. flexneri* 2a, EPEC, and EAaggEC. By using the titers generated against our O139 strains in these control sera, reciprocal vibriocidal titers of ≤ 160 cannot be considered specific for O139 exposure (calculated by taking the reciprocal GMT plus 2 standard deviations of the peak vibriocidal titers).

These nonspecific responses could be differentiated from the specific responses in that they were extremely short-lived and were contained only in the IgG fraction. However, we were unable to demonstrate that these cross-reactive antibodies were directed against the LPS moiety of O139 since these sera lacked any detectable binding to the strain 2L LPS, as determined by Western blotting. It is possible that specifically binding epitopes were altered by the gel conditions since absorption with strain 2L but not strain 6a (which contains no O side chain) removed the vibriocidal activity from these control sera. Other investigators have identified strains of *V. cholerae* O22 and O155 and *Aeromonas trota* that cross-react with the O-antigen determinants of *V. cholerae* O139 (3, 24). For example, hyperimmune rabbit antiserum generated against *A. trota* strains has been shown to bind to the rapidly migrating LPS moiety from *V. cholerae* O139 on immunoblots, suggesting that our difficulties in demonstrating positive immunoblot data for our control sera may be due in part to low levels of cross-reacting antibody (3).

The vibriocidal antibody response against strain 2L produced by volunteers exposed to O1 vibrios and members of the family *Enterobacteriaceae* may represent anamnestic IgG antibody directed against O-side chain epitopes not represented in the blot or, perhaps, to poorly immunogenic shared sugar residues or even to protein membrane determinants. Loss of the O139 capsule has been shown to produce weak agglutination with O1 typing sera, suggesting the presence of shared antigens (5). It is also possible that there may be conservation of O-side chain antigenicity among some of these enteric strains, as demonstrated by hybridization studies linking non-O1 Bengal genes coding for O139 antigen with vibrios other than O139 (5).

It is apparent that the capsular polysaccharide of *V. cholerae*

O139 is chemically similar to its truncated O side chain and that they share some epitopes (27). However, we believe that the O side chain and capsular antigens in these strains also have distinct epitopes since volunteers challenged with wild-type strain AI-1837 or vaccine strain CVD 112 may respond to one and not the other purified LPS or capsular antigens (10, 17). The relatively low magnitude of the vibriocidal response against unencapsulated strain 2L also supports this contention. If the capsule and the O side chain were antigenically similar, the vibriocidal response to strain 2L should be markedly enhanced since removal of the capsule would allow for more effective antibody binding and complement fixation. These data may have relevance for vaccine development, since vaccines directed against LPS may have limited or reduced activity against fully encapsulated strains.

In addition to the problems of the nonspecificity of the vibriocidal response to either the encapsulated or unencapsulated strains, we have been unable to associate the presence of a vibriocidal response to the O139 strains AI-1837 or 2L and protection against wild-type rechallenge. However useful the O1 serum vibriocidal assay has been as a correlate for protective immunity for O1 cholera, there is general consensus that the protective antibody for O1 disease is mucosal rather than systemic. A recent study has demonstrated that anti-capsular polysaccharide serum orally administered to another fully encapsulated O139 strain, MDO-12, resulted in full protection against fluid accumulation and colonization in the suckling mouse challenge model (23). No vibriocidal antibody to the encapsulated strain was demonstrated in this protective serum.

In summary, the modified vibriocidal assay for fully encapsulated *V. cholerae* O139 strain AI-1837 and for the unencapsulated insertion mutant strain 2L produces a very modest vibriocidal response in volunteers challenged with *V. cholerae* O139 that is not specific. It is our opinion that the vibriocidal assay with unencapsulated strain 2L can be used as a marker for exposure to O139 AI-1837 vibrios, but only under well-controlled research conditions. The lack of specificity of this assay would suggest that it would not be appropriate for the routine detection of *V. cholerae* O139 exposure under field conditions. The specificity of a vibriocidal assay with other *V. cholerae* O139 strains with more apparent sensitivity to complement-mediated killing needs to be further evaluated before any O139 vibriocidal assay is applied in field sites.

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