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Factors Influencing Secondary Vibriocidal Immune Responses: Relevance for Understanding Immunity to Cholera

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Although serum vibriocidal activity is used extensively as a marker of immunity to O1 *Vibrio cholerae*, there are limitations in this assay to detect instances of reexposure. We define the conditions operative in producing secondary vibriocidal responses in North American volunteers primed with either wild-type *V. cholerae* or CVD 103-HgR live attenuated oral cholera vaccine and then challenged with wild-type *V. cholerae* 1, 4, or 6 months later. Secondary serum vibriocidal responses occurred under two distinct secondary challenge conditions. The first occurred when secondary challenge produced a breakthrough in clinical protection. Following secondary exposure, 14 of 22 (64%) and 1 of 29 (3%) subjects with and without vibrio stool excretion, respectively, had secondary responses ($P < 0.001$); 5 of 6 (83%) and 10 of 45 (22%) subjects with or without diarrhea, respectively, mounted a secondary response ($P = 0.006$). The second condition occurred in the presence of full clinical protection but was dependent on the time interval between exposures. No subject (0 of 17) vaccinated with CVD 103-HgR and given homologous wild-type challenge within 4 months mounted a secondary vibriocidal response compared with 8 of 11 (73%) vaccinated volunteers challenged at 6 months who developed a secondary vibriocidal response ($P = 0.0009$). The majority of the serum vibriocidal activity was of the immunoglobulin M (IgM) isotype, seen in 96 and 73% of subjects following primary and secondary exposure, respectively. Vibriocidal activity in the IgG fraction following primary and secondary exposures occurred with ≤50% of volunteers; lipopolysaccharide (LPS)-specific IgG1 and IgG3 subclass responses supported the vibriocidal isotype data. However, following primary exposure, IgG4 LPS responses predominated, occurring in 81% of responding volunteers. These data suggest that, under certain conditions of secondary exposure to *V. cholerae* O1 antigens, when there is sufficient active local immunity present, there is a block of vibrio antigen resampling at the M cell level. We discuss the implications of and possible explanations for these findings.

The vibriocidal antibody response is a bactericidal assay requiring the fixation of complement by antibody that is bound specifically to vibrios. Studies done in Bangladesh in the 1960s reported a rise in vibriocidal antibody with age with an impressive inverse relationship between the geometric mean vibriocidal titer and the susceptibility to disease (17, 18). Almost 20 years later, these studies were corroborated and expanded to show an additional association between the magnitude of vibriocidal antibody and intestinal colonization of O1 vibrios (9). These data offered ample evidence for using the vibriocidal assay as a marker for immunity to *Vibrio cholerae*. As a result, this assay became an essential tool in the assessment of exposure and immunity to O1 *V. cholerae.*

However, there are limitations in the usefulness of this assay. Approximately 40% of Bangladeshi family contacts exposed to cholera with asymptomatic or symptomatic infection had no increased vibriocidal response (9). We have previously reported a lack of secondary vibriocidal responses in North American volunteers given wild-type cholera challenges following both natural infection and vaccine-induced immunity (6, 13, 25). Recent vaccine trials of a cholera B subunit–whole-cell vaccine in Bangladesh have shown similar limitations (5). The factors influencing the production of vibriocidal responses have not, heretofore, been studied.

In this report, we have examined the variables operative in eliciting secondary vibriocidal responses in healthy North American volunteers who had been given primary exposure with either wild-type cholera or CVD 103-HgR live attenuated oral cholera vaccine followed by wild-type *V. cholerae* challenge. The immunoglobulin G (IgG) and IgM isotypes responsible for vibriocidal antibody activity in primary and secondary challenge studies were also determined to control for the possibility that an IgM-to-IgG isotype switch occurred between primary and secondary exposures in the absence of a detectable rise in whole serum secondary vibriocidal responses. This could theoretically happen since IgM antibody, which is the immunoglobulin isotype responsible for vibriocidal activity in primary exposure, has an increased efficiency in complement fixation (1, 23). In addition, since most of the vibriocidal activity is directed against the lipopolysaccharide (LPS) moiety of O1 vibrios, we evaluated the usefulness of determining the LPS-specific serum IgG subclass response in primary and secondary challenges (19). Our ultimate aim was to define the variables operative in eliciting primary and secondary serum vibriocidal responses and provide the rationale for the pursuit of alternative approaches to evaluate immunity to cholera that could be applied to field conditions.

MATERIALS AND METHODS

Subjects and volunteer studies. The vibriocidal responses from 69 volunteers participating in 10 different primary and secondary *V. cholerae* O1 exposure studies from 1982 to 1991 were reviewed. The 58 men and 11 women had a mean age of 25 years (range, 18 to 39 years). The screening, procedures, methods of informed consent, description of the volunteers, and treatment regimens (fluids and antibiotics) have been detailed in previous reports (6, 13, 14, 25).

The variables evaluated for possible association with a secondary vibriocidal

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TABLE 1. Primary and secondary O1 *Vibrio cholerae* challenge studies conducted at the Center for Vaccine Development with North American volunteers to evaluate variables important for the production of a secondary vibriocidal response[a]

Challenge
Homologous biotype and serotype strains
Primary and secondary exposure
El Tor Inaba N16961 (n = 4)
El Tor Ogawa E7946 (n = 9)
CVD 103 (*ctxA* mutant classical Inaba strain 569B) followed by wild-type classical Inaba 569B (challenge intervals, 1, 4, and 6 months) (n = 9, 3, and 11, respectively)
Classical Inaba 569B (n = 4)[b]
Homologous biotype and cross-serotype strains
Primary and secondary exposure
El Tor Inaba N16961 followed by El Tor Ogawa E7946 (n = 5)
El Tor Ogawa E7946 followed by El Tor Inaba N16961 (n = 5)
CVD 103 followed by classical Ogawa 395 (n = 7)
Cross-biotype and homologous serotype strains
Primary and secondary exposure
El Tor Inaba N16961 followed by classical Inaba 569B (n = 6)
CVD 103 followed by El Tor Inaba N16961 (n = 6)

[a] n = number of subjects evaluated in each group.
[b] Since this group of four subjects had a primary and secondary exposure interval of approximately 36 months, data were used only for interval analysis.

response were the development of illness and stool excretion of vibrios after secondary challenge, the time interval between primary and secondary exposure, and the nature of the strains used in secondary exposure studies with reference to primary exposure. Three different types of secondary exposure, characterized by biotype and serotype of the strains used, were studied (Table 1). Except for the analyses used to determine the significance of the interval between primary and secondary exposure, all secondary challenges took place 1 month after primary exposure. All challenge inocula contained 10^6 organisms given with $NaHCO_3$ to buffer gastric acidity.

To determine the importance of the time interval between primary and secondary exposures for secondary whole serum vibriocidal antibody responses, we evaluated three studies using 10^8 to 10^9 CFU of live oral *V. cholerae* O1 vaccine strain CVD 103-HgR as the initial exposure. CVD 103-HgR is the live oral *V. cholerae* O1 vaccine strain constructed by deleting 94% of the *ctxA* gene of classical Inaba strain 569B with the introduction of a gene encoding mercury resistance (11). Wild-type classical Inaba secondary challenges took place at 1, 4, and 6 months postvaccination (14, 25). In addition, we analyzed four volunteers given initial clinical infection with wild-type classical Inaba (one subject) or classical Ogawa (three subjects) organisms who were reexposed 3 years later with 10^6 CFU of wild-type classical Ogawa 395 organisms (13).

The IgG- and IgM-specific vibriocidal responses were assessed in volunteers in the primary and secondary exposure studies described in Table 1. Prechallenge and day 7 and day 21 postchallenge sera were evaluated. The IgG subclass-specific LPS responses were then determined in subjects participating in the CVD 103 vaccine studies (Table 1). The IgG vibriocidal responses generated from these studies were compared with the IgG subclass-specific anti-LPS responses.

Stool collection and bacteriology. Every stool produced by volunteers (except for CVD 103-HgR primary vaccination studies) from the time of admission to discharge was collected, the volume was measured, the consistency was recorded, and the sample was cultured as previously described (6, 25). Rectal swabs were taken from any volunteer who failed to produce a stool in a 24-h period. The number of challenge organisms per gram of stool was determined.

Definition of a diarrheal stool. Diarrhea was defined as one of the following: three or more loose stools, irrespective of volume, within 48 h; two or more loose stools within 48 h, totaling ≥200 ml; or one loose stool of at least 300 ml in volume.

Immunology. Sera were collected before and then on days 7 and/or 10 and 21 and/or 28 following each exposure. All sera were stored at −20°C, and all specimens from each individual were assayed simultaneously.

(i) Serum vibriocidal antibody assays. Titers of vibriocidal antibodies were determined in all sera against serotype Ogawa strain 79 and serotype Inaba strain 89 as previously described (6). A fourfold or greater increase between pre- and postexposure titers signified a seroresponse.

To determine the isotype distribution of the vibriocidal responses generated in primary and secondary exposure studies, sera were separated into IgG and IgM-IgA isotype fractions by ion-exchange chromatography, adapting a previously described method (24). Serum samples were fractionated on a QAE Sephadex A-50 gel (Pharmacia, Piscataway, N.J.) packed in a column (2.6 by 20 cm). The column was first washed with ethylenediamine and glacial acetic acid, pH 7.0. A 200-μl serum specimen was applied to the column, which was then washed with 5 ml of wash solution to elute IgG. The column was again washed; the IgM elution buffer (glacial acetic acid, sodium acetate water, and sodium chloride [pH 4.2]) was added. This eluate was brought back to neutral pH 7.4 to allow for functional antibody testing. The recovery of IgG and IgM in both fractions was determined by quantitative radial immunodiffusion (AccraAssay; ICN Immunobiologicals, Costa Mesa, Calif.) and compared with whole serum to assess the relative isotype purity and yield of each fraction. Every IgG and IgM fraction of each serum sample was reconstituted to its original concentrations in whole serum prior to measurement of vibriocidal activity.

(ii) Serum IgG subclass anti-LPS assays. IgG subclass-specific LPS responses were determined by an enzyme-linked immunosorbent assay (ELISA) system using specific antibodies to all four IgG subclasses. Assays were run as previously described for determining IgG antibodies to Inaba or Ogawa LPS except that sheep antiserum at a 1:500 dilution to IgG1, IgG2, IgG3, or IgG4 (ICN Immunobiologicals) was used as detection antibody in each assay (2). Each subclass-specific antiserum was produced by immunization of sheep with purified IgG1, IgG2, IgG3, or IgG4 myeloma proteins. Specificity of antisera for each subclass was determined by ELISA: at levels of IgG1 ≤ 0.1 mg/ml, no cross-reactivity was seen by IgG2, IgG3, or IgG4 antisera; at levels of IgG2 ≤ 0.5 mg/ml, no binding to IgG1, IgG3, or IgG4 antisera was detected by ELISA; at levels ≤ 1.0 mg/ml, no binding by either IgG1 or IgG2 antisera was detected to IgG3 or IgG4. Because of this, the initial starting dilution of test sera was established at 1:80 for IgG1 and IgG2 subclass determinations and at 1:40 for IgG3 and IgG4 subclass determinations. With 20 North American preimmunization serum samples, cutoff absorbance for determining positivity was determined to be 0.1 for each subclass at these dilutions. The limit of detection of these assays was 2 ng/ml. A fourfold rise in titer in sera obtained prior to and following each exposure was considered evidence of a seroresponse.

Statistical methods. Proportions of subjects who seroconverted in response to vaccination or wild-type challenge were compared by chi-square tests or Fisher's exact tests, when appropriate. When more than two groups were compared, resulting probabilities were adjusted by the Bonferroni approximation for multiple comparisons. Only two-tailed hypotheses were evaluated; probabilities of less than 5% were considered significant.

RESULTS

Factors influencing the development of a secondary vibriocidal response. For studies summarized in Table 1, in which the time interval between primary and secondary exposure was 1 month, vibriocidal seroresponse was seen in all volunteers after primary exposure but in only 15 of 51 (29%) after secondary exposure ($P < 0.001$, chi-square test).

(i) Type of secondary challenge. Most secondary vibriocidal responses followed cross-serotype challenges, occurring in 10 of 17 (59%) such exposures compared with 4 of 32 (13%) secondary vibriocidal responses generated after homotypic secondary exposures (Table 2, $P = 0.01$, Fisher's exact test). There was a suggestion that the magnitude of the initial vibriocidal response to the secondary exposure strain was inversely associated with the likelihood that a secondary response was generated when the time between primary and secondary exposure was 1 month. However, these differences were not statistically significant (Table 2).

(ii) Vibrio stool excretion or diarrhea following secondary challenge. Twenty-two of 51 (43%) volunteers excreted vibrios after secondary exposure. The average duration of vibrio excretion was 2.3 days (range, 1 to 5 days). A cross-serotype secondary challenge was more likely to result in stool vibrio excretion: 11 of 17 (65%) subjects given this type of exposure had positive stools compared with 11 of 23 (48%) subjects given either a homologous or a cross-biotype challenge ($P = 0.05$, chi-square test). Fourteen of these 22 subjects (64%) with vibrio excretion in their stools after secondary exposure had a detectable secondary vibriocidal response compared with only 1 of 29 (3%) subjects who had no excretion of vibrios ($P < 0.001$, chi-square test). There was no association with the titer of vibrios excreted and the generation of a secondary vibriocidal seroresponse. All 5 volunteers who had stool excretion at least 4 days after exposure had a secondary vibriocidal response compared with 9 of those 17 (53%) who excreted for 3 days or less ($P = 0.04$, Fisher's exact test).

TABLE 2. Vibriocidal responses in volunteers after primary exposure to CVD 103-HgR live attenuated *V. cholerae* or natural cholera infection and after various secondary wild-type *V. cholerae* O1 exposures 1 month later[a]

Group and type of secondary exposure (no. of subjects)	No. of responses after first exposure (%)[b]	No. of responses after second exposure[c]	Reciprocal vibriocidal GMT following first exposure	
			No. of subjects with a secondary response	No. of subjects without a secondary response
Group I, homologous biotype and serotype (22)	22/22 (100)	2/22 (9)[d]	905	1,631[e]
Group II, homologous biotype, cross-serotype (17)	17/17 (100)	10/17 (59)[f]	368	706[e]
Group III, cross-biotype, homologous serotype (12)	12/12 (100)	2/12 (17)[d]	80	2,744[e]

[a] A fourfold rise in titer postexposure was considered evidence of a seroresponse. Reciprocal vibriocidal GMT was generated against the serotype of the secondary exposure strain.
[b] Vibriocidal responses were measured against the primary exposure strain's biotype and serotype. The number of subjects with a fourfold rise in titer over the total number tested is reported.
[c] Vibriocidal responses were measured against both the primary and secondary exposure strain's biotype and serotype. The number of subjects with a fourfold rise in titer over the total number tested is reported.
[d] Vibriocidal response following primary exposure versus secondary exposure ($P < 0.001$, chi-square test).
[e] Magnitude of primary vibriocidal responses compared between those subjects with and without secondary vibriocidal responses. Comparisons were found not to be significant in groups I, II, and III ($P = 0.79$, 0.35, and 0.25, respectively, by two-tailed t test).
[f] Vibriocidal response following cross-serotype secondary exposure versus either homologous serotype exposure group ($P = 0.01$, Fisher's exact test).

Subjects who developed diarrhea were also more likely to mount a secondary vibriocidal response: 5 of 6 subjects (83%) with diarrhea had a secondary vibriocidal response compared with only 10 of 45 (22%) volunteers without diarrhea ($P = 0.006$, Fisher's exact test). The one volunteer who had diarrhea following secondary exposure but did not develop a secondary vibriocidal response was given a *V. cholerae* El Tor Inaba challenge after receiving *V. cholerae* CVD 103 vaccine. He just met criteria for diarrhea with three stools of 386 ml in total volume.

(iii) Time interval between challenges. The results are seen in Table 3. None of the 32 volunteers developed diarrhea on rechallenge. The serotype-specific vibriocidal antibody titer seen in all the volunteers prior to rechallenge was 4- to 200-fold lower than that generated after primary exposure ($P = 0.004$, Mann-Whitney U test).

There was variability in the production of a secondary vibriocidal response on secondary wild-type cholera exposure that was dependent on the time interval between primary and secondary exposures. All 17 subjects given wild-type *V. cholerae* within 4 months of vaccination had no secondary vibriocidal responses. At 6 months postvaccination, 8 of 11 (73%) rechallenged vaccinees had a detectable secondary vibriocidal response ($P = 0.0009$, Fisher's exact test; Bonferroni adjusted, group A versus group C). Two of four (50%) subjects with wild-type *V. cholerae* infection-derived immunity made a secondary vibriocidal response on wild-type rechallenge 33 to 36 months later. These results suggest that as the time interval between exposures increases, there is an increased likelihood that a secondary vibriocidal response will take place. When pre-secondary exposure vibriocidal activity was evaluated in subjects challenged at 4, 6, and 33 to 36 months, the level of pre-secondary exposure vibriocidal activity was not significantly different in those subjects who mounted a secondary vibriocidal response (pre-secondary exposure geometric mean titer [GMT], 106 [range, 20 to 640]), compared with those subjects who did not mount a secondary response (GMT, 123 [range, 20 to 1,280] [Mann-Whitney U test, $P = 0.5$]).

In order to evaluate whether asymptomatic infection contributed to the production of secondary vibriocidal responses, we examined the vibrio stool excretion data for challenged volunteers. No volunteer challenged within 4 months of primary exposure had a secondary serum vibriocidal response, and yet 33% (4 of 12) excreted vibrios. Seventy-three percent of volunteers (8 of 11) challenged 6 months after primary exposure mounted a secondary response, and yet only 4 of these 8 (50%) secondary seroresponders had evidence of vibrios in their stools after challenge. In all but one case, the titer of vibrios present in the stool was of a low magnitude, i.e., $\leq 10^3$/g of stool, and of short duration, i.e., ≤ 3 days.

Isotype distribution in primary and secondary vibriocidal responses. Results are presented in Table 4. After primary exposure, 43 of 45 (96%) subjects who mounted a detectable whole serum vibriocidal response also had an IgM isotype

TABLE 3. Primary and secondary vibriocidal responses in volunteers initially given CVD 103-HgR live attenuated oral cholera vaccine (groups A, B, and C) or classical biotype wild-type *V. cholerae* (group D) and then exposed to *V. cholerae* classical Inaba strain 569B (groups A, B, and C) or classical Ogawa strain 395 (group D)

No. with vibriocidal response after first exposure[a] (%)	Pre-first exposure GMT[b]	Post-first exposure peak GMT[b]	Time to second exposure (mo)	No. with vibriocidal response after second exposure (%)	Pre-second exposure GMT[c]	Post-second exposure GMT[d]
Group A, 9/9 (100)	47	1,218	1	0/9	320	275[e]
Group B, 3/3 (100)	10	6,450	4	0/3	160	100[e]
Group C, 10/11 (91)	17	4,238	6	8/11 (73)	202	1,451[f]
Group D, 4/4 (100)	42	14,481	33–36	2/4 (50)	67	226

[a] Number of subjects with a positive vibriocidal response over the total number of subjects tested.
[b] Reciprocal geometric mean whole serum vibriocidal titer. Peak antibody responses occurred 7 to 10 days after primary exposure.
[c] Serum sample obtained on the day prior to second exposure.
[d] Peak vibriocidal responses occurred 7 to 10 days after second exposure.
[e] Vibriocidal response of groups A and B after primary vaccination compared with that following second exposure ($P = 0.00001$, chi-square test).
[f] Secondary vibriocidal responses of group C vaccinees versus group A ($P = 0.0009$, Fisher's exact test; Bonferroni adjusted).

TABLE 4. Serum IgG- and IgM-specific vibriocidal responses following primary and secondary cholera exposure

Exposure (time)[a]	Response[b]					
	Whole serum		IgG		IgM	
	No. (%)	Peak GMT	No. (%)	Peak GMT	No. (%)	Peak GMT
First						
El Tor Inaba	4/4 (100)	2,560	1/4 (25)	40	4/4 (100)	2,153
El Tor Inaba	5/5 (100)	1,940	2/5 (40)	80	5/5 (100)	2,229
El Tor Ogawa	8/9 (89)	1,016	3/9 (33)	50	8/9 (89)	947
CVD 103-HgR	9/9 (100)	1,218	3/9 (33)	50	9/9 (100)	1,742
CVD 103-HgR	3/3 (100)	6,450	0/3	20	3/3 (100)	6,451
CVD 103-HgR	10/11 (91)	4,238	4/11 (36)	80	10/11 (91)	2,119
Classical Inaba	4/4 (100)	1,810	2/4 (50)	80	4/4 (100)	1,522
Second						
El Tor Inaba (1 mo)	0/4	1,076	0/4	37	0/4	590
El Tor Ogawa (1 mo)	2/5 (40)	557	0/5	71	2/5 (40)	422
El Tor Ogawa (1 mo)	0/9	508	2/9 (18)	118	0/9	54
Classical Inaba (1 mo)	0/9	275	0/9	47	0/9	160
Classical Inaba (4 mo)	0/3	100	0/3	20	0/3	64
Classical Inaba (6 mo)	8/11 (73)	1,451	5/11 (45)	234	8/11 (72)	182

[a] First, primary challenge strain used; second, strain used in second exposure; time, interval between primary and secondary exposure.
[b] No., number of subjects with a vibriocidal response (defined as a fourfold rise in titer from preexposure to postexposure serum samples) over the total number of subjects challenged; peak GMT, peak reciprocal GMT obtained from all subjects after exposure.

response. An IgG response made up only 33 to 50% of the primary responses following primary exposure to wild-type El Tor, classical, or CVD 103-HgR vaccine. The peak IgG reciprocal GMTs generated by these strains after primary exposure were low, ranging from 20 to 80. The peak IgM GMT was 3- to 300-fold higher than the peak IgG GMT.

Of the 30 subjects challenged within 4 months of primary exposure, only 2 had evidence of a secondary whole serum vibriocidal response. These two subjects were given a cross-serotype secondary exposure and also had a detectable vibriocidal response in the IgM fraction of their serum. We detected an occult IgG vibriocidal antibody response following secondary exposure in only 2 of 28 (7%) subjects who had no evidence of secondary whole serum vibriocidal responses. One of these two volunteers who did have an IgM and an IgG fractionated response had only a twofold rise in whole serum vibriocidal activity following secondary exposure. He, therefore, did not meet our criterion for a whole serum vibriocidal response.

In contrast, 8 of 11 (73%) volunteers challenged 6 months after their initial vaccination mounted a secondary whole serum vibriocidal response; 5 of these 11 (45%) volunteers had a detectable IgG response, and 8 of 11 (73%) had a detectable IgM response. The IgG GMT (234) following secondary exposure was higher than that seen after primary exposure (80), while the IgM activity fell more than 10-fold, suggesting that a maturation of the systemic immune response was taking place. The magnitude of this secondary IgG response, however, was 10-fold less than that of the IgM primary response.

Cholera LPS-specific IgG subclass responses following primary and secondary cholera challenges. Since vibriocidal activity measures the binding of antibody with complement, which is dependent on the valency of the antibody class involved, we further attempted to quantitate IgG vibriocidal activity by determining LPS-specific IgG subclass activity. Following CVD 103-HgR immunization, an IgG subclass LPS was seen in 16 of 23 (69%) vaccinees with the majority of the response occurring in the IgG4 fraction (13 of 23, 57%). Only 8 of 23 (35%) had responses in IgG1, IgG2, or IgG3.

Secondary wild-type homologous exposure within 4 months of vaccination produced an IgG subclass response in only one subject (an IgG2 titer of 1:80), corroborating our whole serum and IgG vibriocidal results. However, comparisons among the IgG subclass responses for subjects challenged 6 months after vaccination show a maturation of their IgG subclass response (Table 5). Whereas only 2 of 11 (18%) subjects had either an IgG1, IgG2, or IgG3 LPS-specific response after primary exposure, 8 of 11 (73%) had an IgG1, IgG2, or IgG3 response after 6-month rechallenge ($P = 0.03$, chi-square test); 5 of 11

TABLE 5. Serum IgG subclass response to Inaba LPS following primary exposure to live attenuated cholera vaccine, CVD 103-HgR, at 10^8 organisms and secondary exposure with homologous wild-type parent strain, at 10^6 organisms, 6 months after vaccination

Exposure	IgG subclass response to Inaba LPS[a]											
	IgG1			IgG2			IgG3			IgG4		
	No. (%)	Peak GMT		No. (%)	Peak GMT		No. (%)	Peak GMT		No. (%)	Peak GMT	
		All	R		All	R		All	R		All	R
CVD 103-HgR[b]	0/11	57		2/11 (18)	66	160	0/11	30		5/11 (45)	52	106
6 mo[c]	5/11 (45)	249	735	2/11 (18)	71	160	4/11 (36)	52	113	2/11 (18)	85	113

[a] No., number of subjects with a positive seroresponse over the total number tested; peak GMT, reciprocal peak GMT of all subjects (All) or only those with detectable antibody rises (responders [R]).
[b] Initial exposure was CVD 103-HgR live attenuated oral *V. cholerae* vaccine.
[c] Time interval between primary and secondary exposure. The secondary exposure was the homologous wild-type parent strain of CVD 103-HgR, classical Inaba 569B.

(45%) and 4 of 11 (36%) challenged volunteers had an IgG1 and an IgG3 subclass response, respectively. The peak IgG1 GMT in all responders was approximately fivefold higher than that seen after primary exposure.

The IgG1 and IgG3 LPS subclass responses were highly associated with the IgG serum fraction vibriocidal response ($P < 0.001$, chi-square test). In fact, the one volunteer who had no evidence of a whole serum vibriocidal response following secondary exposure but had an IgG fraction vibriocidal response also had an IgG1 LPS subclass response. IgG4 LPS responses became less prominent in rechallenged volunteers, occurring in only 2 of 11 (18%).

DISCUSSION

Although the vibriocidal assay has been used in the serologic detection of clinical and subclinical infections with and immunity to O1 *V. cholerae*, we have defined the limitations of its usefulness under certain conditions of secondary O1 *V. cholerae* exposure (9, 16–18). Secondary vibriocidal responses are seen under two seemingly unrelated experimental conditions. The first occurs when there is an obvious break in clinical immunity, as evidenced by prolonged stool excretion of vibrios (which probably reflects intestinal colonization) or by the production of clinical disease. Specifically, we found that cross-serotype challenge was more likely to cause stool excretion of vibrios and that prolonged excretion or clinical diarrhea was highly associated with production of a secondary vibriocidal response. The fact that subtle differences in the O polysaccharide of LPS are responsible for these findings supports the importance of LPS as a virulence and immunoprotective factor in cholera (4, 10).

The second condition that produces a secondary vibriocidal response occurs in the presence of full clinical protective immunity to cholera but seems to be dependent on the interval between primary and secondary exposure. Volunteers given wild-type homologous biotype and serotype challenge within 4 months of vaccination with CVD 103-HgR had no secondary vibriocidal responses; challenge at 6 months, however, stimulated a secondary vibriocidal response in the majority of volunteers. With these time interval studies, we see evidence of an uncoupling of the serum vibriocidal response as a marker for clinical immunity to cholera. In addition, after secondary exposure, low-level stool excretion of vibrios (a variable used as a marker for asymptomatic infection) could be detected in subjects with or without evidence of a secondary vibriocidal response, suggesting that secondary vibriocidal responses cannot be used as a marker for asymptomatic infection, either.

Using isotype-purified preparations of study sera, we demonstrated that an occult IgG secondary vibriocidal response does not occur in the absence of a whole serum antibody response. Whole serum vibriocidal responses, therefore, adequately represent secondary serum vibriocidal activity. It is of special interest that when secondary serum vibriocidal responses occurred, activity was still contained in the IgM fraction in the majority of responding subjects. There was some increase in the numbers of subjects with an IgG response and some suggestion of a maturation of IgG response by an increase in absolute titer produced following secondary exposure. The long-term protective significance of these findings is not known. It has been shown that rabbits challenged with oral doses of *V. cholerae* O1 can be passively protected by intravenous antisera, in proportion not to the vibriocidal titer of the serum but, rather, to the amount of 7S antibody present in the serum (7). Epidemiologic data from areas in which cholera is endemic showing the geometric mean of vibriocidal antibody rising with age with a concomitant fall in the incidence of cholera may support a role for IgG vibriocidal antibody in long-term protection (16, 18). Since vibriocidal activity has been detected within bile fluids, serum antibody may have activity at a mucosal level (3). However, we have conducted natural wild-type cholera challenges 3 years after primary exposure and demonstrated full clinical protection in volunteers with very low serum vibriocidal titers prior to challenge (13).

Following primary immunization, IgG subclass-specific LPS responses were detected by ELISA in 57% of our vaccinees with the majority (81%) of the responses in the IgG4 subclass, not in IgG1 or IgG3, antibody classes principally involved in complement fixation activity. The significance of this primary IgG4 LPS response is not known but is interesting in view of the fact that IgG4 is the smallest IgG subclass constituent in blood and has unknown biological function. It has been shown in studies analyzing human peripheral blood lymphocyte cytokine and antibody levels from atopic individuals that increased levels of IgG4 are associated with interleukin 4 (IL-4) and IL-5 activity, and this probably indicates Th2-regulated responses (8, 20). Since IL-4 and IL-5 are also important cytokines regulating IgA production at a mucosal level, future studies will be needed to determine both systemic and mucosal cytokine production and systemic IgG subclass responses following exposure to cholera vaccine candidates and wild-type challenges to evaluate the significance of these initial IgG subclass findings (12).

Our data suggest that, under certain secondary exposure conditions, vibrio antigen resampling is not occurring, resulting in a lack of a secondary vibriocidal response. This block is probably taking place at the M cell level. *V. cholerae* O1 is processed by M cells and conveyed into intestinal lymphoid tissue for immunologic processing (26). Precedent data from Owen et al. for experimental O1 *V. cholerae* infections in rats have shown that fewer infecting organisms are transported to M cells in immunized animals compared with unimmunized animals, suggesting that, following primary exposure, active mucosal immune processes that interfere with further uptake of antigen are indeed taking place (21).

This blockade of antigen uptake seems to be occurring not only at the systemic level but also at a mucosal level. We have previously reported that initial exposure to CVD 103-HgR *V. cholerae* vaccine followed by wild-type challenge within 4 months of vaccination also produces no secondary cholera-specific LPS or cholera toxin antibody-secreting cell (ASC) responses. However, if the time interval between vaccination and challenge is 6 months, a secondary LPS-specific ASC response of higher magnitude than that seen after primary exposure is detected in the majority (83%) of volunteers (15). Since ASCs originate in the mucosal lymphoid tissue and circulate for a short time, before homing back to the lamina propria, their detection reflects activation of specific mucosal priming at the lymphoid follicle level. Combining our ASC data with that reported here, we find that every subject who had secondary ASC responses at 6-month rechallenge also had a secondary whole serum and/or isotype-specific vibriocidal response. The one volunteer who did not have a detectable secondary LPS-specific ASC response also did not have a secondary whole serum or IgG or IgM vibriocidal response. These data support the concept that, following effective primary exposure to *V. cholerae* O1, active mucosal immunity is interfering with the efficient delivery of bacteria or bacterial antigens to responsive lymphoid tissues. Presumably, this active immunity is contained in the lamina propria by cholera antigen-specific activated plasma cells or memory B cells. Similar observations have been made with the rabbit intestinal challenge

cholera model in which, after priming rabbits with live *V. cholerae*, even at suboptimal immunizing doses, it was impossible to provoke a secondary boosted mucosal plasma cell response in the gut lamina propria unless very high challenge inocula were used (22).

The generation of a secondary vibriocidal response does not necessarily mean that there is no mucosal protection against cholera. We have clearly shown that an individual can be fully protected from clinical disease following secondary cholera exposure and yet mount a secondary vibriocidal response. Obviously, there is some elasticity in the mucosal immune system that allows for the restimulation of a specific immune response, at both the local and the systemic levels, yet provides for the full clinical protection of the host. Presumably, specific local IgA immunity following CVD 103-HgR vaccination has sufficiently decreased by 6 months to allow for vibrio antigen resampling. It is unclear whether a more strenuous challenge of these subjects who mounted a secondary response may have produced clinical illness. It is also presently unknown whether there are differences in the abilities of wild-type *V. cholerae* and CVD 103-HgR vaccine to provide long-term protection against cholera. Only half of the subjects exposed initially with wild-type classical Ogawa strain 395 and given a homologous rechallenge 3 years later had evidence of a secondary vibriocidal response compared with 73% of volunteers given CVD 103-HgR followed by wild-type homologous challenge 6 months after vaccination. Although the numbers of volunteers are small, these results do suggest that active mucosal protection generated by wild-type cholera exposures may be longer lasting than that achieved with CVD 103-HgR. Future studies aimed at long-term challenges of CVD 103-HgR vaccine recipients are obviously needed to answer this question.

These data would also suggest that the generation of a secondary vibriocidal response and our previously reported results on secondary ASC responses would be useful parameters to use in determining the length of complete active mucosal immunity in O1 cholera infections. They can also be used for determination of the timing for secondary boosters of orally administered O1 cholera vaccines in vaccinated populations. In addition to demonstrating the limitations of the serum vibriocidal response to detect secondary exposure to O1 *V. cholerae*, the assay may not even be a reliable indicator of primary exposure to cholera under endemic or epidemic conditions since serum vibriocidal levels fall quite rapidly after primary exposure. For evaluation of cholera vaccines under field trial conditions in regions in which cholera is endemic, there are severe handicaps in using this assay for determining vaccine-induced immunity. Current approaches in our laboratory to develop alternative markers of immunity to O1 cholera challenges involve investigations of cholera antigen-specific fecal antibody responses following primary and secondary exposures and serum IgG subclass-specific LPS responses.

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